

RANDOM CENTROID OPTIMIZATION OF LUTEIN-ENRICHED OIL IN WATER
EMULSION AT ACIDIC pH

A Thesis

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DEDICATION

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ABSTRACT

Age-related macular degeneration (AMD) is a physiological condition that affects the elderly, diabetic patients, and leads to loss of vision. There are no effective therapies for AMD. The role of nutrition in preventing AMD is receiving strong scientific scrutiny. Lutein and zeaxanthin have been identified as dietary strategies that can delay the onset of AMD. Consumption of at least six milligrams of lutein per day has been reported to delay the onset of AMD by as much as 43%. However, most available food sources contain such low levels of lutein that it would be difficult to meet the daily requirement. It would require 2 lbs of corn, 16 lbs of tomatoes, or one salad bowl of kale to meet the minimum daily requirement. As a result, food fortification with lutein extract is appealing to health conscious consumers.

The objective of this study was to prepare an oil in water emulsion containing at least 6 mg of lutein per serving size (one tablespoon) as a daily source of lutein. Random Centroid Optimization was applied to find the best conditions for preparing a stable emulsion. Experimental parameters included: lutein concentration (0.010-0.020 %w/w); pH (3.5-4.7); phosphatidylglycerol (0.018-0.1 %w/w); whey protein (1.5-3.5 %w/w); and corn oil (15-25 %w/w). Emulsions were prepared and turbidity measurements were determined as a function of half-life stability in minutes. Absorbance was measured at 500 nm for 10 min. The same measurements were carried out using the best values for four factors obtained from the RCO program.

Emulsion stability was evaluated by heating samples at 90 °C from 5 to 30 min to check for oil separation. Emulsion samples were also shaken at 700 rpm for 30 min. Size distribution and zeta potential of emulsion droplets were measured, using zetameter.

After 9 experiments in the first cycle and 4 experiments in centroid cycle, optimal conditions for the preparation of a stable lutein-enriched emulsion were: corn oil: 20 %, phosphatidylglycerol (0.018 %w/w), pH: 4.55, lutein concentration: (0.015 %w/w), and whey proteins concentration 2%.

CHAPTER 1

INTRODUCTION

Life expectancy has significantly increased in the western world and in most parts of the third world. As a result, the multitude of debilitating chronic and long-term illnesses such as vision impairment due to age related macular degeneration (AMD) and blindness from diabetes exposes the aging population to increased risk of loss of independence. There are few effective treatments, little prevention and no real cures for these debilitating conditions that already threaten millions of people around the world. The exact cause of AMD is not yet known, but only risks factors have been identified. Inherited and acquired risk factors include age, smoking, family history, race, light exposure, and nutrition (Bernstein, 2002). As standard therapies are limited, costly, and often associated with undesirable pathological side effects, the role of nutrition in protecting against degenerative diseases is intensively under scientific consideration.

AMD is a pathological process in the macula that is a result of genetic and environmental factors. AMD affects about 50 % of people at age 75 ± 5 and rarely affects people below 50 years of age. There are two types of AMD: the "dry form" and "wet form". The dry form is atrophic, consists of yellow pigmentation spots in the macula, and represents about 90% of the AMD cases. The wet form consists of complications of neovascularization or angiogenesis-outgrowth of new blood vessels-beneath the macula and usually progresses to complete loss of central vision. At this point in time, there is no real cure or treatment. Risk factors for macular degeneration include diabetes and all its complications (hypertension, retinopathy, and arteriosclerosis), smoking, chronic sunlight exposure, nutritional deficiencies, and blue/green eyes. Treatment options available today for AMD consist of slowing down the angiogenesis process of AMD by either laser or photodynamic therapies. While laser treatment carries the

possibility of damaging the remaining vision, photodynamic therapy is not an established therapy yet. However, most AMD patients progress to legal blindness.

Standard therapies for AMD are still lacking, prevention of the disease onset and/or progression has drawn a lot of interest among patients and care providers. The role of nutrition using antioxidants, vitamins, and carotenoids has shown that these bioactive compounds may slow down the progression of AMD.

Lutein and zeaxanthin (3, 3'-dihydroxy- α -carotene) differ by the position of one double bond and are the only carotenoids quantitatively identified in the human retina. Lutein and zeaxanthin have been identified and recognized by various interdisciplinary studies as one of the dietary strategies that can delay the onset of macular degeneration (Berendschot et al., 2000). Higher levels of lutein and zeaxanthin were measured in the retina of individuals without known clinical history of AMD while lower levels of both xanthophylls were measured in the eyes of individuals with known clinical history of AMD (Bone et al., 2001). Lutein and zeaxanthin concentration in the peripheral retina and the macular region was shown to be as high as 100 ng (Bernstein, 2002). Other carotenoids such as lycopene and beta-carotene are present in the ocular tissues other than the retina. Carotenoids are excellent antioxidant by virtue of their ability to utilize the long carbon and double bond chain to dissipate or absorb the damaging effects of free radicals.

It was suggested that 6 mg of lutein a day may reduce the risk of AMD by 43 % (Seddon et al., 1994). This concentration is equivalent to consuming 2 lbs of corn, 16 lbs of tomatoes, 2 salad bowls of spinach or one salad bowl of kale a day. Tomato and spinach are high oxalate-containing foods that may cause a significant increase in urinary oxalate excretion and kidney stones (Massey et al., 1993, Hesse et al., 1993). Kale is a low-oxalate vegetable but is not

normally found in sufficient quantities at any local food market for all the households in the community. Lutein enriched vitamin supplements provide up to 250 μg of lutein per tablet. Very few people can have the discipline and consistency to consume 2 lbs of corn or 16 lbs of tomatoes daily for lutein intake. As a consequence, food fortification with lutein extract is convenient and appealing to health conscious consumers. Thus, the aim of this research was to prepare a lutein enriched salad dressing emulsion.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Increasing health awareness and a corresponding lifestyle are the main factors that influence the development of new food products. Especially, carotenoid formulations with processing characteristics and stability to meet the customers' requirements are of great interest (Sambale, 1999).

Xanthophylls were found in algae, and lutein, a component of xanthophylls was found in egg yolks (Surai et al., 2000). Later research confirmed that carotenes and xanthophylls are closely related in molecular structure, and the term "carotenoids" is commonly used to refer to the entire group of diverse, and yet closely related substances (Krinsky et al., 2003). Major known functions of xanthophylls are photoreception and photoprotection. Zeaxanthin and antheraxanthin, other members in the family, are also found to be involved in heat (energy) dissipation by converting themselves to violaxanthin, thus adding additional measures for the protection of photosynthesis systems.

In addition to being an integral part of photosynthesis systems as photoreceptors and protectors, carotenoids are strong anti-oxidants and protect plant tissues from damages caused by free radicals formed by UV-irradiation (Krinsky et al., 2003).

2.1.1 Chemical/Physical Properties of Lutein/Zeaxanthin

Lutein and zeaxanthin are both isomeric dihydroxy-carotenoids with the ionone ring systems being substituted at both the 3 and 3' carbon (Figure 1).

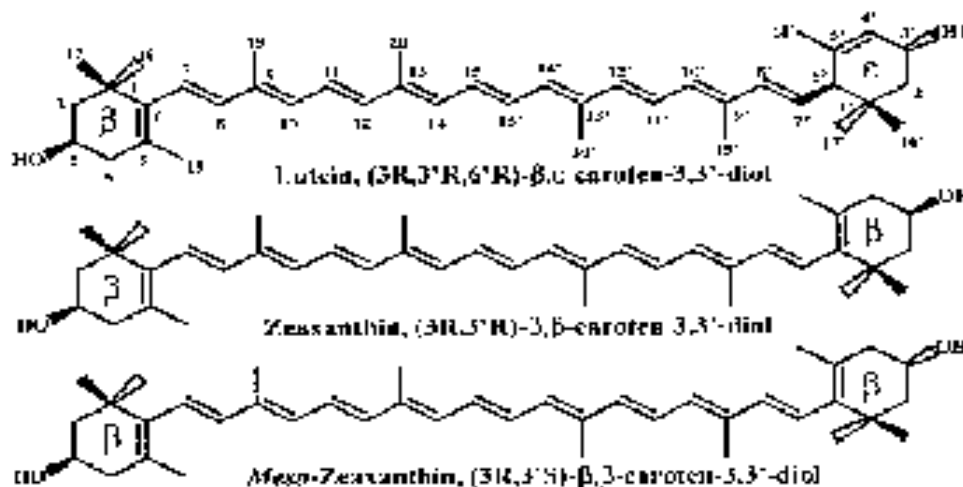


Figure 1 Chemical structure of lutein and zeaxanthin (Krinsky et al., 2003)

In zeaxanthin, the less abundant of these two isomers in most plant sources (Goodwin, 1984), the ionone rings are both β types. The β -ionone ring double bond is found between the C_5 and C_6 carbons, placing it in a position to interact, albeit weakly, with the conjugated polyene chain. A strong steric interaction between the C_{18} methyl found on C_5 of the ionone ring and the hydrogen on C_8 constrains the ring double bond to an angle of about 40° to the plane of the conjugated polyene chain (Buchecker, 1995). The β -ionone ring double bond is therefore functionally isolated and behaves largely independently of the conjugated system. This has spectroscopic as well as chemical implications for zeaxanthin.

Lutein has both a β -ionone ring and a ϵ -ionone ring. The presence of the hydroxyl groups at both the 3 and 3' carbons suggests that a close similarity in physical properties exists between lutein and zeaxanthin. The ϵ -ionone ring has a C_4 - C_5 double bond and an allylic 3'-hydroxyl group. Interestingly, in the predominant form of lutein (3R, 6'R, 3'R) - β -carotene-diol (Andrews, 1974) the designation of the stereochemical configuration of the 8-ring hydroxyl (R) is identical to that of the β -ring because of the Cahn-Ingold-Prelog rules (Eliel, 1962). However,

the ϵ -ring hydroxyl group is oppositely directed with respect to the hydroxyl group in the β -ionone ring. As shown in Figure 1, the 3'-hydroxyl of the zeaxanthin ring projects forward from the surface of the page whereas the 3'-hydroxyl of lutein is folded back away from the plane of the page. This is a major stereochemical distinction between the dominant forms of lutein and zeaxanthin. The relative orientation of the hydroxyls may be a factor of some importance for specific recognition of these two isomers by proteins (Tabunoki et al., 2002) and it may also influence the preference in site selection exhibited by these carotenoids when they are incorporated into membranes (Gruszecki et al., 1999).

The presence of the hydroxyl groups makes lutein and zeaxanthin distinctly more polar than their respective carotene analogs, α and β -carotene. This is demonstrated dramatically by their relative retention times on both normal and reversed-phase chromatographic columns where the difference in retention times is due primarily to polarity (Craft, 1992). The ratio of the retention times of the carotenes to those of the xanthophylls is approximately 4:1 on C-18 (octadecylsilane) derivatized reversed-phase HPLC columns. As would be expected, because the polarities of lutein and zeaxanthin are very similar, baseline chromatographic separation is not easily achieved. This has resulted in a tendency of many researchers to report combined lutein/zeaxanthin values (Schmitz et al., 1993). Lutein and zeaxanthin are most soluble in nonpolar or dipolar solvents such as hexane, benzene, ethers, methylene chloride, and chloroform. They are also generally soluble in alcohols. The solubility of lutein and zeaxanthin in methanol is less than in alcohols having long alkyl chains.

The ability of carotenoids to absorb light arises from the presence of a conjugated polyene chain. The wavelength maximum of the absorption band is related to the extent of the conjugation in the polyene chain (Kohler, 1995). Both lutein and zeaxanthin have nine

conjugated double bonds in the polyene chain. Lutein has an absorption maximum of 445 nm in ethanol whereas that of zeaxanthin is 451 nm. In addition to the length of the polyene chain, the nature of the end-group attached to the polyene chain has significance for the spectral characteristics of carotenoids. The small difference in the wavelength of maximum absorption for lutein and zeaxanthin is due to the interaction of the double bonds in the β -ionone ring(s) with the polyene chain. The β -ring double bond, which might seem to be conjugated with the polyene chain, interacts with it only weakly. Nevertheless the presence of β ring double bonds induces a modest red shift in the carotenoid absorption spectrum.

Lutein and zeaxanthin are constitutional isomers and differ in the position of a double bond in one of the ionone rings. Each has a variety of different stereoisomers. These include the geometrical *Z*- and *E*- isomers (often referred to as *cis* and *trans* isomers). Many are noted to occur in human serum. The presence of a *Z* bond in an otherwise all-*E* polyene chain of the carotenoid causes the molecule to have a pronounced V-shape and alters the visible spectrum. Because of the methyl substitution of the carotenoids, only 9-, 13-, and 15-*Z* isomers (and 9'- and 13'-) are encountered (Zechmeister, 1962). Isomers containing multiple *Z*-bonds are also possible. Minor quantities of lutein *Z* isomers are detectable in human serum and the human retina (Khachik and Bernstein, 1997).

2.1.2 Natural Sources of Lutein

The major food sources of lutein are presented in Table 1 (Huck et al., 2000). Spinach, kale and broccoli have the highest amount of lutein. On the other hand carrot, tomato flakes, tomato granulates and tomato powder has less amount of lutein.

Although lutein and zeaxanthin concentrations of egg yolk are relatively low, recent data suggest that lutein and zeaxanthin from this food source are highly bioavailable (Surai et al.,

2000). Data on the lutein content of foods frequently include zeaxanthin and are reported as lutein + zeaxanthin, making examination of specific effects of dietary lutein difficult. In terms of food sources, human metabolism, and tissue storage, lutein and zeaxanthin are similar.

Table 1 Food Sources of Lutein

Food	LuteinContent (µg/100g wet wt)
Broccoli	2358
Kale	6390
Carrot	280
Spinach	3920
Tomato granulate	226
Tomato powder	39
Tomato flakes	99

Adapted from (Huck et al., 2000)

2.1.3 Lutein in Health and Disease

The first report that the yellow spot in the macula of human retinas might be a carotenoid appeared in 1945. George Wald dissected the foveal region of 10 human retinas, extracted them with chloroform, and reported that the spectrum of the yellow pigment agreed quite well with the visual estimate of the macular pigment, derived from the differences in the log sensitivity of peripheral and foveal cones (Wald, 1945). Fifty years after this observation, carotenoids were also identified in the lens of the human eye (Yeum et al., 1995) and several years later, carotenoids were identified in virtually all of the tissues of the eye (Bernstein et al., 2000). Bone and Landrum (1988) carried out the first chromatographic characterization of the macular pigment using a high performance liquid chromatography (HPLC) analysis to demonstrate that there were actually two xanthophylls present in macula, namely lutein and zeaxanthin (Bone et al., 1988). Shortly thereafter, these investigators and others reported that there was a different ratio of lutein to zeaxanthin between the central fovea and the more peripheral regions, with zeaxanthin predominating in the central fovea and lutein in the periphery (Bone et al., 1988).

More recently, it has been reported that some of the minor peaks observed in the HPLC analysis of the macular pigment consist of oxidation products of both lutein and zeaxanthin, such as 3'-epilutein and 3-hydroxy- β , ϵ -caroten-3'-one, as well as geometric isomers of the major pigments (Khachik and Bernstein, 1997). The presence of *cis*-isomers in the retina is not surprising, since the macula is exposed to bright light, which is known to isomerize carotenoids. However, the presence of oxidative metabolites suggests that the pigments are susceptible to oxidation in the tissue, or that an active metabolic process takes place, with some potential for interconversions from among the reported intermediates.

Dietary carotenoids are thought to provide health benefits in decreasing the risk of disease, particularly eye disease. The evidence supporting a relationship between lutein and zeaxanthin and AMD, which has been reviewed numerous times in the past (Beatty et al., 1999) was based on the presence of lutein and zeaxanthin in the macula, and the relative decrease in these carotenoids in the macula of AMD patients (Bone et al., 2001). It has been suggested that eating leafy vegetables, which are rich in lutein and zeaxanthin, may decrease the risk for eye disease called Age-Related Macular Degeneration (AMD) (Curran-Celentano et al., 2001). AMD is the most common cause of irreversible vision loss and legal blindness among older people. It is a degenerative condition of the region of the retina that is responsible for central vision. These carotenoids are concentrated in the inner retinal layer of macula, where the concentration is high and variable (Bernstein, 2002).

Evidence from human studies suggest that dietary intake of carotenoids can lead to their accumulation in the retina and, therefore, may provide protection against retinal degeneration. In a recent prospective study, 11 subjects modified their usual daily diets by adding 60 grams per day of spinach (containing 11mg lutein) for 15 weeks. Eight subjects had increases in serum

lutein and macular pigment density, 2 subjects showed substantial increases in serum lutein but not in macular pigment density and 1 subject showed no changes at all (Handelman et al., 1988).

Population studies provide evidence to suggest that protection from AMD can be obtained from lutein (Seddon et al., 1994). Investigators from the Eye Diseases Case-Control reported that patients in the group with highest level of plasma lutein/zeaxanthin had a decreased risk for AMD. In a subsequent study, the investigators found that protection from AMD was associated with dietary intake of specific carotenoids (Seddon et al., 1994). The authors then investigated the specific carotenoids responsible for the protection from AMD and associated it with lutein/zeaxanthin.

However, the evidence has continued to grow stronger that there is a relationship between the ingestion of lutein and zeaxanthin, primarily from dark green, leafy vegetables, and the amount of macular pigment in the retina (Curran-Celentano et al., 2001). It has been known for many years that short wavelength light absorbed by lutein and zeaxanthin has a damaging effect on the retina (Ham, 1983).

2.2 Characteristics of Food Emulsions/Definitions

An emulsion consists of two immiscible liquids (usually oil and water), with one of the liquids dispersed as small spherical droplets in the other. In most foods, the diameters of the droplets usually lie somewhere between 0.1 and 100 μ m (Dickinson and Stainsby, 1982, Dickinson, 1992). An emulsion can be conveniently classified according to the distribution of the oil and aqueous phases. A system that consists of oil droplets dispersed in an aqueous phase is called an oil -in- water or O/W emulsion (e.g., mayonnaise, milk, cream etc.). A system that consists of water droplets dispersed in an oil phase is called a water-in-oil or W/O emulsion (e.g. margarine, butter and spreads).

The process of converting two separate immiscible liquids into an emulsion, or of reducing the size of the droplets in a preexisting emulsion, is known as homogenization. It is possible to form an emulsion by homogenizing pure oil and pure water together, but the two phases rapidly separate into a system that consists of a layer of oil (lower density) on top of a layer of water (higher density). This is because droplets tend to merge with their neighbors, which eventually leads to complete phase separation.

Emulsions usually are thermodynamically unstable systems. It is possible to form emulsions that are kinetically stable (metastable) for a reasonable period of time (a few days, weeks, months, or years) by including substances known as emulsifiers and /or thickening agent prior to homogenization.

Emulsifiers are surface-active molecules that adsorb to the surface of freshly formed droplets during homogenization, forming a protective membrane that prevents the droplets from coming close enough together to aggregate. Most emulsifiers are molecules having polar and nonpolar regions in the same molecule. The most common emulsifiers used in the food industry are amphiphilic proteins, small-molecule surfactants, and phospholipids monoglycerides, such as sucrose esters of fatty acids, citric acid esters of monodiglycerides, salts of fatty acids, etc (Krog, 1990).

Thickening agents are ingredients that are used to increase the viscosity of the continuous phase of emulsions, and they enhance emulsion stability by retarding the movement of the droplets.

A stabilizer is any ingredient that can be used to enhance the stability of an emulsion and may therefore be either an emulsifier or thickening agent.

2.2.1 Thermodynamic and Kinetic Stability of Emulsions/ Emulsion instability

An understanding of the properties of food emulsions requires knowledge of the difference between the thermodynamic stability and the kinetic stability of the emulsion systems (Dickinson, 1992). Consider a system that consists of a large number of molecules that can occupy two different states: E_{low} and E_{high} . The state with the lowest free energy is the one that is thermodynamically favorable, and therefore the one that the molecules are most likely to occupy.

The larger the difference between the two energy levels compared to the thermal energy of the system (kT), the greater the fraction of molecules in the lower energy state. In practice, a system may not be able to reach equilibrium during the time scale of an observation because of the presence of an energy barrier (E^*) between the two states. A system in the high-energy state must acquire energy greater than (E^*) before it can move into the low energy state. The rate at which a transformation from a high to a low energy state occurs therefore decreases as the height of the energy barrier increases. When the energy barrier is sufficiently large, the system may remain in a thermodynamically unstable state for a considerable length of time, in which case it is said to be kinetically stable or metastable (Atkins, 1994). In food emulsions, there are actually a large number of intermediate metastable states between the initial emulsion and the separated phases, and there is an energy barrier associated with a transition between each of these states. Nevertheless, it is often possible to identify a single energy barrier that is associated with a particular physicochemical process that is the most important factor in determining the overall kinetic stability of an emulsion.

The term "emulsion stability" is broadly used to describe the ability of an emulsion to resist changes in its properties with time. Food emulsions may become unstable through a variety

of physical processes including creaming, sedimentation, flocculation, coalescence, and phase inversion.

Creaming and sedimentation are both forms of gravitational separation. Creaming describes the upward movement of droplets due to the fact that they have a lower density than the surrounding liquid, whereas sedimentation describes the downward movement of droplets due to the fact that they have a higher density than the surrounding liquid.

Flocculation and coalescence are both types of droplet aggregation. Flocculation occurs when two or more droplets come together to form an aggregate in which the droplets retain their individual integrity, whereas coalescence is the process where two or more droplets merge together to form a single larger droplet. Extensive droplet coalescence can eventually lead to the formation of a separate layer of oil on top of a sample, which is known as "oiling off."

Phase inversion is the process whereby an oil-in-water emulsion is converted into a water-in-oil emulsion or vice versa.

2.2.2 Ingredient Partitioning in Emulsions

Most food emulsions can conveniently be considered to consist of three regions that have different physicochemical properties: the interior of the droplets, the continuous phase, and the interface. The molecules in an emulsion distribute themselves among these three regions according to their concentration and polarity (Wedzicha, 1988). Nonpolar molecules tend to be located primarily in the oil phase, polar molecules in the aqueous phase, and amphiphilic molecules at the interface. It should be noted that even at equilibrium, there is a continuous exchange of molecules between the different regions, which occurs at a rate that depends on the mass transport of the molecules through the system. Molecules may also move from one region to another when there is some alteration in the environmental conditions of an emulsion (e.g., a

change in temperature or dilution within the mouth). The location and mass transport of the molecules within an emulsion have a significant influence on the aroma, flavor release, texture, and physicochemical stability of food products (Dickinson and Stainsby, 1982, Wedzicha et al., 1991, Coupland and McClements, 1996).

2.2.3 Dynamic Nature of Emulsions

Many properties of the emulsions can only be understood with reference to their dynamic nature. The formation of emulsions by homogenization is a highly dynamic process which involves the violent disruption of droplets and the rapid movement of surface-active molecules from the bulk liquids to the interfacial region. Even after their formation, the droplets in an emulsion are in continual motion and frequently collide with one another because of their Brownian motion, gravity, or applied mechanical forces (Melik and Fogler, 1988, Dukhin and Sjoblorn, 1996). The continual movement and interactions of droplets cause the properties of emulsions to evolve over time due to the various destabilization processes such as change in temperature or in time.

2.2.4 Particle Size Distribution

The most important properties of emulsion-based food products include shelf life, appearance, texture, and flavor. These properties are determined by the size of the droplets they contain (Dickinson and Stainsby, 1982, Dickinson, 1992). Consequently, it is important to control, predict and measure, the size of the droplets in emulsions.

If all the droplets in an emulsion are of the same size, the emulsion is referred to as monodisperse, but if there is a range of sizes present, the emulsion is referred to as polydisperse. The size of the droplets in a monodisperse emulsion can be completely characterized by a single number, such as the droplet diameter (d) or radius (r). Monodisperse emulsions are sometimes

used for fundamental studies because the interpretation of experimental measurements is much simpler than that of polydisperse emulsions. Nevertheless, food emulsions always contain a distribution of droplet sizes, and so the specification of their droplet size is more complicated than that of monodisperse systems. Ideally, one would like to have information about the full particle size distribution of an emulsion (i.e., the size of each of the droplets in the system). In many situations, knowledge of the average size of the droplets and the width of the distribution is sufficient (Hunter, 1986).

2.2.5 Emulsion Droplet Charge and Zeta Potential

The bulk physicochemical and organoleptic properties of many emulsions are governed by the magnitude and sign of the electrical charge on the droplets (Dickinson and Stainsby, 1982). The origin of this charge is normally the adsorption of emulsifier molecules that are ionized or ionizable. Surfactants have hydrophilic head groups that may be neutral, positively charged, or negatively charged. Proteins may also be neutral, positively charged, or negatively charged depending on the pH of the solution and their isoelectric points. Consequently, emulsion droplets may have an electrical charge that depends on the types of surface-active molecules present and the pH of the aqueous phase. The charge on a droplet is important because it determines the nature of its interactions with other charged species or its behavior in the presence of an electrical field. All of the droplets in an emulsion are usually coated with the same type of emulsifier, and so they have the same electrical charge (if the emulsifier is ionized). When this charge is sufficiently large, the droplets are prevented from aggregating because of the electrostatic repulsion between them. The properties of emulsions stabilized by ionized emulsifiers are particularly sensitive to the pH and ionic strength of the aqueous phase. If the pH of the aqueous phase is adjusted so that the emulsifier loses its charge, or if salt is

added to "screen" the electrostatic interactions between the droplets, the repulsive forces may no longer be strong enough to prevent the droplets from aggregating. Droplet aggregation often leads to a large increase in emulsion viscosity and may cause the droplets to cream more rapidly (Dickinson and Stainsby, 1982).

The accumulation of charged species at a droplet surface and the rate at which this accumulation takes place depend on the sign of the charge of the species relative to that of the surface, the strength of the electrostatic interaction, the concentration of the species, and the presence of any other charged species that might compete for the surface.

The above discussion highlights the importance of droplet charge in determining both the physical and chemical properties of food emulsions. Based on droplet charge, the stability of emulsions is depends on zeta potential. The surface charge on the emulsion droplet produces a difference in electrical potential, in millivolts, between the surface of each droplet and the bulk of suspending liquid. The difference in each potential is called the “Zeta potential” (van Nieuwenhuyzen and Szuhaj, 1998). Table 2 summarized the typical zeta potential (ZP) values for flocculated and dispersed systems.

Table 2 Zeta Potential (in millivolts) for hydrophobic colloids (van Nieuwenhuyzen and Szuhaj, 1998)

Stability of Hydrophobic Colloids	Zeta Potential (in millivolts)
Threshold of agglomeration	- 11 to -20
Moderate stability (no agglomeration)	- 31 to -40
Good stability	- 41 to - 50
Very good stability	-51 to - 60
Excellent stability	- 61 to - 80

According to van Nieuwenhuyzen (1998), the magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. If all the particles have a large negative or positive zeta potential they will repel each other and there is dispersion stability. If

the particles have low zeta potential values then there is no force to prevent the particles from coming together and there is dispersion instability. A dividing line between stable and unstable aqueous dispersions is generally taken at either +30 or -30mV (van Nieuwenhuyzen and Szuhaj, 1998). Particles with zeta potentials more positive than +30mV are normally considered stable. Particles with zeta potentials more negative than -30mV are also normally considered stable.

The study of Chansiri (1999) showed that the electrostatic repulsive force could be increased in phospholipids stabilized o/w emulsions by the addition of phosphatidylglycerol (PGL). It has been shown that the addition of a small amount of PGL may increase the zeta potential of the emulsions. In addition to increasing the zeta potential of emulsions, the addition of phosphatidylglycerol increases the naturally occurring negative surface charges and therefore emulsions having larger negative surface charge will undergo less coalescence during heat treatment (Chanziri et al., 1999). It has been found recently that phosphatidylglycerol protects human retinal pigment epithelial cells against apoptosis (Shaban et al., 2002) induced by N-retinyl-N-retynildene ethanolamine (A₂E) compound, which is suspected to cause the dry form of AMD. Therefore it is suggested that use of phosphatidylglycerol in combination with the light-absorbing lutein can prevent or stop the AMD (Shaban et al., 2002).

2.2.6 Testing Emulsifier Efficiency

In emulsion-based products the most important ingredient is often the emulsifier. The type of emulsifier largely determines overall appearance of food emulsions. Proteins derived from whey are widely used as emulsifiers (Philips et al., 1994, Dalgleish, 1996). They adsorb to the surface of oil droplets during homogenization and form a protective membrane, which prevents the emulsion droplets from coalescing. Different additives to O/W emulsions like

carbohydrates, carotenoids etc. may increase their emulsion stability. In particular these ingredients may contribute to the reduction of the surface or interfacial tension.

One of the most important decisions a food manufacturer must make when developing an emulsion-based food product is the selection of the most appropriate emulsifier (Fisher and Parker, 1985; Hasenhuettl, 1997). A huge number of emulsifiers are available as food ingredients, and each has its own unique characteristics and optimum range of applications (Hasenhuettl and Hartel, 1997). The efficiency of an emulsifier is governed by a number of characteristics, including the minimum amount required to produce a stable emulsion, its ability to prevent droplets from aggregating over time, the speed at which it adsorbs to the droplet surface during homogenization, the interfacial tension, and the thickness and viscoelasticity of the interfacial membrane. These characteristics depend on the food in which the emulsifier is present and the prevailing environmental conditions (e.g., pH, ionic strength, ion type, oil type, ingredient interactions, temperature, and mechanical agitation) (Sherinan, 1995). For this reason, it is difficult to accurately predict the behavior of an emulsifier from knowledge of its chemical structure (although some general predictions relative to its functional properties are usually possible). Instead, it is often better to test the efficiency of an emulsifier under conditions that are similar to those found in the actual food product in which it is going to be used (Sherman, 1995).

2.2.7 Emulsion Stability and Stability Index

Use of six simple steps to achieve food emulsion stability is crucial (McClements, 1999). The steps are system determination; knowledge of ingredient composition; adequate mechanical emulsifying action; order of addition of ingredients during mixing of emulsions; the need for a smooth emulsion texture; and temperature changes within the product (McClements, 1999).

Food emulsion stability is strongly influenced by the interactions in the adsorbed layer around emulsion droplets. For instance, competitive adsorption of pure milk proteins (beta-casein or beta-lactoglobulin) with nonionic surfactants in oil-in-water emulsions was found to depend on age of the adsorbed protein layer (Demetriades et al., 1998).

An efficient emulsifier produces an emulsion in which there is no visible separation of the oil and water phases over time. Phase separation may not become visible to the human eye for a long time, even though some emulsion breakdown has occurred. Consequently, it is important to have analytical tests that can be used to detect the initial stages of emulsion breakdown, so that their long-term stability can be predicted.

One widely used test is to centrifuge an emulsion at a given speed and time and observe the amount of creaming and/or oil separation that occurs (Smith and Mitchell; 1976, Das and Kinsella, 1990). This test can be used to predict the stability of an emulsion to creaming using relatively low centrifuge speeds or to coalescence by using speeds from $2000 \times g$ to $25000 \times g$, which are enough to rupture the interfacial membranes (Pearce and Kinsella, 1978). The greater the degree of creaming or oil separation occurs, the greater the instability of an emulsion and the less efficient the emulsifier.

An alternative approach that can be used to accelerate emulsion instability is to measure the degree of droplet coalescence when an emulsion is subjected to mechanical agitation (Dickinson and Williams, 1994). The droplet size distribution of the emulsions can be measured either as a function of time as the emulsions are agitated at a constant stirring speed or as a function of stirring speed after the emulsions have been agitated for a fixed time. The faster the increase in droplet size with time, the greater the instability of the emulsion and the lower the efficiency of the emulsifier.

A more quantitative method of determining emulsifier efficiency is to measure the change in the particle size distribution of an emulsion with time. An efficient emulsifier produces emulsions in which the particle size distribution does not change over time, whereas a poor emulsifier produces emulsions in which the particle size increases due to coalescence and/or flocculation. The kinetics of emulsion stability can be established by measuring the rate at which the particle size increases with time.

2.3 Whey Protein Stabilized Emulsions

2.3.1 Physicochemical Properties of Whey Protein-Stabilized Emulsions

In emulsion based food products the most important ingredient is often the emulsifier (Dickinson, 1992, Damodaran, 1996). The type of emulsifier largely determines overall appearance, texture and shelf life of food emulsions. Consequently, it is important to identify optimum conditions at which each emulsifier would be most effective for desired product properties.

In oil-in-water emulsions, proteins are used mostly as surface active agents and emulsifiers. One of the food proteins used in o/w emulsions is whey proteins. The whey proteins include four proteins: β -lactoglobulin, α -lactalbumin, bovine serum albumin and immunoglobulin (Tornberg, 1990). Commercially, whey protein isolates (WPI) with isoelectric point ~ 5 (Tornberg, 1990) are used for o/w emulsion preparation. According to Hunt (1995), whey protein concentrations of 8% have been used to produce self-supporting gels. Later on, the limiting concentrations of whey protein to produce self-supporting gels are known to be reduced to 4 - 5%. It is possible to produce gels at whey protein concentrations as low as 2% w/w, using heat treatments at 90°C or 121°C and ionic strength in excess of 50 mM (Hunt et al., 1995).

Proteins derived from whey are widely used as emulsifiers (Phillips et al., 1994, Dalgleish, 1996). They adsorb to the surface of oil droplets during homogenization and form a protective membrane, which prevents droplets from coalescing. The physicochemical properties of food emulsions stabilized by whey protein isolates (WPI) are related to the aqueous phase composition (e.g., ionic strength and pH) and the processing and storage conditions of the product (e.g., heating, cooling, and mechanical agitation). Emulsions are prone to flocculation around the isoelectric point of the WPI, but are stable at higher or lower pH (Phillips et al., 1994). The stability to flocculation could be interpreted in terms of colloidal interactions between droplets, i.e., van der Waals, electrostatic repulsion and steric forces (Phillips et al., 1994; Dalgleish, 1996). The van der Waals interactions are fairly long-range. Electrostatic interactions between similarly charged droplets are repulsive, and their magnitude and range decrease with increasing ionic strength. Short range interactions become important at droplet separations of the order of the thickness of the interfacial layer or less, e.g., steric, thermal fluctuation and hydration forces (Israelachvili, 1992). Such interactions are negligible at distances greater than the thickness of the interfacial layer, but become strongly repulsive when the layers overlap, preventing droplets from getting closer.

2.3.2 Heat Stability of O/W Emulsions Stabilized by WPI

Many food products based on emulsions are subjected to heat treatment prior to distribution. The heat treatment is applied generally to extend the microbiological shelf life of the product, but may be moderated if the heat treatment destabilizes the emulsion. The effect of heating at 90°C on emulsions made with WPI has been reported (Jost et al., 1986, Yost and Kinsella, 1992). It was demonstrated that gels might be formed under those conditions. Such gels were produced at much lower concentrations than would be required if the protein was

present alone in solution (Matsudomi et al., 1992). It was suggested, therefore, that the emulsified droplets became incorporated into the gel matrix. The formation of gels depends greatly on variables such as ionic strength and pH (Mulvihill and Kinsella, 1987).

Another variable in emulsions made with proteins is the distribution of the protein. It may either be adsorbed at the surface of the emulsion droplet or it may be free in solution.

2.4 Random Centroid Optimization

Random Centroid Optimization (RCO) is a program that uses an algorithm to optimize given conditions for chemical engineering and even biological reactions. It consists of regulated random search (to avoid uneven distribution of the positions to mutate within search spaces), centroid search (sequential simplex less the worst response), and mapping in each cycle (Ogawa et al., 2002). The cycle is repeated until the best response is obtained and therefore, selection of the new, narrowed search space derived from the maps to be used in the subsequent cycle is most critical in finding the global optimum. This approach, when applied to a variety of mathematical models, has been successful in finding the global optimums mostly within 50 interactions (Ogawa et al., 2002). The RCO has proven to be successful for optimizing broadly both chemical and engineering, and even biological reactions (Nakai, 1998). Successful application of RCO in complicated multimodal cases relies on 1) regulated random designs to avoid localized designs, 2) centroid designs to search around the best results found during the preceding random search, and 3) trend curves in mapping directing toward the global optimum. Saito (2003) applied RCO to determine the optimum conditions of the emulsifying activity of soy protein isolate and concluded that RCO method was suitable for the optimization of food processing conditions with a minimum number of measurements.

The objective of this study was to use the multifactor program of random centroid optimization for lutein enriched o/w emulsion formulation and try to optimize and find the best conditions for preparing a stable emulsion.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Whey protein isolate powder (WPI) was supplied by Davisco International (Le Sueur, MN). The certificate of analysis was as follows: protein: 97.5 %, ash: 2.1 %, and moisture: 5.0 %. This adds up to 105 %. L- α -Phosphatidyl-DL-Glycerol was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Corn oil was obtained from a local store and used without further purification. Distilled water was used to prepare the aqueous phase of emulsions. A lutein standard (FloraGlo) was obtained from Kemin Foods (Des Moines, IA). The HPLC column (YMC C₃₀) was a product of Waters (Mildford, MA). All reagents were either HPLC grade or reagent grade.

3.2 Emulsion Preparation

All procedures were carried out at ambient temperature (23°C). A 2 % (w/w) WPI solution was prepared by dissolving the powdered protein in distilled water. Corn oil-in-water emulsions were prepared by homogenizing weighed amounts of oil (20 % w/w) and aqueous phase (80% w/w). A coarse emulsion premix was prepared using a high-speed blender (Waring Commercial, Model 33BL79, New Hartword, Connecticut). The lutein (0.15 % w/w) and phosphatidylglycerol (0.083 % w/w) was added to the coarse emulsion. The WPI solution and coarse emulsion premix were mixed and stirred, using a magnetic stirrer for two hours. The ionic strength of the aqueous phase was then adjusted by adding potassium chloride (100 mM) to the emulsions. The emulsion was adjusted to the desired pH using either NaOH or HCl at concentration of 0.1M or 1M, respectively. The emulsion was homogenized and passed 2 times through high-pressure valve homogenizer (Fort Wayne, Indiana) for 10 min at 1725 rpm and

2000 psi to reduce the droplet size. The emulsion was moved to a container and stirred for 30 minutes. The final concentration of oil in all emulsions was kept constant (19.6 % w/w) by adding different amounts of distilled water, so that the total mass of added salt+acid/alkali + water was constant. During visual observation the following ratings was used for explaining the conditions of emulsions after applying different treatments: 1-liquid emulsion, 2-low viscosity emulsion, 3A-moderate viscosity emulsion, 3B-high viscosity emulsion, 4-gel. An emulsion sample containing all of the ingredients except the lutein was prepared under similar conditions. Emulsions were stored at 4°C from one to four days.

Using the same ingredients and same procedure, small scale (60 g). Emulsion samples were prepared for the RCO program. pH of these emulsions was in the range of 3.8 - 4.8, because all salad dressing emulsions are in the acidic range of pH. Lutein concentration was chosen from 8 to 10 mg, based on daily recommended intake of lutein, which is in the range from 6 to 10 mg (Seddon et al., 1994). Ionic strength was chosen from 80 – 100 mM potassium chloride respectively. According to Demetriades (1999), ionic strength in this range could give greater stability to o/w emulsions. Phosphatidylglycerol was chosen in the range of 9 – 10 mg, based on comparison with another emulsifier, Tween 20, which is used in the range from 0.05 % w/w to 0.5 % w/w (Dickinson, 1999).

3.3 Optimization of Emulsion Stability Using RCO Technique

The computer program for the RCO of Nakai, (1990) was used for finding the optimum conditions to prepare stable oil-in-water emulsions. Four variables with the following formulation conditions pH (3.8 - 4.8), lutein (8 – 10 mg), KCl (670 – 770 mg) and phosphatidylglycerol (10 – 90 mg) were used to enter into RCO program for random design

search. Random design algorithm using deterministic rule was used (by RCO program) to obtain uniform distribution of experimental points.

After obtaining the four most suitable formulations out of nine experiments, the second part of RCO program, centroid search, was used. These four formulations along with nine preceding formulations were entered into RCO program. The centroid search gave the next four most suitable formulations.

After carrying out centroid search experiment, the four most suitable formulations out of thirteen formulations were obtained and the third part of RCO program, which consist of mapping, was used. Mapping gave the final formulation for stable emulsion preparation, which was confirmed by checking half life stability of emulsion and conducting zeta potential and size distribution measurements

3.4 Measurement of Emulsifying Properties

Emulsifying activity and emulsion stability for large scale (150 g) emulsions were estimated according to the modified method of Pearce and Kinsella (1978). Five milliliters of the prepared emulsion was homogenized in a Tissumizer (Tekmar Company, West Germany) at 12,000 rpm for 1 min at 20 °C. Aliquots of 30 µl of emulsion were taken from the bottom of the test tube after standing for 0, 1, 2, 3, 5, 10, 15 and 20 min. Each aliquot was immediately diluted to 5.0 ml with 0.1% sodium dodecyl sulfate (SDS) solution. The absorbance of the diluted emulsion was measured at 500 nm as the emulsifying activity. The emulsion stability was estimated by measuring the half time of the turbidity detected immediately after the emulsion had formed.

3.5 Emulsion Stability Index (ESI) Measurement

A buffer of 10mM imidazole-HCl (pH 7) containing 0.1% SDS was prepared. Within 30 minutes, 30 μ l of the freshly prepared emulsion was taken from three different locations. A disposable polyethylene transfer pipet was used to gently swirl the three 10 μ l aliquots in the 30 ml of dilution buffer. Absorbance was measured at 500 nm. The emulsion was then held at 4 °C for 24 h and reanalyzed. Emulsion stability index was defined, using the formula suggested by Kinsella (1978).

$$ESI = (T \times \Delta t) / \Delta T \quad (\text{Eq. 1})$$

where T is turbidity value at 0 h, ΔT is change in turbidity during 24 h period, Δt is time interval (24 h).

T is defined using the following formula $T = 2.303 \times A/l$ where A is the observed absorbance and l is the pathlength of the cuvette.

3.6 Determination of Particle Size Distribution and Zeta Potential

The weight-average diameters ($d_{4,3}$) of the emulsion droplets, their size distribution (volume fraction as a function of particle size) and zeta potential were determined using a Zetasizer (Malvern Instruments Ltd., Malvern, U.K) and software supplied with the instrument. The sample under investigation is contained within an electrophoresis cell. A known field is applied and the sample illuminated by crossed focused laser beams. Particles moving through the measurement volume scatter light. The intensity of the light fluctuates with a frequency proportional to the velocity of the particles. The velocity is calculated from the measured frequency then expressed as mobility by dividing by the applied field. and converted to zeta potential.

Deionized water was used to disperse the emulsion, with a dilution factor of about 1:400. Ten microliters of emulsions samples were diluted into 4 ml deionized water. Three ml of the diluted solution was transferred into plastic cuvettes. Each cuvette was put into the zetasizer to measure particle size distribution and zeta potential.

3.7 Heat Stability of Emulsions

After preparation of preliminary emulsions, 20 ml of each sample was put in the glass tubes and placed in a water bath at 30, 75 and 90 °C for 30 minutes. After heat treatment, qualitative observation Describe the qualitative evaluation process was carried out. For RCO experiments, 20 ml of each emulsion sample was again put in glass tubes and placed in an oven at 90 °C for 5 minutes. After 5 minutes each sample was analyzed for droplet size distribution, zeta potential measurements and emulsion stability (ESI) index.

3.8 Stability of Lutein in the Emulsion Sample

Two gram of final emulsion was extracted with 80 ml of acetone, in screw-capped containers, at room temperature in the dark for 3 days. The acetone extracts were filtered through Whatman No. 4 filter paper and evaporated under vacuum. The residuals were saponified with 200 ml of 10% KOH with gentle shaking overnight. The saponified solution was extracted with ether: hexane (1:1) mixture, using a separatory funnel. The upper level containing ether/hexane solution and lutein was recovered; the solvent was evaporated using a Buchi Rotavapor R-200 evaporator (Brinkman Instruments, Inc., Westbury, N.Y.) The residue was dissolved in 20 ml of MTBE/methanol (5:95) mixture, filtered through 0.45 µm filter and 20 µl were injected into an YMC C₃₀ carotenoid S 3µ, 4.6 x 250 mm HPLC column. The HPLC separation was carried using a Waters Model 600E solvent delivery system fitted with a model 717A plus autosampler, a Model 486 tunable absorbance detector and Millenium 32 chromatography manager with data

processor (Milford, MA). The flow rate was 1 ml/min, detection was 450nm, and separation was isocratic using MTBE: methanol (5:95) as the mobile phase, with a total separation time of 35 min.

Peaks on a chromatogram were identified by comparing their retention times and spectra with those of lutein standards (all-trans) and by comparison of the absorbance maxima with those in the literature. All-trans-lutein was quantified by using a regression equation obtained from the calibration curve. Percentage of lutein recovered was determined.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Qualitative Observation of Lutein Enriched Emulsions

Preliminary experiments were performed to observe visually the appearance and texture of the emulsion at different pH, ionic strength and heat treatment at 30, 75 and 90 °C for 30 min. The temperature region mentioned was chosen to check emulsion stability under different conditions.

It is known that food emulsions are subjected to heat treatment to extend the microbiological shelf-life of the product. The results showing the appearance of o/w emulsion at different pH and ionic strength after heating are summarized in Table 3.

The experiments showed that separate incorporation of lutein or together with PGL increased the viscosity of emulsions, remaining stable up to 90 °C at pH = 4.5. The visual appearance and texture of the test tubes at pH = 4.5 were almost the same after 2 - 3 days storage at room temperature.

Table 3 Appearance of o/w emulsions at different pH and ionic strength after heating to 30, 75 and 90 °C for 30min.

Ingredients*	pH	30°C		75°C		90°C	
		0mM	100mM	0mM	100mM	0mM	100mM
O/W	3.5	1	1	1	1	1	1
	4.5	2	2	3A	3B	3B	3B
	5.5	1	1	1	3A	1	3A
O/W+lutein	3.5	1	1	1	1	1	1
	4.5	3A	3B	3B	3B	3B	4
	5.5	1	1	3A	3B	3A	3B
O/W+L+PGL	3.5	1	3A	1	3A	1	1
	4.5	3A	3B	3B	3B	3B	3B
	5.5	1	3A	3A	3A	3A	3B

* - Corn oil in water emulsion (~20 %) stabilized with 2 % w/w WPI in presence of either lutein-L (0.055 % w/w) or with surface active emulsifier posphatidyl glycerol-PGL (0.018 % w/w).

The abbreviations are: 1 – liquid, 2-low viscosity, 3A-moderate viscosity, 3B-high viscosity, 4-gel. Ionic strength was kept by adding KCl at 100 mM. The high viscosity emulsions containing lutein and phosphatidylglycerol at pH = 4.5 were converted to weak gel texture after heating in autoclave at 120 °C. In general, the range of pH over which emulsions had high viscosity increased as KCl concentration and temperature increased. These results were almost similar depending on daily concentration of lutein from 0.015 % (equal to the serving size of 6 mg) to 0.055 %.

4.2 Optimization of Emulsion Preparation Using RCO Technique

The RCO technique was used to determine the optimal composition for o/w emulsion stability based on visual observations of O/W (corn oil+Whey Protein+Water) emulsion stability with added ingredients Lutein + PGL + KCl.

RCO was applied to the four-factor model. To optimize this model, the mapping process was automated by selecting narrower search spaces for subsequent search cycles to be one-third the size of search spaces of the previous search cycle around the most suitable response values. The optimum was designed by using the following formulation conditions: pH (3.8 - 4.8), lutein (8 - 10 mg), KCl (670 - 770 mg) and phosphatidylglycerol (10 - 90 mg). The composition of four factors was simultaneously optimized. Each experiment was carried out using turbidity measurements. Half-life stability for each experiment was expressed as time (in minutes), when the optimal density for each experiment decreased by half. The highest half-life stability of emulsions was sought by random and centroid searches (Table 4).

Table 4. Random and Centroid search results to optimize half-life stability of WP based emulsions in the presence of ingredients Lutein + PGL + KCl.

Variables	Time(mean)*	Standard Deviation
Cycle 1, random search		
1)pH(4.12)Lutein(9.17mg)KCl(.76g)PGL(.04g)	3.45	±0.47
2)pH(4.78)Lutein(9.33mg)KCl(.71g)PGL(.06g)	12.64	±2.98
3)pH(4.56)Lutein(8.33mg)KCl(.72g)PGL(.06g)	9.15	±1.93
4)pH(3.95)Lutein(9.87mg)KCl(.71g)PGL(.08g)	2.12	±0.77
5)pH(4.77)Lutein(9.77mg)KCl(.74g)PGL(.09g)	2.32	±1.24
6)pH(4.59)Lutein(9.54mg)KCl(.74g)PGL(.01g)	7.31	±0.86
7)pH(3.86)Lutein(8.24mg)KCl(.67g)PGL(.08g)	1.61	0
8)pH(4.06)Lutein(8.13mg)KCl(.71g)PGL(.04g)	2.17	±0.29
9)pH(4.72)Lutein(9mg)KCl(.71g)PGL(.06g)	12.77	±2.83
Cycle 1, centroid search		
1)pH(4.66)Lutein(9.05mg)KCl(.72g)PGL(.05g)	17.03	±2.05
2)pH(4.55)Lutein(8.96mg)KCl(.73g)PGL(.05g)	17.56	±6.37
3)pH(4.55)Lutein(9.26mg)KCl(.73g)PGL(.04g)	16.1	±1.3
4)pH(4.5)Lutein(9.01mg)KCl(.73g)PGL(.04g)	16.2	±6.23

*- Half-life stability of the emulsions (in minutes).

In each cycle, centroid search was carried out based on experimental results of the preceding random search. The most suitable results for half-life stability of emulsions were obtained from number 2, 3, 6, 9 formulations. The centroid search yielded greater performance for half-life stability of emulsions compared to the random search. Higher stability for emulsions was obtained in centroid search formulation, after entering number 2, 3, 6, 9 formulations into RCO program. The maximal stability was observed in number two formulation of centroid search. To narrow down the formulations of four factors, the mapping step was applied. After a total of nine experiments in random search and four experiments in centroid search, the best

values obtained for the following four factors: pH = 4.55, lutein = 8.96 mg, KCl = 730 mg and phosphatidylglycerol=50 mg. Examples of the maps drawn after 13 experiments in cycle 1 are illustrated in Figure 2 (A, B, C, D). The arrows that appeared at the bottom of the maps show the assigned locations of the optimum, which were used for computing the approximated slope curves of the response surface.

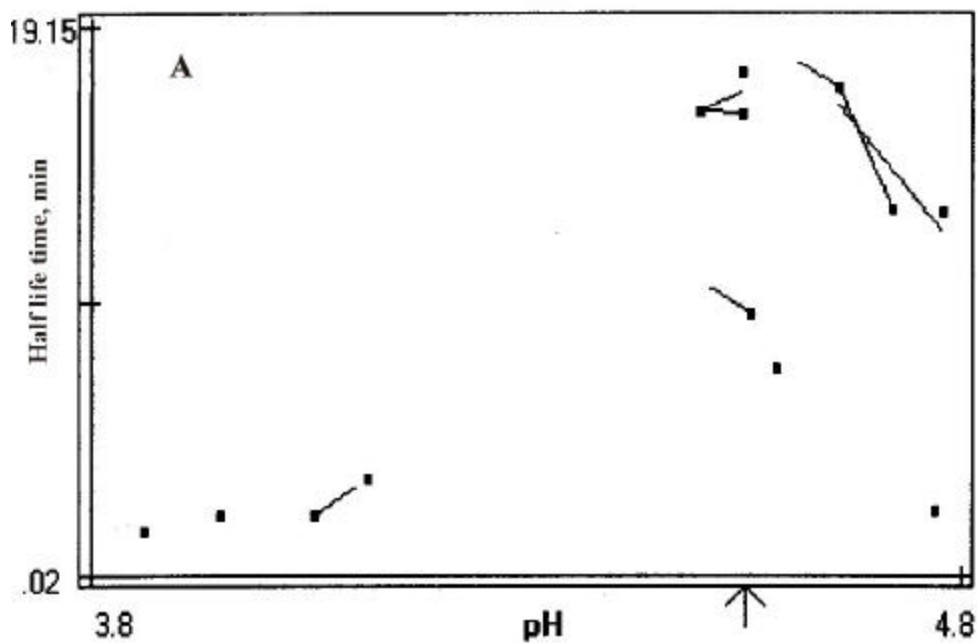
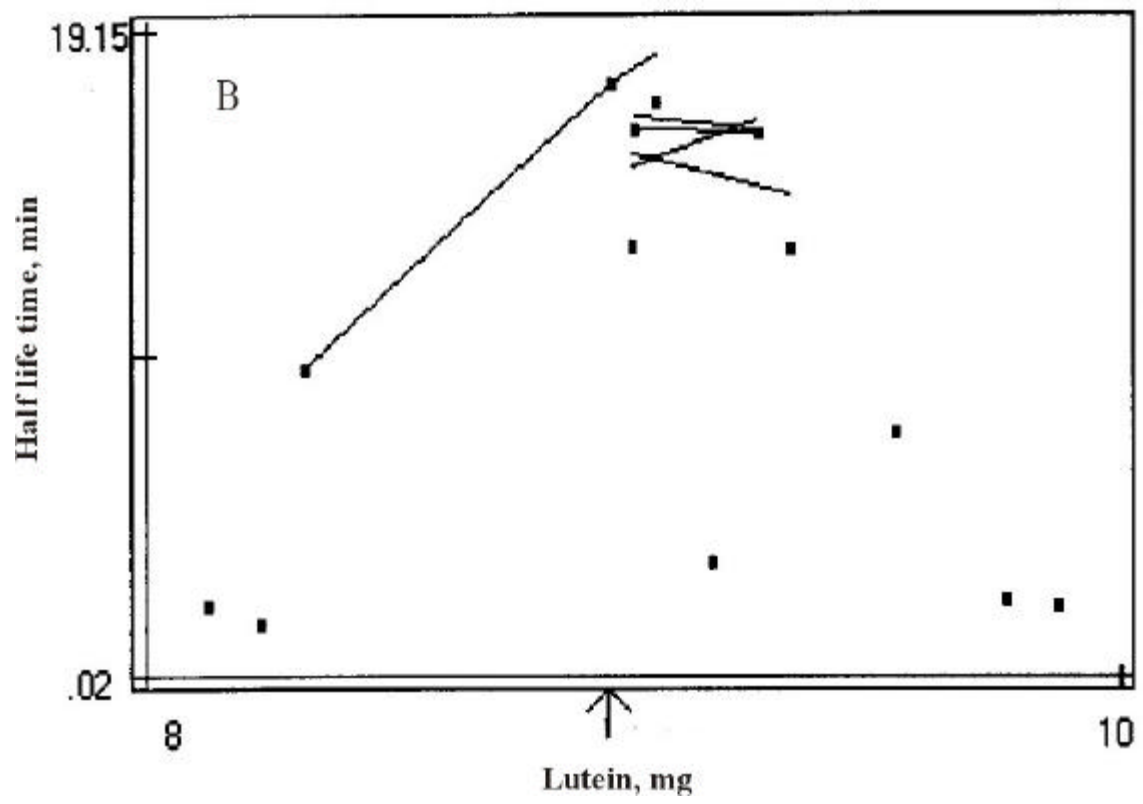
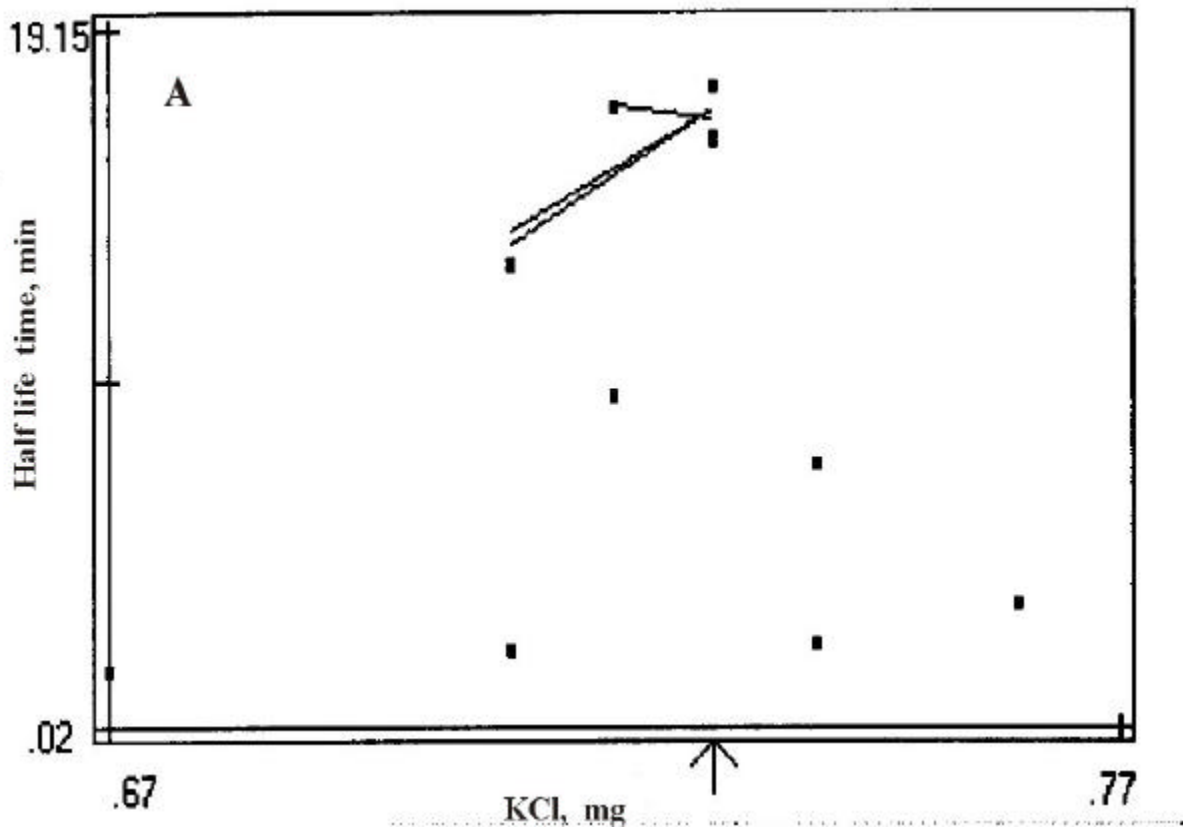


Figure 2 (A, B, C, D). Mapping results of emulsions stability drawn by RCO program (fig. cont.)

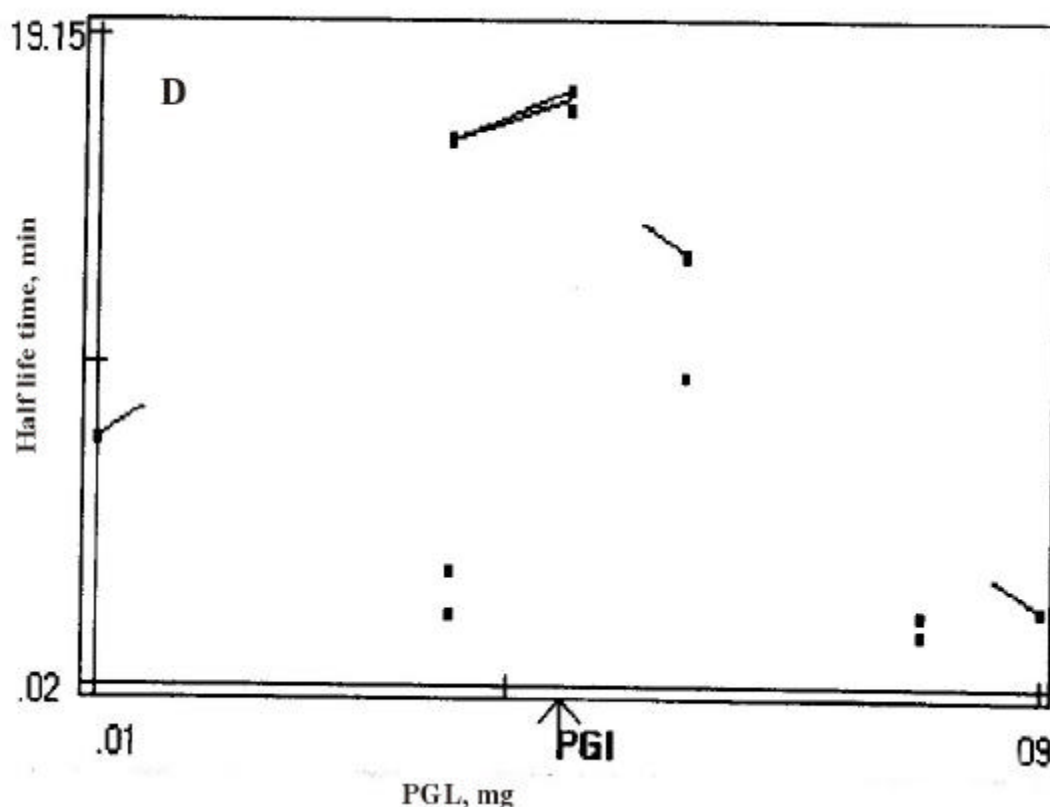
A) Turbidity measurements as half-life time of emulsion vs.pH



B) Turbidity measurements as half-life time of emulsion vs. lutein concentration



C) Turbidity measurements as half-life time of emulsion vs. KCl concentration



D) Stability of emulsion as half-life time of emulsion vs. phosphatidylglycerol (PGL) concentration

These four graphs show more clearly the most appropriate values of emulsion constituents that were obtained from the Random Centroid Optimization design. As it can be seen from figure 2 (A, B, C, D) the most stable emulsion could be formulated using pH = 4.55 (Figure 2A), lutein = 8.96 mg (Figure 2B), KCl = 730 mg (Figure 2C) and phosphatidylglycerol = 50 mg (Figure 2D).

Based on RCO results the following graphs (Fig. 3, A, B, C and D) show the dependence of emulsion stability (as half-life time of emulsions, min) on of pH (A), the concentrations of lutein (B), phosphatidylglycerol (C) and KCl (D).

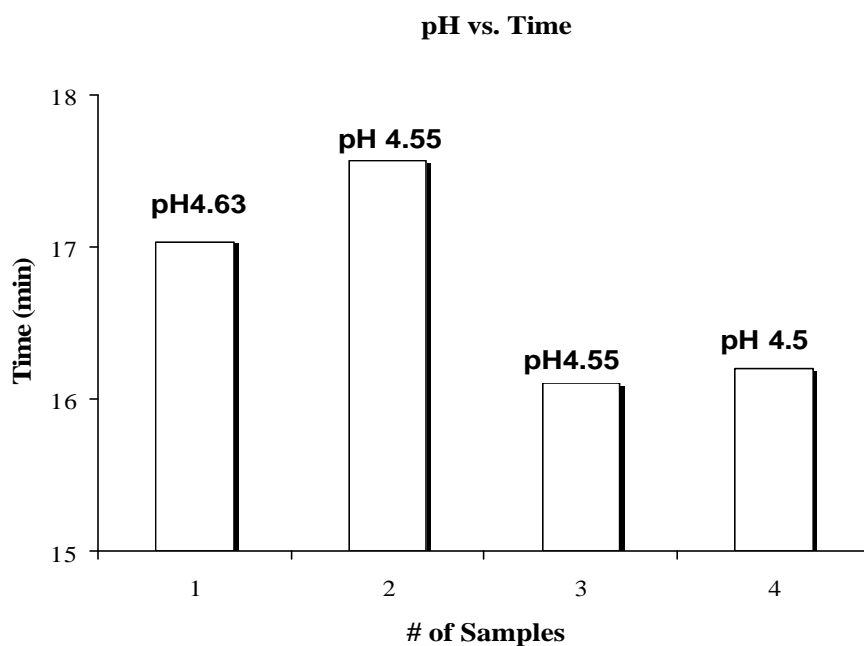
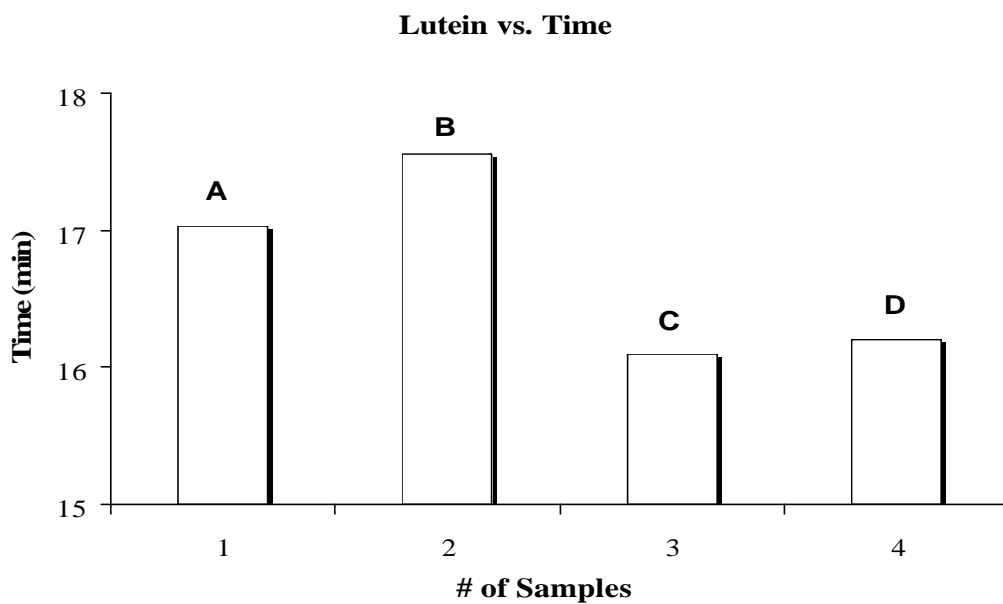


Figure 3 (A, B, C, D)

A) Half life stability of emulsions at different pH during centroid optimization cycle



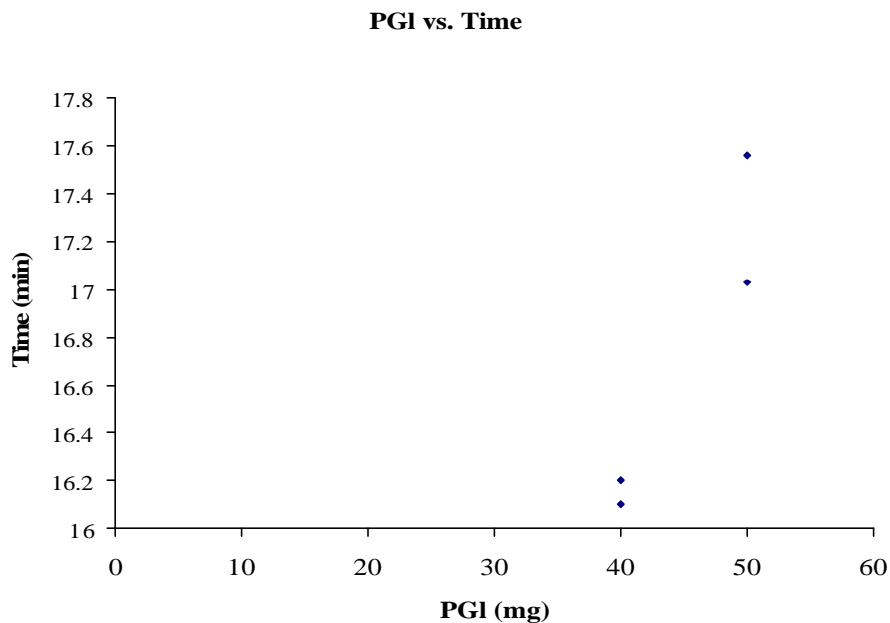
A - Lutein (9.05 mg), KCl (720 mg), pH 4.66, PGI (40mg)

B - Lutein (8.96 mg), KCl (730 mg), pH 4.55, PGI (50mg)

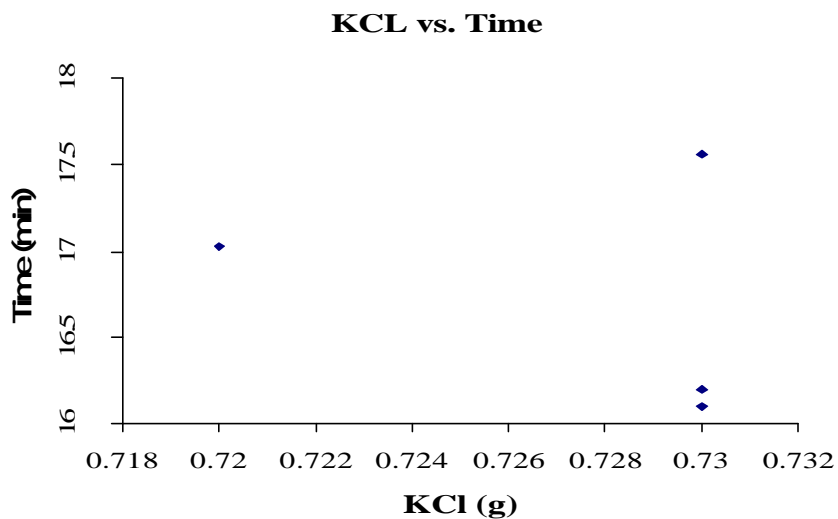
C - Lutein (9.26 mg), KCl (730 mg), pH 4.55, PGI (40mg)

D - Lutein (9.01 mg), KCl (730 mg), pH 4.5, PGI (40mg)

B) Half life time stability of emulsions at different lutein concentrations during centroid optimization cycle



C) Half life time stability of emulsions at different PGI concentrations during a centroid optimization cycle



D) Half life time stability of emulsions at different KCl concentrations during a centroid optimization cycle

Figures 3 (A, B, C, D) showed that the longest half-life stability of emulsion was observed with the combination of four optimized factors: pH = 4.55 (Figure 3A), lutein 8.96 mg

(Figure 3B), phosphatidylglycerol=50 mg (Figure 3C) and potassium chloride = 12.16 mg/ml (Figure 3D).The emulsion prepared under these conditions gave 17.6 min. of half-life stability.

The turbidity measurements in Figure 4 show the half-life stability of emulsion (large scale) using the most appropriate values for four factors obtained from RCO program.

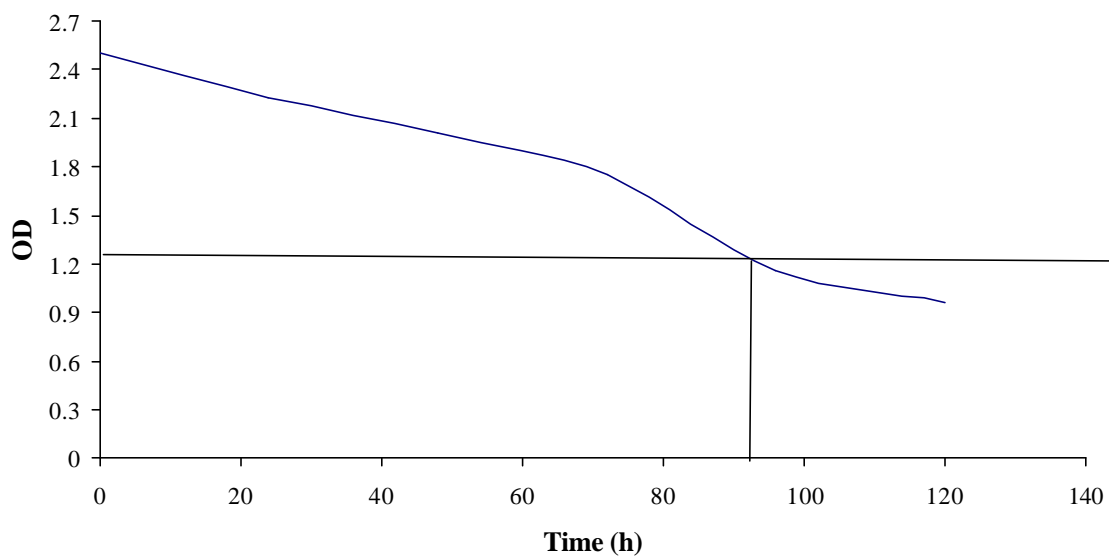


Figure 4 Half-life stability of emulsions (large scale)

The turbidity of emulsion is plotted as the ordinate and standing time after emulsion formation as the abscissa. The value of ordinate at zero time is the relative emulsifying activity, and the half life of initial turbidity reflects the stability of the emulsion. Using formulation obtained from RCO experiments, the half-life stability of large scaled emulsion was ~92 hr.

4.3 Emulsion Stability Index (ESI)

Emulsion stability Index of WPI stabilized emulsion was defined using equation #1

$ESI = (T \times \Delta t) / \Delta T$, presented in section (3.5), where ΔT is the change in turbidity, T, occurring during the time interval Δt .

$$T(0 \text{ h}) = 2.303 \times A/I = 2.303 \times 2.510/1 = 5.78$$

$$T (24 \text{ h}) = 2.303 \times 1.736 / 1 = 3.99$$

$$\text{ESI} = (5.78 \times 24) / (5.78 - 3.99) = 77.5 \text{ h}$$

The ESI value for the optimized emulsion was 77.5 h. The ESI refers to length of time protein is able to maintain a stable emulsion. Different ESI values are reported in the literature, from 25.0 h (for sodium caseinate), 28.0 h (for WPI), 52.0 h (for soy protein isolate) to 294.0 h (for deamidated wheat protein isolate) (Webb et al., 2002). According to Mangino (1994) the increase in apparent viscosity could contribute to the high emulsifying stability of the emulsions. An increased apparent viscosity of the continuous phase will reduce the rate of creaming resulting in a more stable emulsion. The high apparent viscosity of protein solutions can be a result of the high MW fraction, the size of the proteins, the solvent protein interactions and protein-protein interactions (Damodaran, 1996).

It can be seen that, based on Random Centroid Optimization technique, it was possible to obtain experimentally appropriate ESI values for optimized emulsions. In fact, optimized and whey protein based emulsions, because of increased viscosity, gave higher ESI value in comparison with sodium caseinate and soy protein isolate-based emulsions (Webb et al., 2002).

4.4 Zeta Potential (ZP) as Stability Characteristic of Emulsions

In Table 5 the ZP and size distribution measurements of emulsion prepared by RCO recommendation are summarized. It is evident based on zeta potential and size measurement that emulsions prepared at different conditions had adequate stability. One day old emulsions and emulsions after shaking for 30 min. had almost the same trend with ZP values of -50.5 mV and -50.6 mV in comparison with fresh measurements, -52.4 and -53 mV, respectively. The heating effect was also not notable despite of time (5 min at 90 °C) and gave -0.9 millivolt reduction compared with fresh emulsion values.

Table 5 Zeta Potential values and droplet size distribution of optimized emulsions under different treatments.

pH	Condition	ZP(milivolts)	Size (μm)
4.55	Fresh	-53.0	0.73
	90°C for 5min.	-52.1	0.75
	700rpm for 30min.	-50.6	-
	24 h storage	-	1.19
3.8	Fresh	-52.4	0.43
	24 h storage	-50.5	0.65

4.5 Size Distribution as Stability Characteristic of Emulsions

According to Demetriades (1997) the preferred particle size distribution for stable emulsions is in the range of 0.8 to 1 μm . The particle size distribution according the ZP measurements is presented in Table 5. A Twenty-four hour storage of emulsion leads to a little agglomeration from 0.73 to 1.19 μm at pH = 4.55 and from 0.43 to 0.65 μm at pH = 3.8. Heat treatment had almost no affect on particle size, varying from 0.73 to 0.75 μm

Although the emulsion at pH = 3.8 showed adequate stability (ZP = -52.4) and acceptable droplet size (0.43 μm) in any case the emulsion at pH = 4.55 was preferable and most reliable because the droplet size (0.73 - 0.75 μm) and ZP fall into the preferred range as suggested by Demetriades (1997), and van Nieuwenhuyzen and Szuhaj (1998).

The spatial size distribution of emulsions presented in Fig.5 shows more clearly the size distribution of optimized emulsion after different treatments.

It can be seen that freshly prepared emulsion had narrow spatial size distribution. All emulsions usually flocculated around the isoelectric point of WPI (pH ~ 4.9). Proteins have little net charge around the isoelectric point and therefore electrostatic repulsion does not prevent flocculation (Demetriades et al, 1997).

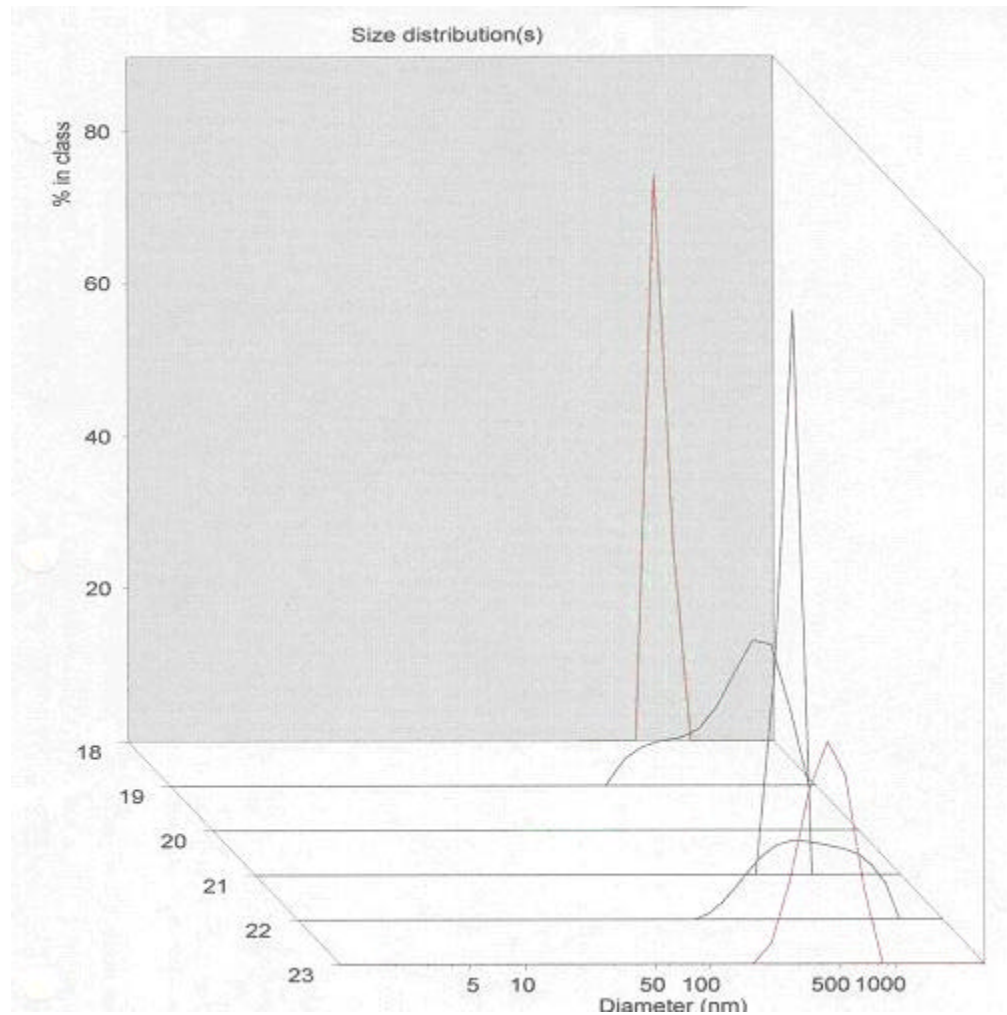


Fig. 5 Droplet size intensity dependence on Zeta potential values

The z - axis is the sample treatment and is labeled as follows: 18 –Fresh, pH=3.8, 19-One day old, pH=4.5, 20-After shaking, 21-After heating, pH=4.55. 22-Fresh, pH=3.8, 23-One day old, pH=3.8

At pH higher or lower than isoelectric point, the protein molecules had a net charge that was adequate to prevent droplets from aggregating. Since optimized fresh emulsion had a pH value of 4.55 it showed a very narrow range of size distribution.

The heat treatment also had almost no effect on size distribution. This may be because at higher temperature the protein became fully unfolded and more flexible so they rearranged and non-polar groups were directed away from the aqueous phase. Hence the droplets had lower surface hydrophobicity and were less susceptible for aggregation.

After one day of storage the aggregation of droplets increased, and as a consequence the spatial size distribution of emulsion was getting broader. The same broad peak appeared at pH 3.8 for both fresh and one day old emulsions. It is relevant to note that based on RCO data the emulsions at pH 3.8 were not considered reliable in comparison with emulsions at pH 4.55.

4.6 The Recovery of Lutein from Emulsions

The percentage of lutein was determined to show whether lutein is degraded in established emulsions. The standard HPLC technique was used to follow the behavior of lutein in different conditions.

A brief comparison of spectra in Figures 6-11 shows that with recovered lutein an unknown intense peak appears at retention time ~3.75 and weak peaks at retention times about 7, 9, 11, 12.5 and 19 min., respectively. For the identification of those peaks pure corn oil and oil-in-water emulsions were extracted and analyzed under the same HPLC conditions. The results are presented in Figures 12-16.

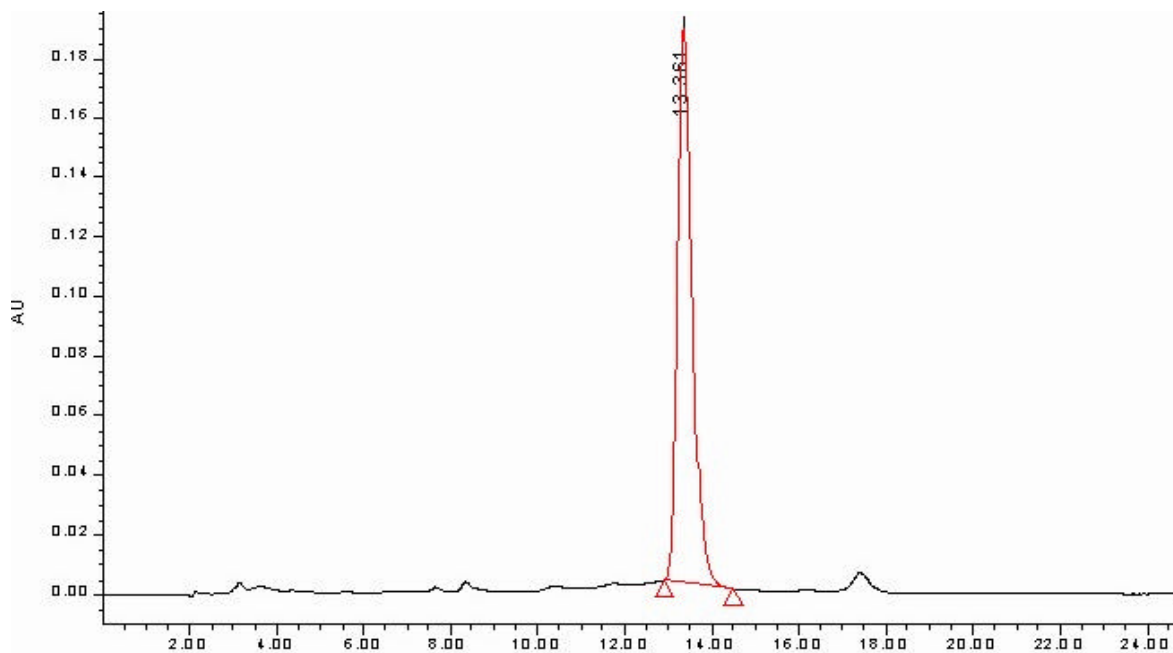


Fig. 6 HPLC chromatogram of 200 ppm lutein in MTBE/Methanol (5:95) solution, elution was performed with an YMC C₃₀ column, at flow rate of 1ml/min, temperature 18 °C. The injection volume was 20 µl, the detection was at 450 nm, and total running time was 30 min. Retention time was 13.361 min.

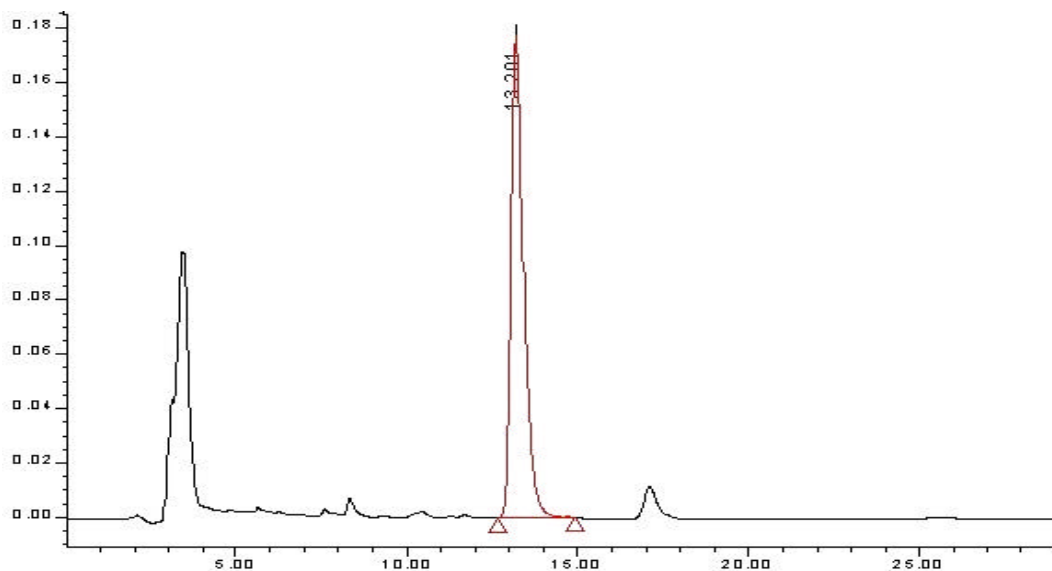


Fig. 7 Lutein recovery from 10mg lutein and corn oil mixture. Elution was performed with an YMC C₃₀ column, at flow rate of 1ml/min, temperature 18 °C. The injection volume was 20µl, the detection was at 450 nm, and total running time was 30 min. Retention time was 13.201 min.

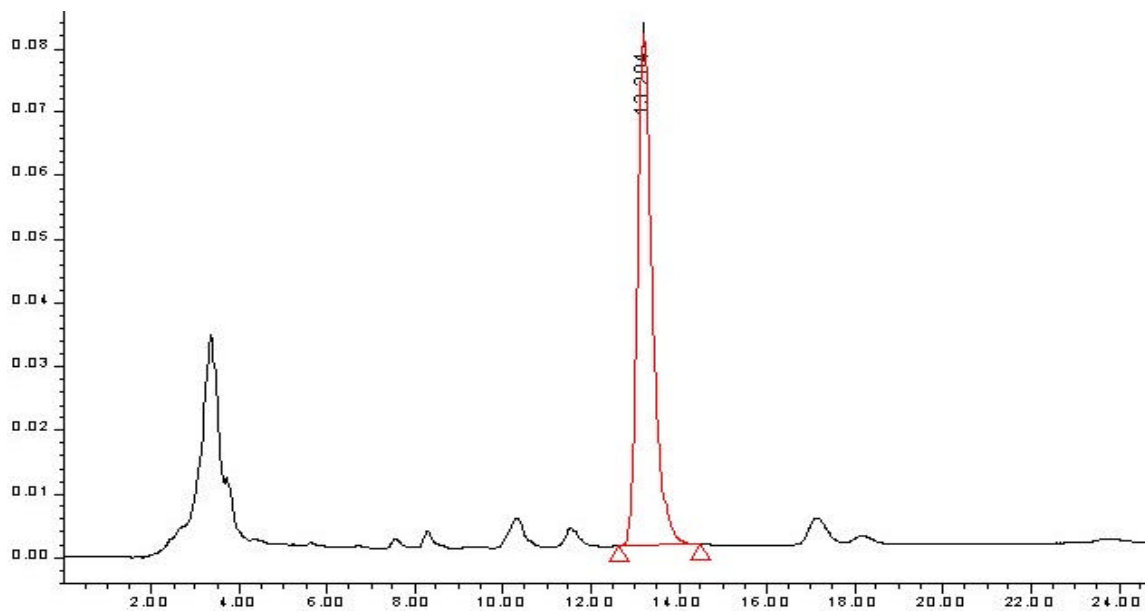


Fig. 8 Lutein recovery from fresh emulsion. Elution was performed with an YMC C₃₀ column, at flow rate of 1ml/min, temperature 18 °C. The injection volume was 20 µl, the detection was at 450 nm, and total running time was 30 min. Retention time was 13.204 min.

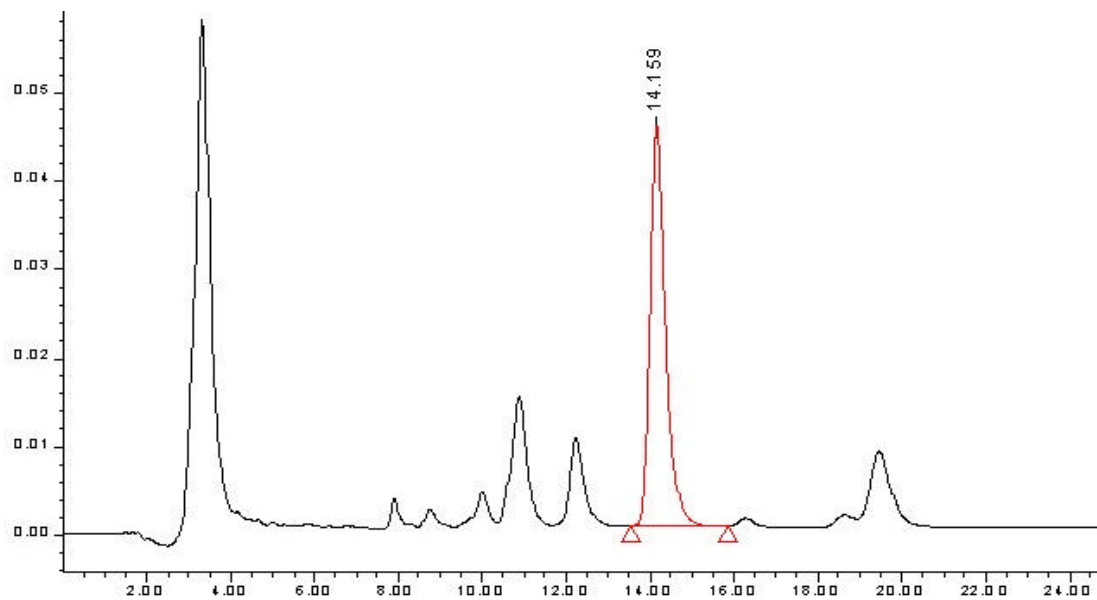


Fig. 9 Lutein recovery from one day old emulsion. Elution was performed with an YMC C₃₀ column, at flow rate of 1ml/min, temperature 18 °C. The injection volume was 20 µl, the detection was at 450 nm, and total running time was 30 min. Retention time was 14.159 min.

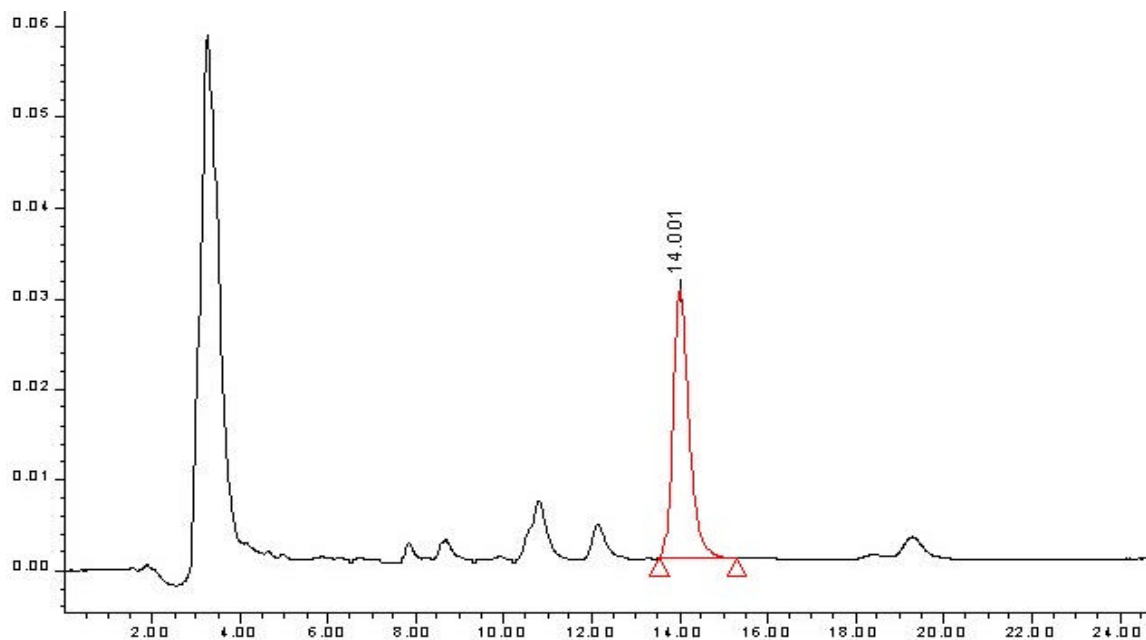


Fig. 10 Lutein recovery from fresh emulsion after heat treatment. Elution was performed with an YMC C₃₀ column, at flow rate of 1ml/min, temperature 18 °C. The injection volume was 20 µl, the detection was at 450 nm, and total running time was 30 min. Retention time was 14.001 min.

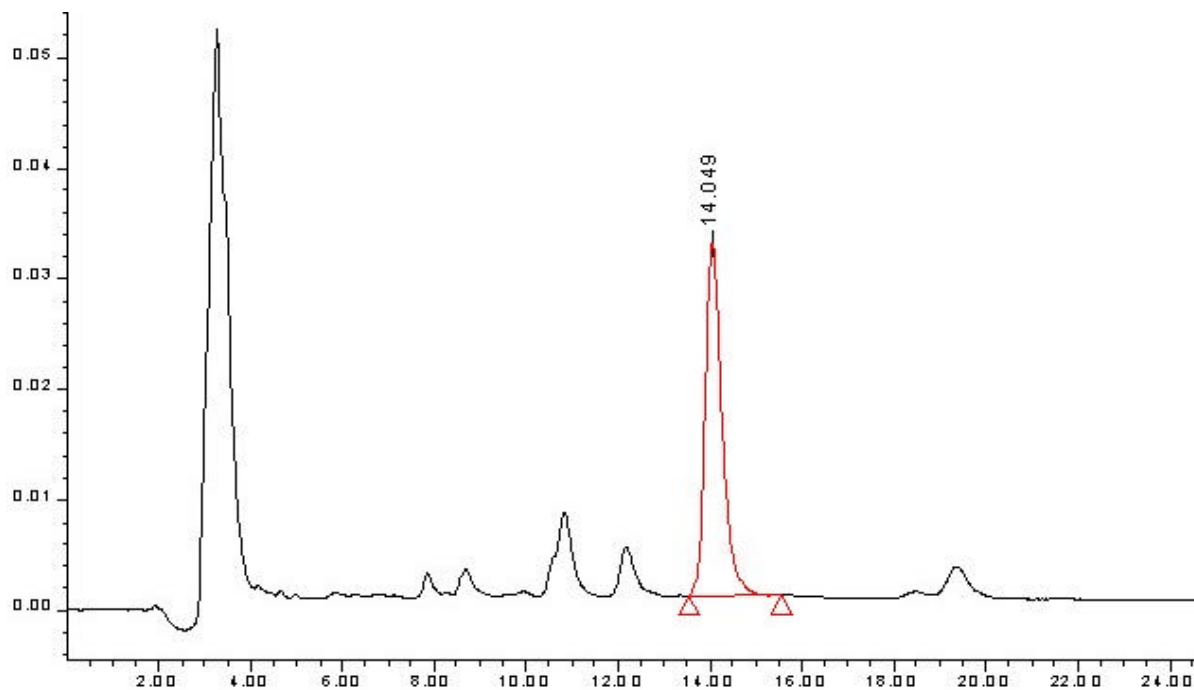


Fig. 11 Lutein recovery from fresh emulsion after shaking. Elution was performed with an YMC C₃₀ column, at flow rate of 1ml/min, temperature 18 °C. The injection volume was 20 µl, the detection was at 450 nm, and total running time was 30 min. Retention time was 14.049 min.

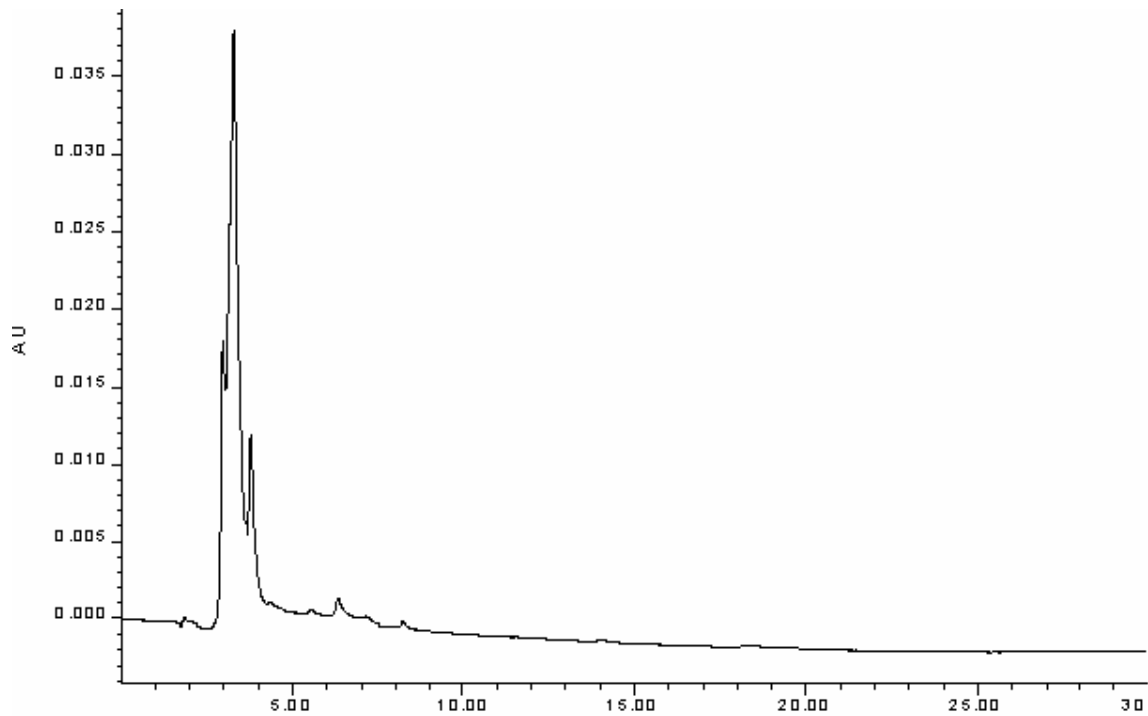


Fig. 12 HPLC chromatogram of saponified corn oil used in o/w emulsion. Elution was performed with YMC C₃₀ column, at flow rate of 1ml/min, temperature 18 °C. The injection volume was 20 µl, the detection was at 450 nm, and total running time was 30 min. Retention time was 3.75 min.

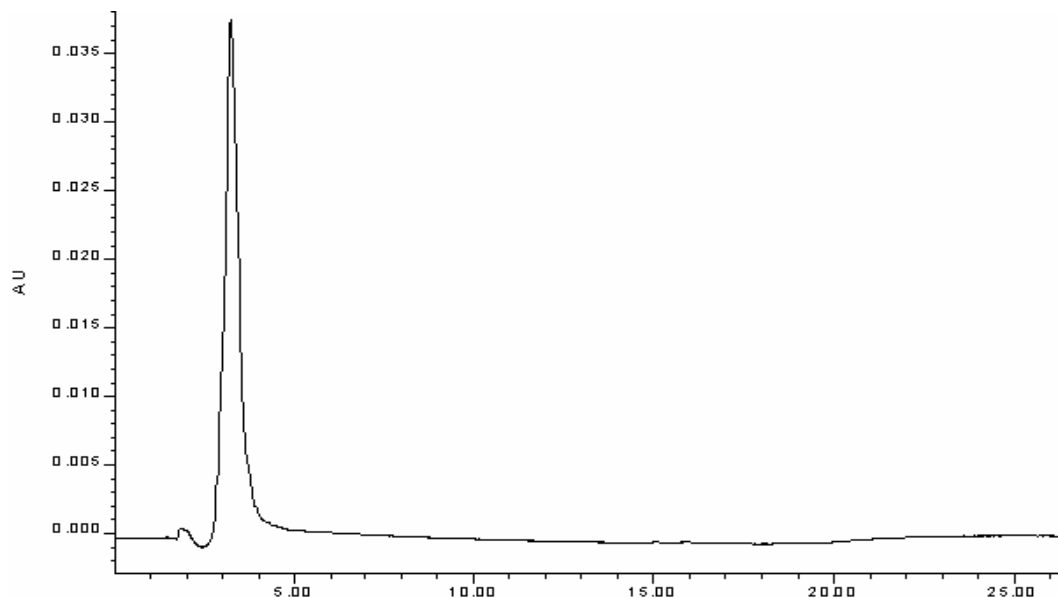


Fig. 13 HPLC chromatogram of fresh o/w emulsion (without lutein). Elution was performed with an YMC C₃₀ column, at flow rate of 1ml/min, temperature 18 °C. The injection volume was 20 μ l, the detection was at 450 nm, and total running time was 30 min. Retention time was 3.75 min.

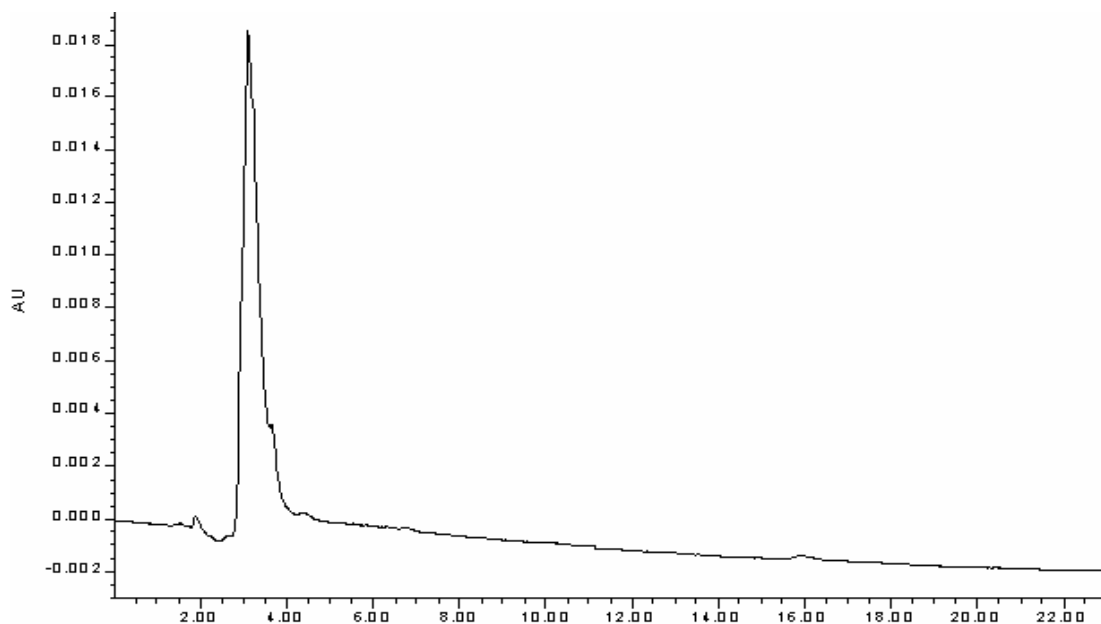


Fig. 14 HPLC chromatogram of one day old o/w emulsion (without lutein). Elution was performed with an YMC C₃₀ column, at flow rate of 1ml/min, temperature 18 °C. The injection volume was 20 μ l, the detection was at 450 nm, and total running time was 30 min. Retention time was 3.75 min.

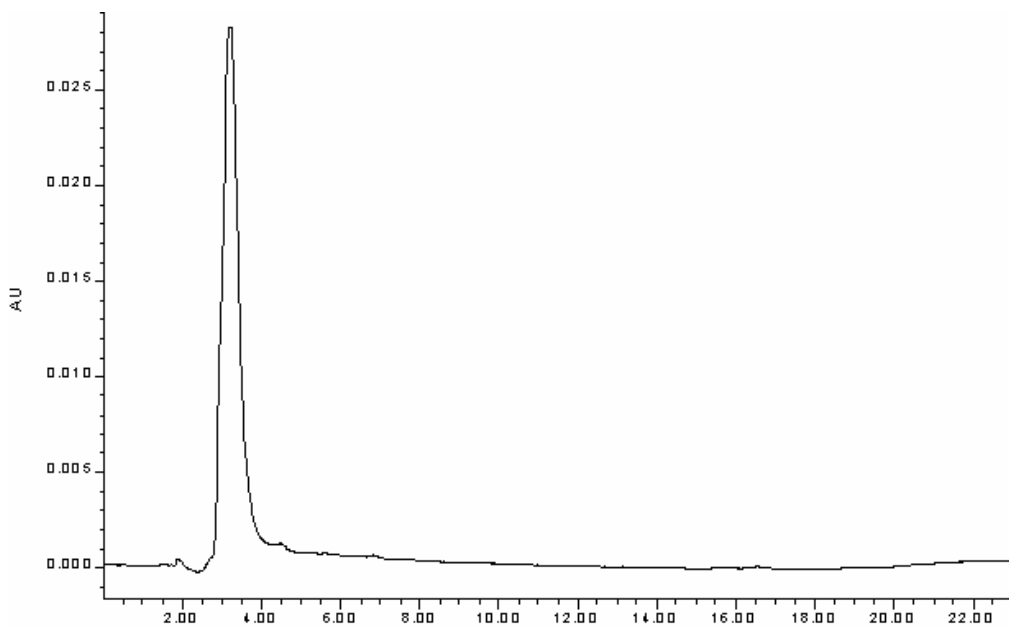


Fig. 15 HPLC chromatogram of o/w emulsion (without lutein) after shaking. Elution was performed with an YMC C₃₀ column, at flow rate of 1ml/min, temperature 18 °C. The injection volume was 20 µl, the detection was at 450 nm, and total running time was 30 min. Retention time was 3.75 min.

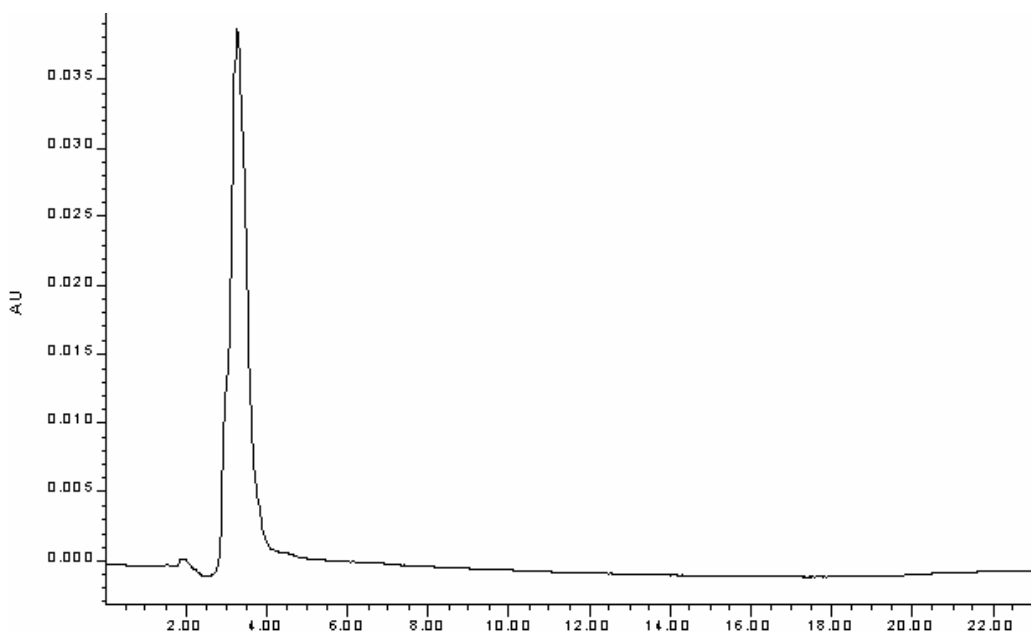


Fig. 16 HPLC chromatogram of o/w emulsion (without lutein) after heat treatment. Elution was performed with an YMC C₃₀ column, at flow rate of 1ml/min, temperature 18 °C. The injection volume was 20 µl, the detection was at 450 nm, and total running time was 30 min. Retention time was 3.75 min.

The results show that the peak that appeared at retention time ~ 3.75 minute was from pure corn oil extract. The retention time was the same for saponified fresh and one day old o/w emulsions (Fig. 13 and 14) as well as for saponified o/w emulsions after shaking and heat treatments (Fig. 15 and 16). The data in Figures 12-16 show that there are no peaks coming out at retention times about 7, 9, 11, 12.5 and 19 min. Therefore it can be concluded that the small peaks are probably the result of lutein degradation. No attempt was made to identify the composition of these unknown peaks. They can be representatives of xanthophyles (for instance all trans neoxanthin, 9'-cis- neoxanthin etc) in presence of lutein, which had been identified by mass spectra from the extract of the green vegetable (Khachik et al., 1986), and in extracts of Tinta Roriz press wine identified by Paula (2001).

The recovery percentages of lutein from fresh, one day old, heated and shaken emulsions were obtained. The data showed that lutein recovery from fresh emulsion was 40.6%, from one day old emulsions it was 31.2%, from heated emulsion it was 21.34% and from shaken emulsion it was 17%. The decreasing recovery percentages of lutein can be explained based on instability of the carotenoid stereoisomers which can be accelerated during aging, heating or shaking processes (Dachtler et al., 1998) and the chromatograms on the Figures 8-11 showed small peaks which are the results of lutein degradation. Shaking has the highest effect on lutein instability, because after shaking treatment the emulsion droplets bound with the lutein molecules were braking down from each other and lutein was more likely to be under light exposure and therefore it might be degraded.

4.7 A Possible Role of Lutein in Stability of Emulsion

Emulsions were prone to flocculation around the isoelectric point of the WP, but they usually are stable at higher or lower pH. Using RCO mathematical approximation and additional

experimental procedures we have found that the pH = 4.55 is optimum to keep very acceptable stability of emulsion containing phosphatidylglycerol, potassium chloride and lutein. Based on well known O/W (corn oil+whey protein+water) emulsion characteristics we were able to incorporate along with phosphatidylglycerol and KCl one more ingredient – lutein. In fact, lutein was not only functional as a compound that can prevent age related macular degeneration, but it also may increase viscosity of emulsion (Table 1)

The role of lutein for establishment of viscous emulsion can be explained by the theory of colloidal interactions. The stability to flocculation could be interpreted in terms of colloidal interactions between droplets, i.e., van der Waals, electrostatic and steric forces.

Because lutein has active terminal hydroxyl groups it can interact with protein by building intermolecular hydrogen bonds. Under specific conditions (proper ionic strength and pH) lutein can interact with WP. According to Bassi (1993), it was confirmed that xanthophyll lutein was able to bind with proteins. Therefore the lutein-protein binding will lead to additional steric stabilization due to generation of a longer hydrophilic portion of protein + lutein adduct. The adduct will cover the droplet surface more efficiently and as a consequence the viscosity of emulsion should be increased, which we have seen experimentally. According to Yanisheva (2001), various antioxidants such as carotenoid can stabilize edible oil based emulsions.

On the other hand, lutein (in general xanthophylls as oxygenated hydrocarbon derivatives) has the alternating double and single bonds that form the central part of the molecule. This constitutes a conjugated system in which the π -electrons are effectively delocalized over the entire length of the polyene chain. It is this feature that gives lutein chemical reactivity and light absorbing properties. Thus, lutein can bond with protein easily by van der Waals forces using flexible π -electrons. It has been suggested that Xanthophylls are able to bind

to a protein (Moros et al., 2002) and as a consequence the viscosity of emulsions should increase. Therefore it can be concluded that lutein has some role in stabilization of emulsions. It has been proven by Dahl (1993), based on visual inspection and lipid globule size distribution, that carotenoids are able to stabilize o/w emulsions.

CHAPTER 5

CONCLUSIONS

During this study a perspective background was established to prepare an o/w emulsion containing at least a serving size of lutein (6 mg or one tablespoon of dressing) as a daily source of lutein.

Based on Random Centroid Optimization experiments, incorporation of lutein in food emulsions in the range from 0.015 %w/w (equal to the daily serving size of 6 mg) to 0.055 %w/w could be formulated. The RCO method gave optimum conditions for the preparation of a stable lutein enriched o/w emulsion, including pH = 4.55, lutein = 8.96 mg, KCl = 730 mg and phosphatidylglycerol = 50 mg.

The stability of emulsions was confirmed by Zeta measurements (ZP~-50, -53 mV) and size distribution between ~0.4 – 1.2 μm . The most suitable stable emulsion was determined by half-life stability measurements ~ 3 days long. The additional ESI measurements were carried out to check the stability index of emulsions defined as high quality of stabilized emulsions.

Experiments on lutein recovery from emulsions were conducted to determine the stability of lutein after subjecting emulsions to different treatments. The data showed a gradual decrease in lutein recovery from fresh emulsion (40.6 % recovery) to one day old (31.2 % recovery), heated (21.34 % recovery) and shaken (17 % recovery) emulsions. The decrease may be associated with lutein degradation as shown by the chromatograms of lutein extracted after storage.

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