CHITOSAN-BASED ENCAPSULATION FOR TUNA-OIL

by

WANWIMOL KLAYPRADIT

(Under the Direction of Yao-Wen Huang)

ABSTRACT

Tuna oil is easily subject to oxidation due to the high susceptibility to oxygen of the highly unsaturated fatty acids. To overcome this drawback, chitosan-based encapsulation may help to maintain stability and nutritional value of tuna oil. The overall objectives were to develop a new encapsulation process for tuna-oil oil and to evaluate the properties of the encapsulated powder. Encapsulation was conducted in three stages; (1) dissolution of wall material, mixing with core and emulsification; (2) atomization into a reactive broth; and (3) Freeze drying. The effects of preparation variables such as concentration and ratio of wall materials components (chitosan, maltodextrim, and whey protein isolate), tuna oil concentration (10%, 20%, 30%), amplitude of atomizer (50, 55, 60, 65) were evaluated. The emulsion was evaluated for droplet size and stability while antioxidant activity was measured over time of storage at 25 °C and 50°C. The powders were characterized after freeze drying in terms of tuna-oil content, EPA and DHA fatty acids composition, and microstructure of formed microspheres. The results showed that the optimum concentration of chitosan-maltodextrin and chitosan-whey protein isolate ratio were found to be 1:10 and 1:1, respectively. Emulsification process was at 5,000 rpm, 30 minutes followed with atomization at the amplitude 55. By varying the preparation conditions, dispersed phase particles in the emulsions were significantly different in size ($P<0.05$) with the

chitosan-maltodextrin wall material giving the smallest particle size and the highest emulsion stability. There was significantly improved oxidative stability of all samples with or without α-topherol compared to bulk tuna-oil. The encapsulated powder had EPA and DHA levels > 100 mg/g. Moisture content, water activity and encapsulation efficiency were acceptable. Scanning Electron Microscope showed particle with no pores or surface oil droplets. The results suggest that chitosan mixed with maltodextrin or whey protein isolate have the potential to be used as the wall material for encapsulating tuna oil or other oils using ultrasonic atomizer. However, all condition that can increase the rate of oxidation must be avoided in every step of the process because once the oil is oxidized the process can not be reversed by microencapsulation.

INDEX WORDS: Tuna-oil, Oxidation, Encapsulation, Chitosan, Ultrasonic Atomizer

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DEDICATION

I gratefully dedicate this dissertation to my parents and my brother for their love and support.

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CHAPTER 1

INTRODUCTION

Fish oils are excellent sources of omega-3 polyunsaturated fatty acids (PUFA) [Eicosapentaenoic acid (EPA, C20:5 n-3) and Docosahexaenoic acid (DHA, C22:6 n3)]. Incorporation of fish oils into food products might have health benefits. Many studies indicate that fish oils may have cardioprotective effects that include anti-arrhythmic, anti-inflammatory, hypotriglyceridemic effects, and lowered blood pressure (Balk, Lichtenstein, Chung, Kupelnick, Chew, & Lau, 2006). However, fish oils have strong odors and without protection they are easily oxidized due to the high susceptibility to oxygen of the highly unsaturated fatty acids. One of the methodologies proposed for protection of fish oil is microencapsulation, which is the technology of a continuous thin coating is formed around solid, liquid or gaseous materials in miniature sealed capsules (Pothakamury & Barbosa-Canovas, 1995; Barbosa-Canovas & et al., 2005). This technology makes it possible to transform fish oil from a liquid to a solid ingredient with enhanced oxidative stability.

Microencapsulation by spray-drying is most widely used in the food industry. The process is relatively well established, rather inexpensive, straightforward and the encapsulated product can be incorporated into dry products and powders (Shefer & Shefer, 2003). One limitation of the spray drying technology is the limited number of wall materials available. Since almost all spray drying processes in the food industry are carried out from aqueous feed formulations, the wall material must be soluble in water at an acceptable level. In recent years, wall materials used for oil encapsulation are caseinate and lactose (Keogh & et al., 2001);

sodium caseinate and maltodextrin (Kagami & et al., 2003); hydroxy methyl cellulose or methylcellulose with maltodextrin (Kolanowski, Laufenberg, & Kunz, 2004); acacia gum/maltodextrin (Turchiuli & et al, 2005); Lecithin-chitosan and corn syrup solids (Klinkesorn & et al., 2006). Drying process at low temperature such as freeze-drying is another potential method for sensitive oils. Not much research has been reported about using freeze drying for encapsulation. Heinzelmann & Franke **(**1999); Heinzelmann & et al. (2000) indicated acceptable oxidative stability of encapsulated oil. In contrast, Marquez-Ruiz & et al. (2000) reported that bulk oil showed better oxidative stability than microencapsulated fish oils.

An ultrasonic atomizer has been used for moistening solids and powder, adding liquids during stirring and mixing operations, injecting gas into liquids, degassing liquids and medical technology applications. Unlike conventional atomizing nozzles that rely on pressure and high-velocity motion to shear a fluid into small drops, the ultrasonic atomizer uses only low ultrasonic vibrational energy for atomization. The liquid can be dispensed to the atomizing probe (nozzle) by either gravity or a small low-pressure metering pump, and atomized continuously or intermittently. The benefits of ultrasonic atomizers include low energy cost, uniform atomization at any flow rate and non-clogging for reliable performance (Cole-Parmer Instrument Company, 2005; Lefebvre, 1989). The ultrasonic atomizer exhibits a promising alternative method for fish oil encapsulation. To date, there is no report on the use of this method for oil encapsulation.

Chitosan is derived from chitin by the N-deacetylation process. It has been receiving worldwide interest for food applications, which include microbial activity inhibition, edible film coatings of food, clarification and deacidification of fruit juices, control of enzymatic browning in fruits, and purification of water (Shahidi & et al.1999). Little information on encapsulation of fish oil using chitosan as a wall material is available.

The overall objectives of the following experiments were to develop a new encapsulation process for tuna-oil oil by ultrasonic atomization and freeze-drying and to evaluate the properties of the encapsulated oil powder. The first part of the study investigated the feasibility of encapsulating tuna-oil in a chitosan microcapsule using an ultrasonic atomizer to disperse the coated oil droplets into a medium that solidified the wall material. The second part was examined the oxidative stability of tuna oil in emulsions and in the microcapsules. In addition, the relationship between emulsion structure and lipid oxidation was elucidated. The last phase; (a) explored oil localization in emulsion by using CLSM; (b) examined the outer-surface of the encapsulated particles by means of Scanning Electron Microscopy; and (c) investigated the relationship between FTIR and the ability of encapsulation.

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CHAPTER 2

LITERATURE REVIEW

Fish oil

The world food market is currently interested on foods that provide not only nutritive values but also health benefits to human. One such category of food products is a so-called "functional foods" which is now rapidly growing in market share and scientific research in this area has greatly proliferated. Fish oils are considered functional foods due to the fact that they are excellent dietary sources of the important ω-3 fatty acids especially polyunsaturated fatty acid (PUFA) [EPA (Eicosapentaenoic acid, C20:5 n-3 and DHA (Docosahexaenoic acid, C22:6, n-3)]. Much research has shown that supplemental fish oil may be beneficial for the healthy function of the heart, brain and nervous system (Lands, 2005). Whereas n-6 fatty acids deficiency has been recognized and considered in directory recommendations, the effects of n-3 fatty acids deficiency is just being realized (Table 2.1) (Furst & Kuhn, 2000).

Chemical composition of fish oils

Generally speaking, oils in ocean fish are complex mixtures containing many fatty acids, some with as many as six double bonds. Nevertheless, for practical purposes, eight fatty acids comprise the majority of fish oil fatty acids. These fatty acids fall into four pairs of particular structure and origin, namely, 14:0 and 16:0, 16:1 and 18:1 (n-7 and n-9), 20:1 and 22:1 (n-9 and n-11), and 20:5 and 22:6 (n-3). These eight fatty acids make up to 80-85% of the total fatty acids in fish oils, and normally can be used to determine the type of oil and its value in different markets. The n-3 PUFA of fish oil such as the nonessential 14:0, 16:0, 16:1 n-7, 18:1n-7, and

Table 2.1. Symptoms of a lack of n-6 and n-3 fatty acids (Furst & Kuhn, 2000)

Deficit in	Clinical symptoms
n-6 fatty acids	Skin lesions
	Anaemia
	Thrombocytopenia
	Fatty liver
	Delayed healing of wounds
	Increased susceptibility to infections
	Delayed growth
	Diarrhea
	Increased thrombocyte aggregation
n-3 fatty acids	Neurological symptoms
	Reduced visual acuity
	Skin lesions
	Delayed growth
	Reduced learning ability
	Abnormal electoretinogram

18:1n-9 are of nutritional interest here only as substrates for the acyl-CoA pathway to CO_2 and energy. All of the nonessential fatty acids can be formed by vertebrate tissues from simple amino acid or carbohydrate precursors, and the acids have no recognized role in modifying the metabolism of Eicosanoids. The average values for the polyunsaturated fatty acid content of fish oils listed in Table 2.2 indicate that much more 20:5n-3 relative to 22:6n-3 occurs in Atlantic menhaden than in most other species. This fish provides a major portion of the fish oil produced in the United States. Canadian tuna, however, has relatively more 22:6n-3 (Lands, 2005).

As mentioned, tuna-oil is rich in ω-3 fatty acids, making it easily subject to oxidation (more details are in the oxidation section), so it is difficult to corporate it in food products. The oxidation affects the sensory properties of the food in which the oil is incorporated by imparting a 'fishy' flavour and odor. Therefore, to produce foods with physiologically bioactive lipid components, methods must be developed to control oxidative reactions. Encapsulation technique has been suggested as a means of preserving reactive, sensitive or volatile additives to be turned into stable ingredients (the details are in encapsulation section).

Official recommendations for ω-3 fatty acids

The first official medical recommendation that people should eat fish regularly was made in the United Kingdom in 1994. Twice a week was specified, with one meal being fatty fish. The American Heart Association (AHA) followed belatedly with committee statements in 2002 and again in 2003 with the following recommendations (Table 2.3) (Ackman, 2006).

In 1997, FDA affirmed, as GRAS, menhaden oil as a direct human food ingredient with specific limitations of use to ensure that the total daily intake of EPA and DHA would not exceed 3.0 grams per person per day (g/p/d) (62 FR 30751; June5, 1997; 21 CFR 184.1472). EPA and DHA are the major omega-3 fatty acids in fish oils and together comprise about 20

Table 2.2 Polyunsaturated fatty acids in fish oils[a] (Adapted from Lands, 2005)

Fatty acids	Atlantic menhaden[b]	Marine sardine[b]	Canadian tuna[b]	Atlantic herring[b]	Atlantic mackeral[c]	Maxican	Anchovy[b,c]
16:2n-7	0.8	0.3	-	1.1	-	0.9	-
16:3n-4	1.7	-	-	1.1	-	1.0	-
16:4n-4	2.9	0.7	1.0	1.0	0.4	0.9	1.3
18:2n-6	1.0	1.6	1.6	3.8	1.2	1.2	1.1
18:3n-6	1.1	1.3	0.8	1.2	-	0.9	-
18:4n-3	2.1	4.7	0.8	3.0	1.3	2.0	1.5
20:3n-3	0.2	-	-	-	-	0.2	-
20:4							
n-3/n-6	1.7	0.5	2.0	4.0	4.0	0.9	4.5
20:5n-3	16.7	7.4	6.9	11.5	11.5	16.7	14.3
22:5n-3	0.9	0.6	2.2	1.8	1.8	1.1	1.8
22:6n-3	7.0	5.2	19.7	15.1	15.1	15.6	13.1

[a]Express as percentage of total fat

[b]Unpublished data from Zapata Haynie Corp., United States

[c]Unpublished data from Fishing Industries Research Institute, Republic of South Africa

Table 2.3. Recommendations for ω-3 fatty acids consumption (Ackman, 2006)

Population	Recommendation
Patients without documented chronic Heart disease (CHD)	Eat a variety of (preferably oily) fish at least once a week, include oils and foods rich in α-linolenic acid (flaxseed, canola, and soybean oils; flaxseed and walnuts)
Patients with documented CHD	Consume ≈ 1 g of EPA + DHA per day, preferably from oily fish, EPA + DHA supplements could be considered in consultation with the physician
Patients needing triglyceride lowering	Consume 2 to 4 g of EPA + DHA per day provided as capsules under a physician's care

percent by weight of menhaden oil. FDA then published a tentative final rule (69 FR 2313; January 15, 2004) to additionally require that menhaden oil not be used as an ingredient in foods in combination with other added oil that is a significant source of EPA and DHA to ensure that total intake from conventional food sources do not exceed 3.0 g/p/d. (U.S.FDA., www.fda.gov)

Oxidation

One of the most common forms of instability for foods that contain fats is lipid oxidation, which resulting in alter flavor and nutritional quality of food and may produce toxic compounds. All of the oxidation changes can make the foods less acceptable or unacceptable to consumers (Boff & Min, 2002). Autoxidation is known as the most common process leading to oxidative deterioration. Fish oils, which have high polyunsaturated fatty acids (PUFA) content, have the potential for being broken down into smaller molecule by this process.

Mechanism of lipid autoxidation

There are three main phases of autoxidation; initiation, propagation, and termination (Figure 2.1) (Gordon, 2001; Erickson, 2002; Eitenmiller, 2003).

Initiation

Initiation occurs as hydrogen is abstracted from an unsaturated fatty acid, resulting in a lipid free radical, which in turn reacts with molecular oxygen to form a lipid peroxyl radical. Abstraction of a hydrogen atom by a reactive species such as a hydroxyl radical may lead to initiation of lipid oxidation. However, in oils there is often a trace of hydroperoxides, which may have been formed by lipoxygenase action in the plant prior to and during extraction of the oil. Secondary initiation by hemolytic cleavage of hydroperoxides is a relatively low energy reaction, and it is normally the main initiation reaction in edible oils. This reaction is commonly catalysed by metal ions.

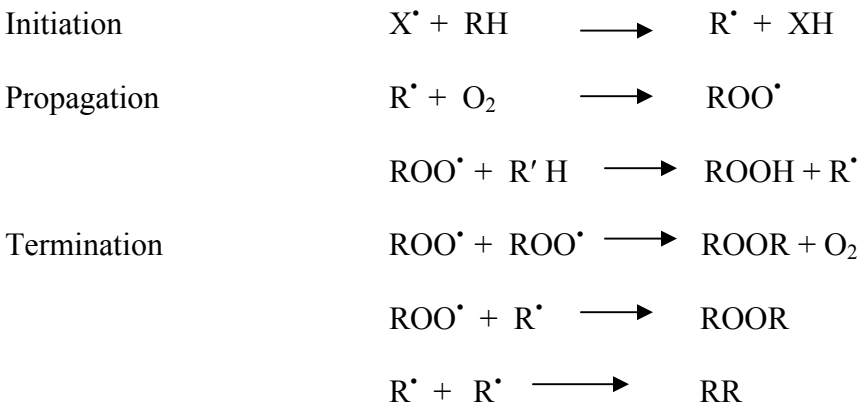
Propagation

After initiation, propagation reactions occur in which one lipid radical is converted into a different lipid radical. These reactions commonly involve abstraction of a hydrogen atom from a lipid molecule or addition of oxygen to an alkyl radical. The enthalpy of the reaction is relatively low compared with that of the initiation reactions, so propagation reactions occur rapidly compared to the initiation reactions. At the normal atmospheric pressure of oxygen, the reaction of alkyl radicals with oxygen is very rapid, and the peroxy radicals are present at much higher concentrations than the alkyl radicals. The lipid peroxyl radical abstracts hydrogen from an adjacent molecule, resulting in a lipid hydroperoxide and a new lipid free radical. Interactions of this type continue 10 to 100 times before two free radicals combine to terminate the process.

Actually, lipid hydroperoxides are not considered harmful to food quality; however, they are further degraded into compounds that are responsible for off-flavors. The main mechanism for the formation of aldehyde from lipid hydroperoxides is hemolytic scission (β cleavage) of the two C-C bonds on either side of the hydroperoxy group. This reaction proceeds via the lipid alkoxy radical, with the two odd electrons produced on neighboring atoms forming a carbonyl double bond. Two types of aldehydes are formed from the cleavage of the carbon bond: aliphatic aldehydes derived from the methyl terminus of the fatty acid chain and aldehydes still bound to the lipid parent molecule. Since unsaturated aldehydes can be oxidized further, additional volatile products may be formed.

Termination

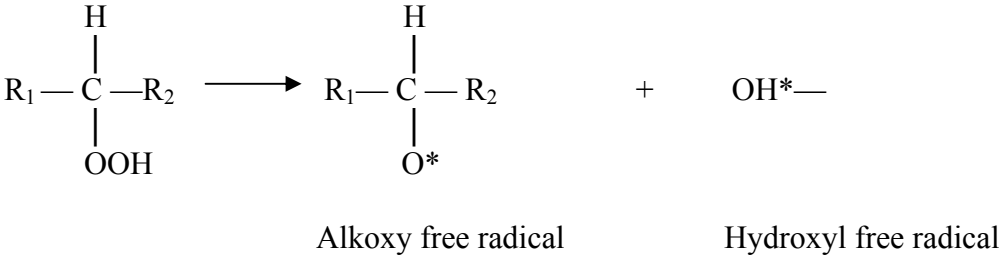
In this step, the free radicals combine together to form molecules with a full complement of electrons and low energy reactions so that the products do not autocatalyze or propagate the reaction.



Hydroperoxide Decomposition Secondary breakdown

Hydroperoxides are readily decomposed by high energy radiation, heat, metal catalysis

(1). Homolytic Decomposition



Homolytic cleavage

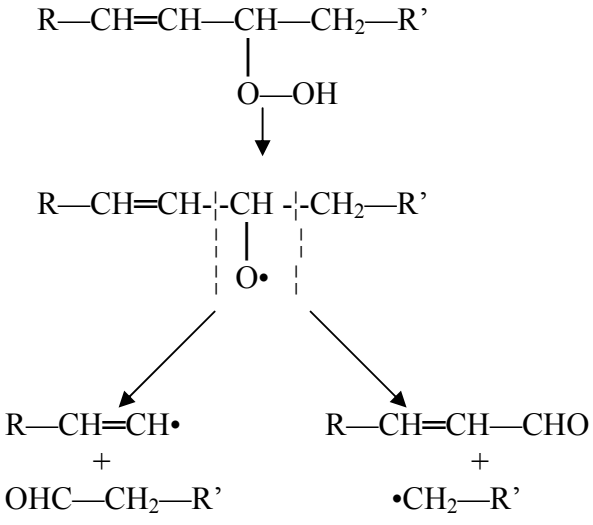
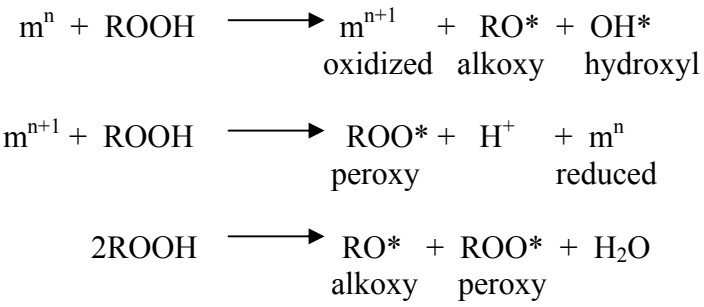


Figure 2.1. Mechanism of lipid autoxidation (Gordon, 2001; Eitenmiller, 2003)

(2) Metal Catalysis



Decomposition of alkoxy free radical

- Alkoxy free radical can cleave on either side of the carbon atom bearing the oxygen atom.
- Subsequent free radical interactions lead to a wide variety of smaller molecular weight organics which contribute to off-flavor and odor.

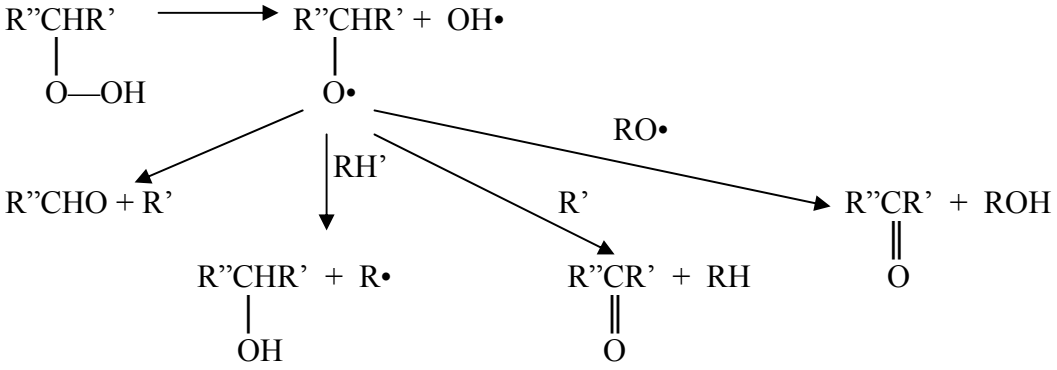


Figure 2.1. (continued)

Measurement of oxidative rancidity (Shantha & Decker, 1994; Gunstone, 1996; Shahidi & Wanasundara, 2002; O'Brien, 2004)

There is no uniform and standard method for detecting all oxidative changes suitable for all food systems. The available methods to monitor lipid oxidation in foods and biological systems may be divided into two groups. The first group measures primary oxidative changes and the second determines secondary changes that occur in each system.

Primary measurement

(1). *Changes in reactants*

This method is not widely used in assessing lipid oxidation because it may require total lipid extraction from food and subsequent conversion to derivatives suitable for gas chromatographic analysis. On the other hand, it cannot be used in more saturated oils because this indicator reflects only the changes that occur in unsaturated fatty acids during oxidation

(2). *Weight gain*

It is generally accepted that addition of oxygen to lipids and formation of hydroperoxides can be reasonably quantified during the initial stages of autoxidation. Therefore, the measurement of change during the induction period from weight gain data is theoretically sound.

(3). *Peroxide value (PV)*

PV is the most commonly used method to assess primary oxidation. There are numerous analytical procedures for measuring PV. In all cases the results and accuracy of the test depend on the experimental conditions. The AOAC official Method and the American Oil Chemists' Society (AOCS) method, both determine peroxide by iodometric titrations, measuring the iodine liberated from potassium iodide by the peroxides present in the oil. However, the methods lack sensitivity and require a large amount of lipid sample. The International Dairy Foundation (IDF)

standard method based on the spectrophotometric determination of ferric complexes is a promising technique for the determination of the PV for fish oils, meat, poultry, dairy, vegetables. This method is simple, rapid, sensitive, and can detect peroxide concentrations as low as 0.1 mequiv/kg sample. The small sample size required makes this assay convenient for the study of a large number of samples.

(4). *Active oxygen method (AOM)/Oil stability index (OSI) and Rancimat methods*

The AOM is the most common accelerated test for estimating the oxidative stability of fats and oils products. AOM employs heat and aeration to accelerate deterioration of the fat and oil sample for different time intervals and the PVs are determined and then plotted against time and the induction period is determined from the graph. OSI and Rancimat are automated versions of the AOM apparatus since both employ the principle of accelerated oxidation.

(5). *Conjugated Dienes*

Oxidation of PUFA is accompanied by an increase in the ultraviolet absorption of the product. Lipids containing methylene-interruptured dienes or polyenes show a shift in their double-bond position during oxidation due to isomerization and conjugate formation. The resulting conjugated dienes exhibit an intense absorption at 234 nm; similarly conjugated trienes absorb at 268 nm.

Secondary measurement

(1). *2-Thiobarbituric acid value (TBA)*

TBA is one of the most frequently used tests for assessing lipid oxidation. It is expressed as milligrams of malonaldehyde (MA) equivalents per kilogram sample or as micromoles MA equivalents per gram sample. MA is a relative minor product of oxidation of polyunsaturated

fatty acids that react with the TBA reagent to produce a pink complex with an absorption maximum at 530-532 nm.

(2). *p-Anisidine value* (*p*-AnV)

p-AnV is defined as 100 times the optical density measured at 350 nm in a 1.0-cm cell of a solution containing 1.0 g of oil in 100 ml of a mixture of solvent and reagent, according to the International Union of Pure and Applied Chemist (IUPAC) method. This method measures the amount of aldehyde in the oil. It is based on the fact that in the presence of acetic acid, *p*-Anisidine reacts with an aldehydic compound in an oil, producing yellowish reaction products.

(3). *Totox value*

The *p*-AnV is often used in the industry in conjunction with PV to calculate the so-called total oxidation or TOTOX value:

Totox value = 2PV + 2 *p*-AnV

There is an increasing interest in oxidation of food emulsions during the process of encapsulation. As mentioned above, oxidation leads to the development of undesirable 'off-flavors' and potentially toxic reaction products. In addition, it may promote the physical instability of some emulsions (Mcclements, 2005). Most food emulsions can be considered to consist of three distinct regions that have different physicochemical properties: the interior of a droplet, the continuous phase, and the interfacial membrane. The interfacial region is potentially very important in lipid oxidation since it represents the region where lipid- and water-soluble prooxidant can interact and it is where surface-active compounds such as lipid peroxide and chain breaking antioxidants concentrate (Mancuso, McClements, & Decker, 1999). The publications reporting lipid oxidation in food emulsions represent a considerable amount of research being carried out in this area over the past few years (Silvestre, Decker, & McClements,

1999; Mancuso, McClements, & Decker, 1999; Chaiyasit, Silvestre, McClements, & Decker, 2000; Silvestre, Chaiyasit, Brannan, McClements, & Decker, 2000; Tong, Sasaki, McClememt, & Decker, 2000; Nuchi, Hernandez, McClements, & Decker, 2002; Richards, Chaiyasit, McClements, & Decker, 2002; Hu, McClements, & Decker, 2003a,b; Hu, McClements, & Decker, 2004; Osborn & Akoh, 2004; Laplante, Turgeon, & Paquin, 2005a,b; Klinkeson, Sophanodora, Chinachoti, McClements, & Decker, 2005; Hu, Decker, & McClements, 2005; Alamed, McClements, & Decker, 2006; Surh, Decker, & McClements, 2006). The main objective of these efforts is to develop effective strategies for retarding lipid oxidation in an emulsion by incorporating antioxidant or combination of continuous phase, controlling storage conditions, or oil droplet interfacial properties. The determination of extent of oxidation in encapsulated oil powder is also very important. Much of the research on encapsulation has included the measurement of oxidative changes as a part of the experiment.

α-Tocopherol

The addition of antioxidant to food containing lipids is one of the approaches used to reduce or prevent oxidation. In the recent years, attention has been given to the use of natural antioxidants because of the worldwide trend to avoid or minimize the use of synthetic food additives. Vitanmin E is one of the most important commercial natural antioxidants to be used in food products. It is the collective term for fat-soluble 6-hydroxychroman compounds. Naturally occurring vitamin E consists of α-, β-, γ- and δ- Tocopherols and the corresponding α-, β-, γ- and δ- Tocotrienols. α-Tocopherol is the most active vitamin E homolog.

The antioxidative mechanism of α-Tocopherol is well understood. α-Tocopherol donates a hydrogen to a peroxy radical resulting in a α-tocopheryl semiquinone radical. This radical may further donate another hydrogen to produce methyltocopherylquinone or react with another

tocopheryl semiquinone radical to produce an α-tocopherol dimer. Higher polymeric forms can then form. The methyltocopherylquinone is unstable and will yield α-tocopherylquinone. The α-tocopheryl dimmer continues to possess antioxidant activity (Reische, Lillard, & Eitenmiller, 2002; Eitenmiller & Lee, 2004).

$ROO^{\bullet}$ + α-tocopherol ⟶ ROOH + α-tocopheryl $semiquinone^{\bullet}$

α-tocopheryl $semiquinone^{\bullet}$ + $ROO^{\bullet}$ ⟶ ROOH + methyltocopherylquinone

α-tocopheryl $semiquinone^{\bullet}$ + α-tocopheryl $semiquinone^{\bullet}$ ⟶ α-tocopherol dimer

Recent studies of α-tocopherol both in oil-in-water emulsion and encapsulation have been reported (Turchiuli & et al, 2005; Gunaseelan, Romsted, Gallego**,** González-Romero & Bravo-Díaz, 2006; Fuchs & et al., 2006; Yoo, Song**,** Chang**,** & Lee, 2006; Zuta, Simpson, Zhao, & Leclerc, 2007).

Encapsulation

Encapsulation technology has been used in the food industry for more than 60 years as a way to provide liquid and solid ingredients with an effective barrier against environmental and/or chemical interactions until a release is desired. In effect, nearly any material that needs to be protected, isolated, slowly released over time, or released at a certain time can be encapsulated. Those food ingredients that may benefit from encapsulation include flavors, acids, alkalis, buffers, lipids, enzymes, microorganisms, artificial sweeteners, vitamins, minerals, preservatives, antioxidants, cross-linking agents, leavening agents, colorants, and nutrients (Barbosa-Canovas, Ortega-Rivas, Juliano, & Yan, 2005).

Microcapsules

Microcapsules can be divided into three main classes in terms of their conformation: single particle structure (regular or irregular), aggregate structure, and multi-walled structure (Figure 2.2). A sphere of the active ingredient surrounded by a thick uniform wall or membrane, resembling the shell of a hen's egg, is called a single particle structure. An aggregate structure is formed when several distinct core particles are enclosed within the same capsule wall. When the capsule is a multi-walled structure, different concentric layers of the wall may have the same, or quite different, composition. In this case, multiple wall layers are placed around the core to achieve multiple purposes related to the manufacture of the capsules, their subsequent storage, and controlled release (Shahidi & Han, 1993). In this way, the microcapsule will have the ability to preserve a substance in a finely divided state and to release it as the occasion demands. Encapsulation is most widely used in the flavor industry.

Microcapsules perform two main functions following the formation of a wall around the core material: (a) keeping and protecting the core material inside the shell during storage, and (b) releasing the core material at the right time. The interior contents of the capsules can be released under four different mechanisms: fracturation, diffusion, dissolution or melting, and biodegradation (Barbosa-Canovas, Ortega-Rivas, Juliano, & Yan, 2005). The type of ingredients that can be used for wall materials are presented in Table 2.4.

Benefits and application of microencapsulations in the food industry

In addition to the great advantage of the physical state change from liquid to solid, other typical advantages of using encapsulation in food ingredients include (Shefer & Shefer, 2003);

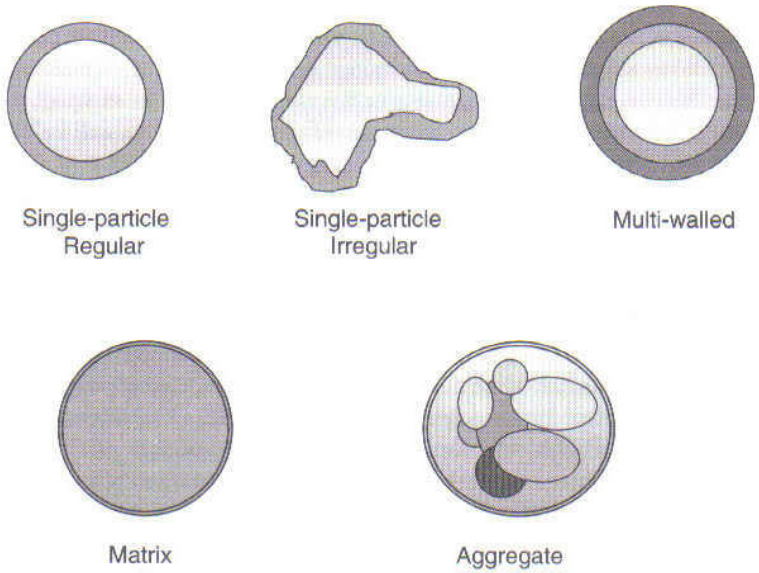


Figure 2.2. Various forms of capsules (Barbosa-Canovas, Ortega-Rivas, Juliano, & Yan, 2005).

Table 2.4. The type of ingredients that can be used for wall materials (adapted from Toledo, 2003)

Wall materials	Example
Polysaccharides	Modified starch, maltodextrin, corn syrup solids, gum arabic Agar (form gels on cooling at concentration as low as 0.04%) Carageenan (anionic-form gel with calcium) Alginates (produces thermally stable gels with calcium) Coat core material/ simply adsorb Make dry product free flowing Produce nonhygroscopic product
Proteins	Whey protein isolate Low methoxy pectin (produces thermally stable gels with calcium) Gelatin Sodium caseinate
Cyclodextrin	Entrap active ingredients inside the molecular structure
Liposomes	Hollow tubules of phospholipids may hold active ingredient inside the core (usually used for essence in cosmetic preparations)

1. Controlled release ingredients
2. Protection of labile ingredients from oxygen, essential oils and vitamins are prevented from undergoing oxidation.
3. Nutraceuticals from natural sources; prevent hygroscopy, possible inclusion into meats and other food products without detracting from the natural color or water binding properties.
4. Prevention of undesirable smells or flavors from ingredients until food is swallowed or until the ingredient is thoroughly incorporated in the food.
5. Probiotics, prolongs viability of microorganisms, protects microorganisms from stomach acids until food reaches the lower intestines.
6. Enzymes, releases enzyme at a specific point in the process and not before
7. Permits production of dry ingredients from normally liquid forms e.g. smoke flavors
8. Production of ingredients that can be incorporated in the packaging material and slowly released to the product.
9. Enhanced bioavailability and efficacy of functional ingredients

Methods of encapsulation

The growing interest by food technologists in the enormous potential of microencapsulation is demonstrated by the exponential increase in the number of publications (non-scientific, scientific articles, and patents) published over the years since the mid 1950s, as illustrated by Figure 2.3. Liposome entrapment and spinning disk, as well as coacervation to lesser extent, have experienced the most rapid growth in interest from researchers and technologists (Gouin, 2004).

Number of published articles

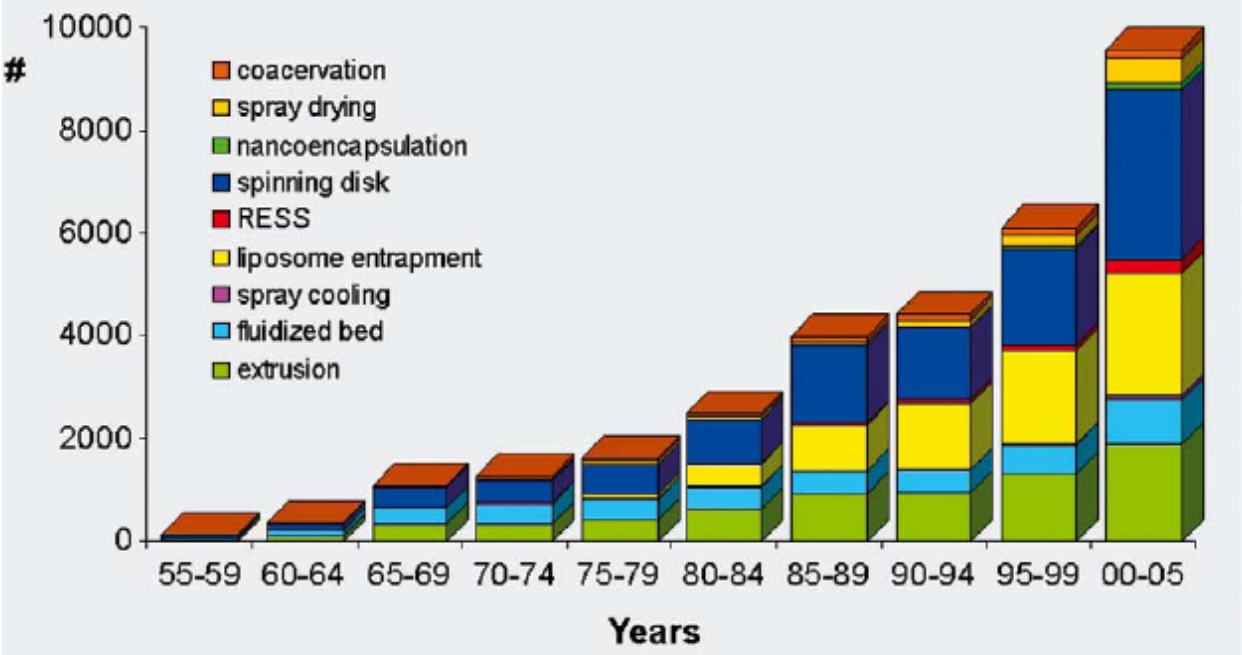


Figure 2.3. Trends in microencapsulation technologies (Gouin, 2004)

(1). ***Spray drying***

Spray drying accounts for the majority of commercial encapsulated materials in food products. The process is relatively well established, rather inexpensive, straightforward and the encapsulated product can be incorporated into dry products and powders (Shefer & Shefer, 2003). One limitation of the spray drying technology is the limited number of wall materials available. Since almost all spray drying processes in the food industry are carried out from aqueous feed formulations, the wall material must be soluble in water at an acceptable level. Typical wall materials include maltodextrins, gum acacia, hydrophobically modified starch and mixtures thereof. Other polysaccharides (alginate, guar gum carboxymethylcellulose) and proteins (whey proteins, soy proteins, sodium caseinate) can be used as the wall materials in spray-drying, but their usage becomes very tedious and expensive because of their low solubilities in water. There are some limitations for using spray-drying. The heat may drive off volatile components which make up the essence of the flavor and also damage the shell by causing microcracks that can lead to decomposition of the capsule and poor flavor retention. The cost may be high if an expensive carrier is used. In addition, explosion risks must be carefully considered before scaling up (Barbosa-Canovas, Ortega-Rivas, Juliano, & Yan, 2005). Currently, encapsulation of fish oils and related oils has been studied. Publications reporting on some oil encapsulation by the spray-drying method were summarized in Table2.5.

(2). ***Coacervation***

It is considered to be the true microencapsulation process since the coating material completely surrounds the core with a continuous phase (Soper, 1995). The basic physical and polymer chemistry behind the coacervation process is well understood. There are three major

stages in the process: (1) formation of the three immiscible phases while mixing under controlled conditions, (2) deposition of the coating material around the core material, and (3) shrinkage and solidification of the liquid coating to form the solid microcapsules. Despite the effectiveness of coacervation compared to the other encapsulation processes food scientists face problems when it comes to commercializing a coacervated food ingredient; (1) the high cost of the process; (2) the solidification step usually involves glutaraldehyde, which must be carefully used according to the country's food law; (3) sensitive multistep batch process; (4) regulations limiting the number of coating materilas. (Dziezak, 1988; Graves & Weiss, 1992). However, the process is widely used in the pharmaceutical, cosmetic, fragrance, and specially products industries. The publications reporting oil encapsulation by the coacervation method are also summarized in Table2.5.

(3). ***Extrusion***

An extruder is employed to form the carrier melt in a continuous process. Then the ingredients to be encapsulated are either mixed with or injected into the molten carbohydrate carrier. Normally, this method has been used exclusively for the encapsulation of volatile and unstable flavors in glassy carbohydrate matrices. The main advantage of the process is the very long shelf life because of its outstanding protection of flavors against oxidation. The limitations include its relatively high cost, high process temperature, low solubility in cold water and low flavor loading, typically below 20% (Reineccius, 1994). Krasaekoopt, Bhandari & Deeth (2003) reported encapsulation of lactic acid and probiotic bacteria by the extrusion technique for their use in fermented and other dairy products. The product had higher stability during storage, and higher productivity than non-encapsulated bacteria. Not much research has been done about oil

encapsulation by this technique. To date, the encapsulation of sunflower oil in a starch matrix via extrusion has been studied (Yilmaz, Jongboom, Feil, & Hennink, 2001).

(4). ***Fluidized bed***

The fluidized bed process, also called air suspension coating or spray coating, is generally used for solid material encapsulation. It accounts for the second largest commercial production of encapsulated products for the food industry (Barbosa-Canovas, Ortega-Rivas, Juliano, & Yan, 2005). In the basic process, solid particles to be encapsulated enter the air stream and are lifted by the upward moving air stream to the top of the chamber. A fine mist of coating material, which is melted or dissolved in volatile solvent, is atomized through the spray nozzle into the air chamber and deposited on the particles while they are suspended. Each particle is gradually covered with a film of coating material. The encapsulated product is removed from the chamber, cooled and put through a final drying procedure prior to packaging (DeZarn, 1995). The advantages of this technique include; (1) suitability for many kinds of wall material (polysaccharides, proteins, fats, emulsifiers, etc.), (2) the encapsulated product may consist of very fine particles. However, cost is high because of the long batch process. The food ingredients that have been encapsulated by fluidized bed coating are ascorbic acid (Knezevic, Gosaki, Hraste, & Jalsenjak, 1998), high amylose corn starch (Dimantou, Greenberg, & Kesselman, 2004). To date, there is no report on the use of fluidized bed technique for fish oil.

(5.) ***Spray chilling***

Spray chilling typically refers to matrix encapsulation in the literature because the particles are more adequately described as aggregates of active ingredient particles buried in the fat matrix. 'True' encapsulation in contrast is usually reserved for processes leading to a core/shell type of microencapsules. Porzio (2004) indicated that the lipid-encapsulation process

is referred to as spray chilling. The process begins with mixing liquid ingredients with molten fat to create a solution/dispersion. The resulting mixture is then atomized into a chamber where it is contacted with an air stream cool enough to cause the atomized droplets to solidify, forming a crude encapsulated product (Gouin, 2004). Some functional ingredients such as enzymes, flavors, vitamins, antimicrobials, etc. have been prepared using this method to improve heat stability and to delay release in wet environments.

(6). ***Spinning disk and centrifugal coextrusion***

These are both atomization methods that can be used in modified spray cooling/chilling encapsulation. Spinning disk involves the formation of a suspension of core particles in the coating liquid and the passage of this suspension over a rotating disk under conditions that result in the formation of a film of the coating much thinner than the core particle size. Centrifugal coextrusion is based on a modified double fluid nozzle where the active ingredient is pumped through the inner part of the nozzle while the shell material is pumped through the outer part of the nozzle. At the end of the nozzle, round beads constituted of the active ingredient in the core and an outer layer of the shell material are formed.

(7). ***Freeze-drying***

Freeze-drying is a desirable process for the dehydration of almost all heat sensitive materials. In the system, water is first removed from the frozen material as a vapor by sublimation in a vacuum chamber and then the vapors are removed from the chamber by mechanical vacuum pumps or steam jet ejector. Freezing of the product, sublimation of ice and removal of water vapor are the three main steps in a freeze-drying operation. The frozen material is loaded on the top of heated plates that provide the energy for ice sublimation and “bound” water desorption. Heat transfer occurs by conduction from the heated plates, convection from the

air inside the drying chamber to the exposed surface, and radiation. The vacuum pump is used to evacuate the drying chamber at the start of the operation and to remove the non-condensing gases and any air that leaked into the system. The absolute pressure inside the chamber is determined by the temperature at which the vapor trap is maintained. When the ice starts to sublime, the water vapor is transported through the chamber to the refrigerated condenser that prevents the return of the water vapor to the product and also reduces the volume of gases to be removed from the system by the vacuum pump (Barbosa-Canovas, Ortega-Rivas, Juliano, & Yan, 2005). The publications reporting oil encapsulation by freeze-drying method are also summarized in Table2.5.

A summary of commercial encapsulated products and their applications is shown in Table 2.6.

Ultrasonic Atomizer

Ultrasonic Atomization is the phenomenon of having a liquid in the form of thin film flow on a vibrating surface (frequency > 20 kHz) which breaks up the fluid film into fine droplets. Two major hypotheses have been proposed to explain the mechanism of liquid disintegration during ultrasonic atomization, namely capillary wave hypothesis and cavitation hypothesis. Capillary wave hypothesis is based on the Taylor instability criteria. The strong correlation between mean droplet size and capillary wavelength favors the capillary wave theory. Cavitation hypothesis is generally applied to high frequency (16 kHz-2 MHz) and high-energy intensity (W/m^2) system (Avvaru, Patil, Gogate, Pandit, 2006).

Principle of ultrasonic atomizer

The ultrasonic power supply converts line voltage to high frequency electrical energy. This high frequency electrical energy is transmitted to the piezoelectric transducer within the

converter, where it is changed to mechanical vibrations. Amplification of the electrical energy is necessary to realize the vibrational levels necessary to effect atomization. The mechanical vibrations from the converter are intensified and focused by the probe. When liquid comes in contact with the probe tip, it disintegrates into micro-droplets and creates a low-viscosity mist.

Unlike conventional atomizing nozzles that rely on pressure and high-velocity motion to shear a fluid into small drops, the ultrasonic atomizer uses only low ultrasonic vibrational energy for atomization. The liquid can be dispensed to the atomizing probe (nozzle) by either gravity or a small low-pressure metering pump and atomized continuously or intermittently. An ultrasonic atomizer is typically used for product moistening, adding liquids during stirring and mixing operations, injecting gas into liquids, degassing liquids or for medical technology. The benefits of ultrasonic atomizers include low energy cost, uniform atomization at any flow rate and non-clogging for reliable performance (Cole-Parmer Instrument Company, 2005; Lefebvre, 1989). To date, no work has been reported on the use of ultrasonic atomizer for tuna-oil encapsulation. A related work has been done on lysozyme encapsulation by Yeo & Park (2004) who successfully studied the use of an ultrasonic atomizer to prepare reservoir-type microcapsules that allowed lysozyme to be encapsulated without loss of functional integrity and to be released with near zero-order kinetics for over 50 days.

Table 2.5. Scientific publications reporting of oil encapsulation

Encapsulation methods	Encapsulants	Wall materials	Remarks	References
Co-crytallization	Orange peel oil	Sucrose syrup		Beristain & et al (1996)
Spray-drying		Whey protein and maltodextrin		Neil & Younger (1998)
Emulionfreezing and subsequent freeze drying	Fish oil (Sandeel oil)	Sodium caseinate with lactose or maltodextrin		Heinzelmann & Franke (1999)
Freeze-drying	Fish oil	Lactose and sodium caseinate	Bulk oil was better in oxidative stability than microencapsulated fish oils	Marquez-Ruiz & et al. (2000)
Complex coacervate	Neutral oil	Gelatin/arabic gum		Lamprecht & et al. (2000)
Freeze-drying	Fish oil	Sodium caseinate and maltodextrin	Acceptable oxidative stability	Heinzelmann & et al. (2000)
Spray-drying	Fish oil	Caseinate and lactose		Keogh & et al. (2001)
Complex coacervation and spray-drying		Gelatin and acacia gum		Lamprecht & et al. (2000b)
Spray-drying	Cardamom essential oil	Mesquite gum		Beristain & et al. (2001)
Spray-drying	Fish oil	Maltodextrin		US patent 6,444,242
Spray-drying	Fish oil	Sodium caseinate and maltodextrin (DE=18)	Induction of browning and agglomeration	Kagami & et al. (2003)
Complex coacervate	Shark liver oil	Linseed pectin-alginate with chitosan		Diaz-Rojas & et al (2003)
Spray-drying	Fish oil	Hydroxy methyl cellulose or methylcellulose with maltodextrin,	Methylcellulose showed a better coating material than hydroxy methyl cellulose	Kolanowski & et al. (2004)
Complex coacervate	Shark liver oil	Calcium alginate/chitosan		Peniche & et al. (2004)

Table 2.5. (continued)

Encapsulation methods	Encapsulants	Wall materials	Remarks	References
Spray-drying and fluid bed agglomeration	Vegetable oil	Acacia gum with maltodextrin	Reduction in oil oxidation	Turchiuli & et al (2005)
Spray-drying and freeze-drying	Safou pulp oil	Maltodextrin (DE=6)		Dznodo-Gadet & et al. (2005)
Spray-drying	Fish oil	Lecithin-Chitosan and corn syrup solid	Good physicochemical properties	Klinkesorn & et al. (2006)
Spray-drying	Olive leaf extract	Chitosan		Kosaraju & et al. (2006)
Spray-drying	Fish oil	Lecithin-Chitosan and corn syrup solid	Good physicochemical properties	Klinkesorn & et al. (2006)
Spray-drying	Fish oil	Maillard reaction products: protein source: Na caseinate, whey protein isolate, soy protein isolate, or skim milk carbohydrate source: glucose, dried glucose syrup, oligosaccharide		Augustin & et al. (2006)
Spray-drying and fluidized bed agglomeration	Vegetable oil	Maltodextrin and acacia gum		Fuchs & et al. (2006)
Spray-drying	Mackerel oil	n-octenylsuccinate-derivatized starch gucrose syrup (DE=18) or trehalose	Trehalose was better than glucose syrup as wall material	Drusch & et. al (2006)

Table 2.6. Commercial encapsulated products and their applications

Type of products	Product applications
Potassium chloride	Processed and cured meats, confections and gums, Yeast leavened baked goods, microwaveable food products, nutritional pre-mixes, feed
Choline chloride	Dry food mixes, health food, Protein health drink mixes, infant food formulations, replacement of crystalline choline chloride, aqualculture
Salt	Meat patties, pretzel products, dough, sausages, yeast leavened baked goods
Vitamin C	Processed and cured meats, confections and gums, Yeast leavened baked goods, microwaveable food products, nutritional pre-mixes, feed
Sodium acid pyrophosphate	Dry cake and biscuit mixes, refrigerated doughs, yeast leavening baked goods microwaveable products, frozen dough
Sodium bicarbonate	Pro-bake batter mix, refrigerated doughs, pancake batter, microwaveable products frozen dough, muffin mixes, liquid bread mixes
Low calorie starch	Reduced calorie gravies, sauces, microwaveable food products, nutritional bake-mixes
Flavors	Surface dusting for barbecued beef and pork, sauces, baked goods
Ready to use batter	Muffin, varieties of pancake
Bromate replacer	Bromate frre sponge and dough bread mix, bromate free continuous Bread mix, microwaveable food products, nutritional pre-mixes
Omega-3 fatty acids	Bread, muffins, salad dressing, beverages, cheese spread, margarine, frozen dessert, sausages, cookies

Emulsion

An emulsion is a dispersion of droplets of one liquid in another liquid with which it is completely immiscible. In foods, the two immiscible liquids are oil and water. The diameter of the droplets in food emulsions are typically within the range 0.1- 50 μm. A system that consists of oil droplets dispersed in an aqueous phase is called an oil-in-water (O/W) emulsion. A system that consists of water droplets dispersed in an oil phase is called a water-in-oil (W/O) emulsion. The material that makes up the droplets in an emulsion is referred to as the dispersed or internal phase, whereas the material that makes up the surrounding liquid is called the continuous or external phase (McClement, 2002).

Emulsion formation

The process of converting two immiscible liquids into an emulsion is known as homogenization, and a mechanical device designed to carry out this process is called a homogenizer. The most important types of homogenizers used in food industry are the high speed blender, colloid mills, high pressure homogenizers, ultrasonic homogenizers, microfluidization, and membrane homogenizers.

Emulsion properties

(1). *Particle size distribution*

The most important properties of emulsion-based food products are determined by the size of the droplets that they contain, for example, shelf life, appearance, texture, and flavor. Consequently, it is important for food scientists to be able to reliably control, predict, measure, and report the size of the droplets in emulsions. If all the droplets in an emulsion are of the same size it is referred to as a monodisperse emulsion, but if there is a range of droplet sizes present it is referred to as a polydisperse emulsion. Monodisperse emulsions are sometimes prepared and

used for fundamental studies because the interpretation of experimental measurements is much simpler than for polydisperse emulsions. Nevertheless, real food emulsions always contain a distribution of droplet sizes, and so the specification of their droplet size is more complicated than for monodisperse systems (McClements, 2005).

(2). *Droplet charge*

The origin of the droplet charge is normally the adsorption of emulsifier molecules that are inionized 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 compared to their isoelectric point. Surface-active polysaccharides may also have an electrical charge depending on the type of functional groups along their backbone. 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 interaction with other charged species (McClements, 1999).

The electrical charge on the droplet can be characterized in a number of different ways that is, the surface charge density (σ); the electrical surface potential (Ψ_0), and the zeta-potential (ζ). The surface charge density is the amount of electrical charge per unit surface area, whereas the surface potential is the free energy required to increase the surface charge density from 0 to σ. The ζ-potential is the effective surface potential of a droplet suspended in a medium, which takes into account those charged species in the surrounding medium may adsorb to the surface of the droplet and alter its net charge. The ζ-potential can be conveniently measured in the laboratory using commercially available analytical instrumentation (Hunter, 2001; Hunter, 1981).

(3). *Interfacial properties*

The droplet interface consists of a narrow region (usually a few nanometers thick) that surrounds each emulsion droplet, and contains a mixture of oil, water, and surface-active molecules (Hunter, 2001; Hunter, 1981). The interfacial region only makes up a significant fraction of the total volume of an emulsion when the droplet radius is less than about 1 μ. Even so, it plays a major role in determining many of the most important bulk physicochemical and organoleptic properties of food emulsions. For this reason, food scientists are particularly interested in elucidating the factors that determine the composition, structure, thickness, rheology, and charge of the interfacial region, and in elucidating how these interfacial characteristics are related to the bulk physicochemical and sensory properties of emulsions.

Emulsion stability/instability

Emulsion stability refers to the ability of an emulsion to resist changes in its properties with time, the greater the emulsion stability, the longer the time taken for the emulsion to alter its properties. Emulsions are thermodynamically unstable systems that tend, with time, to separate back into individual oil and water phase of which they were made. Changes in the properties of emulsions may be the result of physical processes that cause alterations in the spatial distribution of the ingredients. The dominant mechanisms of instability are gravity creaming, Oswald ripening, flocculation and droplet coalescence. Table 2.7 gives the main factors affecting stability. The state of droplet flocculation is dependent on factors such as the biopolymer surface coverage, the layer thickness, surface charge density, and the aqueous solution conditions (especially pH, ionic strength, and divalent ion content). For a freshly prepared fine triglyceride oil-in-water emulsion, the most obvious initial manifestation of instability is creaming, which typically leads on to macroscopic phase separation into separate discernible regions of cream and

serum. This may then be followed by droplet coalescence within the cream and 'oiling off' at the top of the sample. All of these processes can be affected by interactions of the hydrocolloid stabilizer, both in the bulk aqueous phase and at the surface of the emulsion droplets (Dickinson, 2003). A schematic representation of the emulsion instability is presented in Figure 2.4. Recent studies about oxidation of emulsions have been interesting. Some reports are summarized in the oxidation section.

Chitin and chitosan

Chitin is the second most abundant natural biopolymer after cellulose. It is derived from various organisms including crustaceans (especially the exoskeletons of crabs and shrimps), cell wall of fungi and insects. Chitin is a cationic amino polysaccharide composed of β (1,4)-linked *N*-acetyl-D-glucosamine residues (Shahidi, Arachchi, & Jeon, 1999). Chitin exhibits structural similarity to cellulose and differs from it with the replacement of C-2 hydroxyl residues by acetamide groups (Figure2.5). In nature, as many as one in six *N*-acetyl-D-glucosamine residues of chitin is deacetylated. It is known that most commercially prepared chitin is a linear copolymer composed of approximately 70-90% *N*-acetyl-D-glucosamine and 10-30% D-glucosamine units (Synoweiecki & Al-Khateeb, 2003). The occurrence of chitin in various organisms is given in Table 2.8. Chitosan, a copolymer of approximately 80% D-glucosamine and 20% *N*-acetyl-D-glucosamine units, is a product derived from *N*-deacetylation of chitin to different degrees in the presence of hot alkali. Most commercial grades of chitosan contain 5-25% *N*-acetyl-D-glucosamine and 75-95% D-glucosamine units (Winterowd & Sandford, 1995). Chitin and chitosan are known to exhibit polymorphism designated as α-, β- and γ-chitin where the α-form is prevalent and the highest stability. The hydrogen bonds between >NH groups of the one molecule and >C=O groups of the adjacent chain account for the formation of fibrils

Table 2.7. Principle factors affecting oil-in-water emulsion stability (Dickenson, 2003)

Doplet-size distribution	Initially determined by Emulsification equipment Concentration of emulsifier Type of emulsifier Oil/water ratio Other factors (temperature, pH, viscosity)
Nature of interfacial layer	Determined by Concentration and type of emulsifier Interactions of adsorbed species Competition between adsorbed species
Nature of continuous aqueous phase	Rhology, solvent quality, ionic environment, unadsorbed polymers and amphiphilics
Nature of dispersed oil phase	Solid/liquid content Solubility in continuous phase

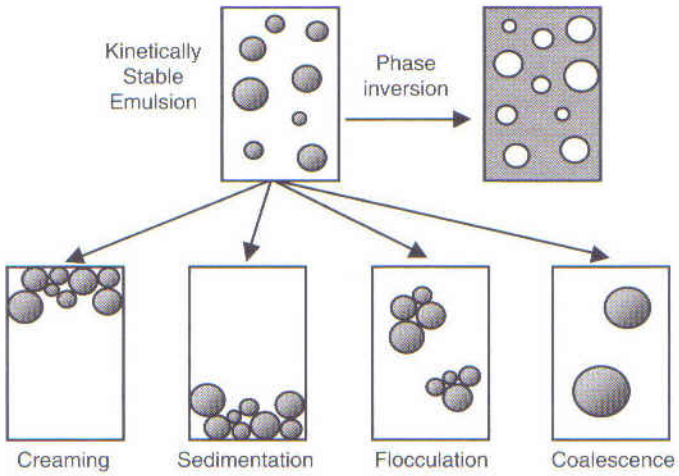


Figure 2.4. Schematic representation of the break-down process in emulsions (McClements, 2005)

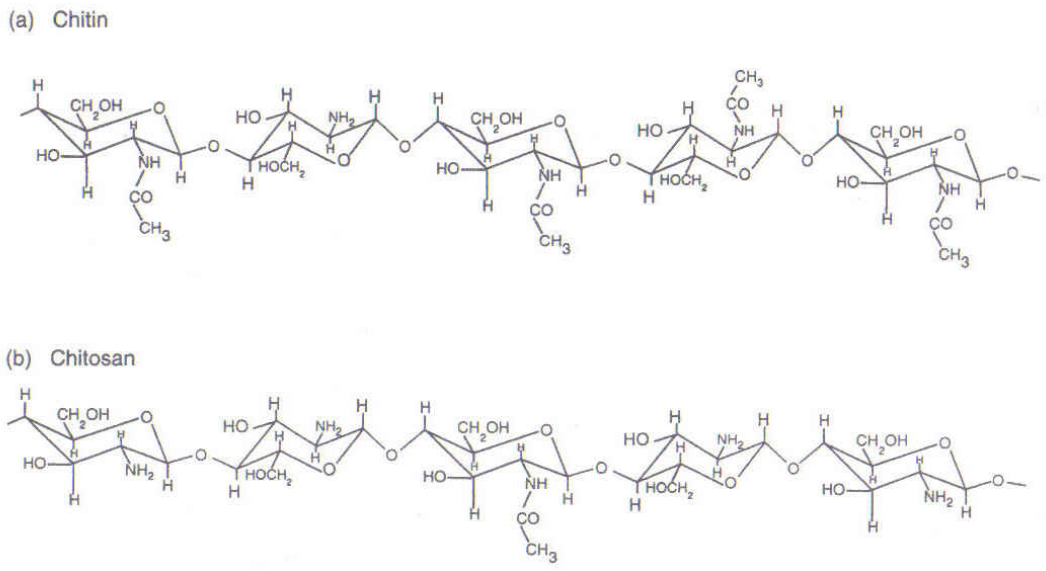


Figure 2.5. Structure of Chitin and Chitosan (Winterowd & Sandford, 1995)

Table 2.8. Chitin content of selected organisms (adapted from Tharanathan & Kittur 2003; Synowieki & Al-Khateeb 2003)

Type		% chitin content
Crustacean	Crab (Cancer)	72.1[c]
	(Carcinus)	64.2[b]
	King crab (Paralithodes)	35.0[b]
	Blue crab (Callinectes)	14.0[a]
	Shrimp (Crangon)	69.1[c]
	(Alasakan)	28.0[d]
	Lobster (Nephrops)	69.8[c]
	(Homarus)	60-75[c]
	Barnacles (Lepas)	58.3[c]
Insects	Cockroach (Peripleneta)	2.0[d]
	(Blatella)	18.4[c]
	Beetle (Colcoptera)	27-35[c]
	Truefly (Diptera)	54.8[c]
	Sulfur butterfly (Pieris)	64.0[c]
	Silk worm (Bombyx)	44.2[c]
	Wax worm (Calleria)	33.7[c]
Molluscan organs	Clamshell	6.1
	Oyster shell	3.6
	Squid, Skeletal pen	41.0
	Krill, deproteinize shell	40.2
Fungi	*Aspergillus niger*	42.0[e]
	Aspergillus phoenicis	23.7[e]
	Blastomyces dermatidis	13.0[e]
	Histoplasma capsulatum	25.8-26.4[e]
	Lactarius vellereus (mushroom)	19.0
	Mucor rouxii	44.5
	Neurospora crassa	8.0-11.9[e]
	Penicillium notatum	18.5[e]
	Penicillium chrysogenum	19.5-42.0[e]
	Paracocidioides brasiliensis	11.0[e]
	Saccharomyces cerevisiae	2.9[e]
	Saccharomyces gutulata	2.3[e]
	Tremeliamesenterica	3.7[e]
	Trichoderma viridis	12.0-22.0[e]

[a] Wet body weight, [b] Dry body weight, [c] Organic weight of cuticle, [d] Total dry weight of cuticle, [e] Dry weight of the cell wall.

occurring differently in each polymorphic form. The degree of *N*-acetylation and the degree of polymerization (dp), which in turn determines the polymer molecular weight, are two important parameters dictating the use of chitosan for various applications (Tharanathan & Kittur, 2003). The molecular weight of these polysaccharides can be as high as 10^6, unless some special treatment is used to degrade them (Maghami & Roberts, 1988).

Production of value-added products such as chitin, chitosan, and their derivatives and application of products in different fields is currently of importance because of the necessity of solving the problem of shell waste accumulation from seafood processing industries. Much chitosan is available and may be employed in a number of commercial applications including water treatment, pulp and paper, textiles, medical, food, agriculture, cosmetics, biotechnology and membranes (Tharanathan & Kittur, 2003).

Chemical and physical properties

Basically, the molecular structures of chitin and chitosan are quite similar but the chemical reactions they undergo and their physical properties are distinct. Hydroxyl and primary amine are reactive groups found in both compounds. The primary amino group is involved in the majority of the reactions but the hydroxyl group can be selectively modified to be derivatized preferentially as detailed in Winterowd & Sandford (1995). As compared to chitin, chitosan has the higher concentration of primary amine groups, which makes it more nucleophilic and basic and is less crystalline, which presumably makes it more accessible to reagents. Upon heating, both compounds decompose prior to melting. Thus, these polymers have no melt point.

Chitin and chitosan are considered to be weak bases where the nonbonding pair of electron on the primary amine group can accept proton to become cationically charged. When a solution carrying multiple negative charges is added, coagulation of chitosan occurs. This might

be of use in the hypocholesterolemic and hypolipidemic effects of chitosan. Previous research demonstrated that chitosan solution forms a gummy precipitate when passed through the acidic stomach to the alkaline small intestine and appears to entrap the cholesterol-containing micelles (Ebihara & Schneeman, 1989). Chitosan is insoluble in neutral or alkaline aqueous solutions but is soluble in acidic solutions. On the other hand, chitin is insoluble in water even under acidic conditions due to a lack of sufficient glucosamine units. Most of the solvents for chitin are toxic e.g. dimethylformamide, lithium chloride while the chitosan solvents are safe to consume e.g. a mixture of 80% water and 20% vinegar.

The degree of *N*-acetylation of chitin and chitosan indicates the reaction rate, the greater the faster. Lysozyme, chitinase or chitosanases can degrade the crystallinity of the polysaccharides and thus decrease the rate of reaction.

Potential applications of chitin and chitosan in foods

The potential broad range of chitin and chitosan products for food applications seems to be promising for manufacturers of food ingredients. The useful features are (1) from a natural resource and biologically reproducible; (2) biodegradable and do not pollute the environment; (3) biocompatible not only in animal but also in plant tissues; (4) almost nontoxic; (5) biologically functional; and (6) changeable in molecular structure (Tharanathan & Kittur, 2003). Their many properties make chitin and chitosan very suitable for innumerable applications in commercial products.

(1). ***Antioxidative properties***

There has been a growing interest to identify natural antioxidants from many natural sources to overcome the deleterious radical mediated effects of oxidation in biological systems. Many biological compounds including carbohydrates, peptides and some phenolic compounds

have been identified as potent radical scavengers. Recently, the antioxidant activity of chitosan and its derivatives has attracted attention (Chiang, Yao, & Chen, 2000). Even though the precise mechanism of radical scavenging activity of chitosan is not clear, it is suggested that amino and hydroxyl groups attached to C-2, C-3 and C-6 positions of the pyranose ring react with unstable free radicals to form stable macromolecule radicals (Kim & Rajapaske, 2005). Some related work have proposed (Xie, Xu, & Liu, 2001) that the NH_2 groups of chitosan act as antioxidant by scavenging OH radical to form stable macromolecule radicals. However, there are some discrepancies about hydroxyl radical scavenging activities of chitosan and some of their derivatives. The latest studies of Huang, Mendis, & Kim (2005) revealed that metal ion uptake ability of chitosan has a great influence on their hydroxyl radical scavenging ability. According to their results, the hydroxyl radical scavenging potency of chitosan is partly due to the chelating ability of transition Fe^{2+}, molecular charge properties and proton donation via hydroxyl and amino groups. The uptake of metal ions by chitosan can proceed through different mechanisms including chelation via lone electron pairs of amino groups and ion exchange mechanisms of protonated amino groups (Guzman, Saucedo, Revilla, Navarro, & Guibal, 2003). However, there is not much information about the relationship between ion chelation and antioxidant properties of chitosan.

The application of chitosan as an antioxidant in some food products has been reported. Weist & Karel (1992) used chitosan powders in a fluorescence sensor to observe lipid oxidation in muscle foods and found that a stable fluorosphere is formed between malonaldehyde, volatile aldehydes derived from the break down of fats, and the primary amino groups of chitosan. According to the latter study, the addition of chitosan at 1% decreased the 2-thiobarbituric acid (TBA) values of meat by 70% after 4 days of storage at 4°C. Kim & Thomas (2007) reported

reduced lipid oxidation following incorporation of chitosan with various molecular weights into ground salmon. Kamil, Jeon, & Shahidi (2002); Klinkeson, Sophanodora, Chinachoti, McClements, & Decker (2005) also indicated the potential of chitosan to be used to engender oxidative stability in food systems high in lipid content.

(2). ***Antimicrobial activity***

At a pH below 6, the glucosamine monomer has a positive charge on C-2 that increase both chiosan's solubility and antimicrobial properties relative to chitin (Chen, Liau, & Tsai, 1998). This finding increased the considerable attention to the antimicrobial activity of chitin, chitosan and their derivatives against different group of microorganisms, such as bacteria, yeast and fungi. The exact mechanism of the antimicrobial action of chitin, chitosan and their derivatives is still unknown, but different mechanisms have been proposed. Interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents (Chen, Liau, & Tsai, 1995; Fang, Li, & Shih, 1994). Chitosan also acts as a chelating agent that selectively binds trace metals and thereby inhibits the production of toxins and microbial growth. Binding of chitosan with DNA and inhibition of mRNA synthesis occurs via chitosan penetrating the nuclei of the microorganisms and interfering with the synthesis of mRNA and proteins (Shahidi, Arachchi, & Jeon, 1999).

(3). ***Modification of physical properties***

Chitosan has been proposed as a texturing, emulsifying, foaming, gelling, and coating agent in a number of recent food-related patents and publications. Cucumber pickled in a seasoning mixture containing chitosan was claimed to have a better taste than those pickled in the seasoning mixture alone (Winterowd & Sandford, 1995). Chitosan is a useful emulsifier that

yields stable water/oil/water (w/o/w) multiple emulsions. The emulsion stability is proportional to the chitosan concentration (Rodríguez, Albertengo, & Agullo, 2002). Gel strength and shelf life of tofu was increased by addition of chitosan (Chang, Lin, & Chen, 2003). The gel strength of tofu increased with increased molecular weight (189, 720 and 2780 kDa) of chitosan while the shelf life of tofu increase with increasing degree of deacetylation (54, 73 and 91%).

(4). ***Nutritional effects***

Chitin and chitosan in food systems are considered dietary fibre and functional ingredients. The United States Food and Drug Administration (USFDA) approved chitosan as a feed additive (Knoor, 1986). Chitosan is also used in the food industry as a food quality enhancer in certain countries such as Japan, Italy and Norway. Chitosan has the potential to be used in foods in order to lower blood serum cholesterol such as in vinegar manufactured and sold in Japan, dietary cookies, potato chips and noodles. Razdan & Pettersson (1994) observed increased high density lipoprotein (HDL) concentrations after feeding chitosan containing diet to broiler chickens. This could be attributed to enhanced reverse cholesterol transport in response to intestinal losses of dietary fats. The effective hypocholesterolemic potential of dietary fiber include no digestibility in the upper gastrointestinal tract, high viscosity, polymeric nature and high water binding properties together with low water binding in the lower gastrointestinal tract. Chitosan shows most of these criteria and has a highly characteristic property in relation to other dietary plant fiber. Its ability to form ionic bonds at low pH permits chitosan to bind *in vitro* with different types of anions such as bile acids or free fatty acids, allowing a large proportion of these bound lipids to be excreted. Bound triacylglycerols would escape hydrolysis by lipase, promoting the excretion of fatty materials including cholesterol, sterols and triacylglycerols. Inside the digestive tract, chitosan forms micelles with cholesterol, both endogeneous and from

dietary sources, in the alkaline fluids in the upper part of the intestine, resulting in the depression of absorption of dietary cholesterol and circulation of cholic acid to the liver (Muzzarelli, 1996).

(5). ***Water purification***

The largest single application of chitosan is in the clarification of waste water by chelating heavy metal ions and radionuclids. More than that, chitosan has a high sorption capacity. In particular, the NH_2 group and thus been used to purify industrial waste water by forming coordinate covalent bonds with metal ions. In addition, chitosan in both powder and dried film forms release most of its free amino group above the pKa of the NH_2 group of chitosan which make it of great use in metal ion complexing (Shahidi, Arachchi, & Jeon, 1999). At present, the United States Environmental Protection Agency (USEPA) approves the use of commercial chitosan at 10 mg/L for water purification (Knorr, 1986).

(6). ***Shelf-life extension of fruit and vegetables***

Chitosan-coated film has been reported to effectively control the decay in peaches, pears, kiwifruits, cucumbers and bell peppers, strawberries, raspberries, litchis, longans, tomatoes, and apples (Zhang & Quantick, 1997; Reddy, Belkacemi, Corcuff, Castaigne, & Arul, 2000; Jiang & Li, 2001; Dong, Cheng, Tan, Zheng, & Jiang, 2004; Han, Zhao, Leonard, & Traber, 2004). Possible mechanisms include decreased respiration rate, inhibition of fungi and delaying of ripening due to the reduction of ethylene and carbon dioxide evolution. Chitosan has been applied to the processing of apple juice, grape, lemon and orange juices (Chattergee, Chattergee, & Guha, 2004).

(7). ***Encapsulation***

Chitosan can be used for encapsulation because of its emulsification capacity (Rodriguez, Albertengo, & Agullo, 2002). In recent years, Chitosan has been applied as a wall material for

encapsulation for some sensitive core ingredients such as hydrophilic drug (Genta, Perugini, Conti, & Pavanetto, 1997), lipophilic drugs (Ribeiro, Neufeld, Arnaud, & Chaumei, 1999), vitamin D2 (Shi & Tan, 2002), bovine serum albumin (Xu & Du, 2003), astaxanthin (Higuera-Ciapara, Felix-Valenzuela, Goycoolea, & Argüelles-Monal, 2004), and olive oil extract (Kosaraju, D'ath, & Lawrence, 2006). Some reports about using chitosan for encapsulation are in Table 2.5.

Maltodextrin

The Food and Drug Administration (FDA) defines maltodextrins as non-sweet, nutritive saccharide polymers that consist of D-glucose units linked primarily by alpha-1-4 bonds, having a dextrose equivalent (DE) less than 20. DE is a quantitative measure of the degree of starch polymer hydrolysis. It is a measure of reducing power compared to a dextrose standard of 100. The higher the DE the greater the extent of starch hydrolysis indicated. As the product is further hydrolyzed (higher DE), the average molecular weight decreases and the carbohydrate profile changes accordingly. Materials are prepared as a white powder or as a concentrated solution by partial hydrolysis of corn starch with safe and suitable acids and/or enzymes. Maltodextrin is generally recognized as safe (GRAS) as direct human food ingredients at levels consistent with current good manufacturing practices.

In general, carbohydrates cannot be used as wall materials in the absence of a surface-active wall component because they generally have no emulsification properties. They are known to have beneficial stabilizing properties by their thickening or gelling effect on the continuous phase. However, incorporating carbohydrates into wall systems has been shown to improve the drying properties of the wall matrix probably by enhancing the formation of a dry crust around the drying droplets (Kagamil, Sugimura, Fujishima, Matsuda, Kometani &

Matsumura, 2003; Laplante, Turgeon, & Paquin, 2005a,b). Maltodextrin has been used in the microencapsulation of food components (Table 2.5).

Whey Protein Isolate

Whey protein is the name for a collection of globular proteins that can be isolated from whey, a by-product of cheese manufactured from cow's milk. It is a mixture of beta-lactoglobulin (~65%), alpha-lactalbumin (~25%), and serum albumin (~8%), which are soluble in their native forms, independent of pH. Whey has the highest biological value of any known protein. Whey protein typically comes in three major forms: isolate, concentrate and hydrolysate. Whey protein concentrates contain fat, lactose, carbohydrates, and bioactive compounds. Isolates are processed to remove the fat, lactose, and carbohydrates, yet are usually lower in bioactive compounds as well. They are nearly 90% protein by weight. Hydrolysates are predigested, partially hydrolysed whey proteins which consequently are more easily absorbed, but their cost is generally higher.

Whey protein isolate has been used as wall material for encapsulation because of its positive emulsifying properties in oil-in-water emulsion. Surface-active biopolymers rapidly adsorb to the surface of the oil droplets created during homogenization to form a protective membrane that prevents the droplets from aggregating (Silvestre, Decker, McClements, 1999). Whey protein isolate has been found to inhibit lipid oxidation in oil-in-water emulsions when it is either at the emulsion droplet surface or in the aqueous phase. The antioxidant mechanisms of Whey protein isolate has been attributed to its ability to (1) form cationic charges on the surface of emulsion droplets, which repel transition metals; (2) form thick viscoelastic films at emulsion droplet interfaces, which physically minimize lipid hydroperoxide-transition metal interactions; (3) chelate prooxidative metals; and (4) inactivate free radical through their sulfhydryl groups

and other amino acids (Hu, McClements, & Decker, 2003a). Some reports about using whey protein isolate in encapsulation also listed in Table 2.5.

Microscopy techniques

Light microscopy

Light microscopes are the most widely used instruments for visualization of structures down to the scale of a few micrometers. These instruments are available in numerous variants, suitable for selection of phase contrast, fluorescence, changing polarity of light, etc. The big advantage of light microscopes is their mechanical stability, allowing their operation under varying environments, and assuring their ease of operation. The fact that fully hydrated specimens may be visualized under natural environmental conditions (room temperature, atmospheric pressure etc.) remains the most important feature of light microscopic applications in biomedical research. Visible light as an imaging medium does not usually induce artificial changes in the specimen (Hoppert, 2003). Bright-field, polarizing, and fluorescence microscopy techniques are used most frequently. In conventional bright-field microscopy, illumination is transmitted sequentially through a condenser, the specimen, and the objective, producing a real image that is upside down and reversed as well as magnified within the microscope tube. The real image is then magnified within by the ocular lens, which produces either a virtual image that appears to be ~25 cm from the eye, or a real image on photographic film placed above the microscope tube. If the specimen is not highly colored, contrast must be introduced to make it visible. This is commonly achieved by the use of dyes or satins of known specificity for different components of the specimen.

Confocal Laser Scanning Microscope (CLSM)

CLSM has recently emerged as a significant new technique with exhibits several advantages over conventional optical microscope. The most important is that CLSM provides only an image of the in-focus plane with the out-of-focus parts appearing as black background so that not only the resolution is improved but also elimination of out-of-focus light allows observation of thick samples by enabling scanning in the Z-axis without interference from light above and below the focal plane. This leads to the possibility of generating three-dimentional (3D) images of thick transparent objects such as biological cells and tissues. In a similar way, it allows profiling of the surfaces of 3 D objects and multi-layer structures such as integrated circuits deposited on silicon, again by a non-contacting and non-destructive method (Sheppard & Shotton, 1997; Hoppert, 2003).

A typical arrangement of a CLSM is shown in Figure 2.6, where the system is built around a host conventional microscope. The essential components are a mechanism for scanning the light beam (usually from a laser) relative to the specimen and an appropriate photodetector to collect the reflected or fluorescent light. In the beam-scanning confocal configuration of Figure 2.6, the scanning is typically achieved by using vibrating galvanometer-type mirrors or acousto-optic beam deflectors. The use of the latter gives the possibility of TV-rate scanning, whereas vibrating mirrors are often relatively slow when imaging an extended region of the specimen (Wilson, 2002). Optical paths of CLSM are designed so that when the laser beam is focused in the specimen, it will be confocal to the point of light focused at the pinhole in the front of the photodetector. Then only information from the focal plane of interest reachs the photodetector. The laser provides the high degree of monochromaticity, small divergence, high brightness, high degree of spatial and temporal coherence, plane-polarized emission, and Gaussian beam profile

that makes it an almost ideal light source for use in confocal. However, if the pinhole size is sufficiently large, illumination is no longer confocal, and the system behaves as a wide field microscope (Takeuchi & Frank, 2001). A limitation in using CLSM compared to conventional microscopy and electron microscopy is that the laser used in CLSM produces light in limited narrow-wavelength bands, which are not available for excitation of all fluorophores. Most commonly used is the argon-ion laser that gives a strong excitation at 488 nm, which is suitable for the excitation of fluorescein and its derivatives. A mix-gas argon-krypton-ion laser provides lines at 488, 568, and 647 nm also used to analyze samples labeled with multiple dyes having different excitation and emission spectra (Pawley, 1995).

Scanning Electron Microscopy (SEM)

SEM is the technique for visualization of surfaces at the cellular and subcellular level. Its images have a great depth of focus, relatively easy to understand and have a strong three-dimensional plasticity (Kalab, Allan-Wojtas, & Miller, 1995). SEM routinely operate between the magnification rang of a stereo light microscope up to 100,000 fold (which is a

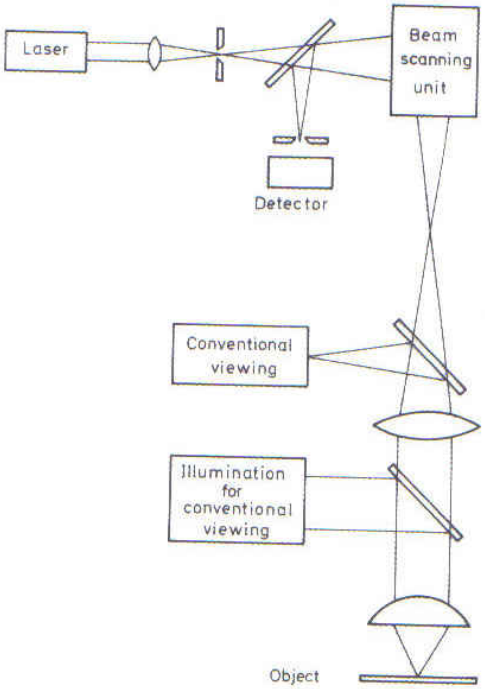


Figure 2.6. Schematic diagram of a confocal (Wilson, 2002)

comfortably large range), with a resolution limit of 2.5 μm. The SEM employs a finely focused electron beam to scan across the specimen surface. The diameter of the focused beam on the specimen surface determines the resolution of the image. The structures of interest must be stable after exposure to the electron beam, and also vacuum stable. For visualization in standard instruments, the specimen must remain stable after exposure to the electron beam.

In order to obtain good images of most non-conductive specimens under the SEM the sample must first be covered with a thin metal coating. This increase conductivity, and also reduces thermal damage and increase secondary and back-scattered electron emission (Hoppert, 2003). However, the drawback of conventional SEM is dimensional changes and shrinkage of soft biological specimen (Aguilera & Stanley, 1999) caused by sample dehydration or freezing prior to examination under the electron microscope the object has to be dehydrated or frozen. For most complex samples this requires chemical fixation and treatment with organic solvents or rapid freezing.

Microscopy techniques and the study of oil-in-water emulsion and encapsulation

Recently, studies on the structure of oil microencapsulated powder using microscopy techniques have been extensively reported. Heinzelmann, Franke, Jensen, & Haahr (2000) found that the fish oil encapsulated particles containing sodium caseinate with maltodextrin or lactose were very porous both in non-ground and ground powder. Kagami, Sugimura, Fujishima, Matsuda, Kometani, & Matsumura (2003) also found dents on the outer surfaces of fish oil microcapsules when maltodextrin (DE18) / sodium caseinate were used as wall materials. Díaz-Rojas, & et al (2004) investigated the surface of shark liver oil encapsulated in a matrix comprising linseed Na-pectate, alginate and chitosan. SEM revealed that the surface topography of the different formulations vary greatly. Some showed a rough tight structure with irregular

ridges, while others showed a somewhat smoother wall layer. Kolanowski, Laufenberg, & Kunz (2004) observed the microspheres of fish oil containing modified cellulose and maltodextrin as coating materials. The SEM images showed that particles formed spherical structures with an inside void. They also used the light microscope to observe fish oil emulsions with different oil concentrations. Klinkeson, Sophanodora, Chinachoti, Decker, & McClements (2006) observed the outer-surface of tuna-oil encapsulated in lecithin-chitosan/corn syrup solids. All samples contained spherical particles with surface wrinkles or dimples on there. Heertje, Van Aalst, Blonk, Don, Nederlof, & Lucassen-Reynders (1996) used CLSM to examine emulsifier adsorption on the interface between water and triacylglycerol. Lamprecht, Schafer, & Lehr (2000a) used CLSM to localize the encapsulated oil phase with Nile red distinguish the wall material structure by labeling with FITC. Macierzanha & Szelag (2006) observed the w/o droplet stabilized by APG-Zn77 emulsifier. Surge, Decker, & McClements (2006) showed the microstructures of oil-in-water emulsions coated by fish gelatin obtained by an optical microscope.

Light Scattering

This technique works by measuring the light scattered from particles as they pass through a laser beam. Particles scatter light at an angle that is directly related to their size. During a laser diffraction measurement, particles pass through a focused laser beam and scatter light at an angle that is inversely proportional to their size. As the particle size decreases so the scattering angle increases logarithmically, while the scattering intensity diminishes according to the particle's volume. Large particles therefore scatter light at narrow angles with high intensity whereas small particles scatter at wider angles but with low intensity. The resulting scattering pattern is used to calculate the particle size distribution (Malvern Instruments Ltd.). The system can analyze

emulsions, suspensions and dry powders and is therefore suitable for a wide range of applications.

Fourier Transform infrared (FTIR)

FTIR is a fast and dynamic technique for collecting infrared spectra of an enormous variety of compounds for a wide range of industries. There are two kinds of information that are typically obtained from the IR spectrum. These are qualitative and quantitative results. Qualitative analysis is defined as the molecular identification of unknown samples. Quantitative analysis is defined as the determination of component concentrations for known sample material.

FTIR relies on the fact that most molecules absorb light in the infra-red region (4,000 – 400 cm^{-1}) of the electromagnetic spectrum, this absorption corresponds specifically to the bonds present in the molecule. The sample is irradiated by a broad spectrum of infra-red light and the level of absorbance at a particular frequency is plotted after a fourier transformation of the data. The resulting spectrum is characteristic of the organic molecules present in the sample. Because chemical bonds absorb infrared energy at specific frequencies (or wavelengths) so that the basic structure of compounds can be determined by the spectral locations of their IR absorptions. In FTIR spectroscopy, the light is directed onto the sample of interest, and the intensity is measured using an infrared detector. The intensity of light striking the detector is measured as a function of the mirror position, and this is then Fourier-transformed to produce a plot of intensity vs. wavenumber (Thermo Electron Corporation; Maddams & Hendra, 1996). The FTIR spectra of chitosan and chitosan derivatives has been investigated (Osman, & Arof, 2003; Xing, Lui, Yu, Guo, Li, & Li, 2005). Kim & Thomas (2007) have shown the FTIR spectra of degraded chitosan and native chitosan. The main characteristic peaks of chitosan were at 3455 (O-H stretch), 2867 (C-H stretch), 1598 (n-H bend), 1154 (bridge O stretch), and 1094 cm-1 (C-O stretch). In the

spectrum of degraded chitosan, the peaks between 1610 and 1410 cm^{-1} were from amine residues. Kagami, Sugimura, Fujishima, Matsuda, Zaleska, Ring, & Tomasik (2001) showed the IR spectra of whey protein isolate (WPI) and WPI-starch that the spectra of complexes presented almost precise superposition of the spectra of both components. Kometani & Matsumura (2003) used FTIR to measure the surface oil of the microcapsules. Loret, Schumm, Puden, Frith, & Fryer (2005) have shown the pure spectra of maltodextrin and agarose. Kosaraju, D'ath, & Lawrence (2006) used FTIR to study the structural interactions of polyphenolic compounds in olive oil leaf extract following chitosan encapsulation.

The overall objectives of the following experiments were to develop a new encapsulation process for tuna-oil oil by ultrasonic atomization and freeze-drying and to evaluate the properties of the encapsulated oil powder. The first part of the study investigated the feasibility of encapsulating tuna-oil in a chitosan microcapsule using an ultrasonic atomizer to disperse the coated oil droplets into a medium that solidified the wall material. The second part was examined the oxidative stability of tuna oil in emulsions and in the microcapsules. In addition, the relationship between emulsion structure and lipid oxidation was elucidated. The last phase; (a) explored oil localization in emulsion by using CLSM; (b) examined the outer-surface of the encapsulated particles by means of Scanning Electron Microscopy, and (c) investigated the relationship between FTIR and the ability of encapsulation.

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CHAPTER 3

CHITOSAN-BASED TUNA OIL EMULSION, ENCAPSULATED POWDER AND THEIR CHARACTERIZATION

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Abstract

The new encapsulation technique using an ultrasonic atomizer was completed in three stages: (1) emulsification (2) atomization (ultrasonic atomizer), and (3) freeze drying. The variables in the emulsion preparation process such as concentration of wall materials [chitosan, maltodextrin, and whey protein isolate] and tuna oil were optimized. The emulsions were evaluated for droplet size and stability. After freeze drying, the characteristics of the encapsulated powders were determined. The optimum ratios of chitosan to maltodextrin and chitosan to whey protein isolate at 20 % (w/w) tuna oil were 1:10 and 1:1, respectively. There was a significant difference ($P<0.05$) in particle sizes of the emulsions when the preparation conditions were varied with the combination of chitosan and maltodextrin giving the smallest particle size and the highest emulsion stability. The encapsulated powder had excellent properties for a functional food ingredient including adequate EPA and DHA content level > 100 mg/g, low moisture content and water activity, acceptable appearance and encapsulation efficiency. The novel microencapsulation technology developed could have numerous applications in the food industry to improve stability of tuna or other oils.

Keywords: encapsulation, emulsion, ultrasonic atomizer, tuna oil, chitosan

Introduction

Fish oils are an excellent source of omega-3 polyunsaturated fatty acids (PUFA) [Eicosapentaenoic acid (EPA, C20:5 n-3) and Docosahexaenoic acid (DHA, 22:6 n-3)]. Studies indicate that fish oils may have health benefits for the heart, brain, and nervous system. Potential mechanisms for the cardioprotective effect of omega-3 fatty acids include anti-arrhythmic, anti-inflammatory, hypotriglyceridemic effects, lowered blood pressure, and improved endothelial function (Balk, Lichtenstein, Chung, Kupelnick, Chew, & Lau, 2006). However, fish oil has a strong odor and unless protected it is easily oxidized.

Encapsulation is the technology of packing solid, liquid or gaseous materials in miniature sealed capsules for release at controlled rates using desired release triggers. The simplest microcapsule consists of a core surrounded by a wall or barrier. The core is the component requiring protection that may be composed of one or more ingredients. The wall may be single or multi-layered (Pothakamury & Barbosa-Canovas, 1995). This technology has been applied in recent food and beverage research with respect to: 1) controlling the release of active ingredients and protecting ingredients from the environment until used in a product, 2) reducing rancidity development in lipid bearing foods and ingredients, 3) reducing flavor loss during the product shelf life, 4) extending flavor perception and mouthfeel over a longer period of time, and 5) enhancing bioavailability and efficacy (Berry, 2004; Shefer & Shefer, 2003).

Little information is available on encapsulation of fish oil using chitosan (CS) as a wall material. CS, a derivative produced by N-deacetylation of chitin, is receiving worldwide interest for food applications because it is from a natural resource, biologically renewable, biodegradable, biocompatible, almost nontoxic, and biologically functional. In the food industry, its applications include use as antimicrobial, edible film, clarification and deacidification of fruit

juices, control of enzymatic browning in fruits, and purification of water (Shahidi, Arachchi, & Jeon, 1999). In recent years, CS has been used as a wall material for encapsulation of sensitive core ingredients such as lipophilic drugs (Ribeiro, Neufeld, Arnaud, & Chaumei, 1999), vitamin D2 (Shi & Tan, 2002), astaxanthin (Higuera-Ciapara, Felix-Valenzuela, Goycoolea, & Argüelles-Monal, 2004), and olive oil extract (Kosaraju, D'ath, & Lawrence, 2006). Recently, encapsulation of fish oil by spray-drying with various wall materials has been studied. Keogh & et al., (2001) used a casein/lactose wall material. Kagami & et al., (2003) used combinations of a highly branched cyclic dextrin or 18 DE (dextrose equivalent) maltodextrin with sodium caseinate. Kolanowski, Laufenberg, & Kuna (2004) used modified cellulose, especially methylcellulose as wall material. Drusch, Serfert, Heurel, & Schwarz, (2006) indicated that trehalose was better than glucose syrup as wall material. However, one limitation of the spray drying technology is the need for water soluble wall materials. Almost all spray drying processes employ aqueous feed dispersion (Barbosa-Canovas, Ortega-Rivas, Juliano, & Yan, 2005). Microencapsulated fish oil produced by freezing and subsequent freeze drying gave a product with high quality and oxidation stability (Heinzelmann & Franke**,** 1999). This was in accordance with the findings of Heinzelmann, Franke, Jensen, & Haahr, (2000) on the effect of formulation and freeze drying process variables on the oxidative stability of microencapsulated fish oil. Another encapsulation process used a double emulsification and an enzymic gelation method with microbial transglutaminase to cross-link proteins to prepare protein-based microcapsules containing fish oils with improved stability and controlled release (Cho, Shim, & Park, 2003).

An Ultrasonic atomization of CS stabilized fish oil emulsion into a reactive broth that sets the CS wall around the oil is proposed as a novel microencapsulation process investigated in this study. The use of CS as the wall material takes advantage of the structural integrity of the CS

wall, its antioxidative properties, and dissolution under aqueous acidic condition. We hypothesize that ultrasonic atomization will be mere gentle to the polymer than centrifugal atomizer resulting in an intact wall with maximal protective effect on the fish oil. In the present study, parameters affecting the formulation, emulsification and atomization process were optimized.

Materials and methods

Materials

Chitosan (CS) with a degree of deacethylation (DD) = 80 and tuna oil were purchased (T. C. Union Company, Samutsakorn, Thailand). Maltodextrin (MD), M040, M100, M150 and M180 with an average dextrose equivalent (DE) of 5, 10, 15 and 18, respectively, was provided by GRAIN Processing Corporation (Muscatine, IA). Whey protein isolate (WPI) was supplied by AMPC, Inc., (Ames, IA). Fatty acids (GLC-68 D) standard and methyl tricosanoate (C23:0) standard were purchased from Nu-chekprep, INC., (Elysian, MN). Boron trifluoride and acetate buffer were purchased from Sigma-Aldrich Company (St. Louis, MO). The emulsifier used was Tween 80 (Fisher Scientific, FairLawn, NJ). Acetic acid, methanol, isooctane, petroleum ether, isopropanol, hexane, and sodium hydroxide were purchased from Mallinckrodt Baker, Inc., (Phillipsburg, NJ).

Analysis of fatty acids

Tuna oil methyl ester was prepared according to the AOAC Official Method 991.39 (AOAC, 2000). The fatty acid composition was then analyzed by a gas chromatography (Hewlett Packard 5890 series II, Ramsey, MN) equipped with a flame ionization detector and a fused silica capillary (25 m × 0.2 mm ID × 0.33 μm) (J&W Scientific, Alltech Associates Inc., Deerfield, IL.). Operating conditions were as follows: temperature-injection port 250°C;

detector temperature 270°C; oven programmed from 70 to 240°C at 20°C/min. Nitrogen was the carrier gas.

Optimization of emulsion

Parameters for optimization included: DE of MD (5, 10, 15, 18), concentrations of MD (5, 10, 15, 20 % (w/w)), concentrations of WPI (0.5, 1.0, 1.5, 2.0 % (w/w)), time (20, 30, 40, 50, 60 min) and rpm (3000, 3500, 4000, 4500, 5000) of homogenization, and the amplitude of atomizer (50, 55, 60, 65). The optimal combination of parameters was found to be: 10 % (w/w) MD and 1 % (w/w) WPI with homogenization speed at 5,000 rpm for 30 min and with an amplitude of 55.

CS with various concentrations (0.5, 1.0, 1.5 % (w/w)) was first dispersed in 0.25% (v/v) aqueous acetic acid and continuously stirred at room temperature until the mixture was completely dissolved by visual examination. The 10 % (w/w) MD or 1 % (w/w) WPI was slowly added. When total dissolution was observed visually, tuna oil (10, 20, 30 % (w/w)) previously mixed with Tween 80 (2.5 % (w/w)) was added to the solution. The mixture was then emulsified using a homogenizer (OMNI International, Waterbury, CT) at 5,000 rpm for 30 min.

Particle size distribution

The size distribution of emulsion droplets was determined by a light scattering instrument (Mastersizer, Model MSS, Malvern Instruments Ltd., Worcestershire, UK).

Emulsion stability

Each emulsion (10 ml) was placed in a test tube and stored at 25°C for 1 month. Each tube was visually evaluated daily by recording the depths in centimeters of a distinctive clear serum lower phase. The results were compared and expressed as the creaming index (%) of the total emulsion height in the tubes. Creaming index = 100 X (the height of formed serum

layer/total height of the emulsion) (Klinkesorn, Sophanodora, Chinachoti, & McClements, 2004).

ζ-Potential measurement

Each emulsion was centrifuged at 8,000 rpm for 30 min then the cream layer was collected and diluted with acetate buffer to a ratio of 1:50 (emulsion:buffer). Diluted emulsion was poured in a cuvette and then put into a chamber of a particle size analyzer (BTC Brookhaven Instruments Corporation, Holtsville, NY). The ζ-Potential measurement are reported as the average and standard deviation of measurements with five reading made per sample.

Microstructure

The microstructure of the emulsions was determined using a light microscope (Nikon Eclipse E600, Southern MicroInstruments, Marietta, GA). The emulsion was diluted 1:5 from the original concentration to avoid the particles clumping together. A drop of emulsion was observed at an objective magnification of 100X. Images of the structure for all emulsions were acquired using image processing (MagnaFire software, Optronic Laboratories, Inc., Goleta, CA) with a CCD camera and stored on a computer.

Ultrasonication process

An ultrasonic atomizer (Model CPX134PB; Cole-Parmer instruments, Vernon Hills, IL), equipped with an ultrasonic probe with a 1/32 inch diameter was used in this study. It was operated at 40 kHz with 130 W high-intensity, and at 120 V. The amplitude of probe oscillation was set at 55 in order to effectively optimize the solution. Each emulsion sample was pumped into the ultrasonic atomizer through the ultrasonic probe using a peristaltic pump (Model 7523-50, Cole-Parmer Instrument, Barrington, IL). The liquids were dispersed as droplets from the vibrating tip into a NaOH solution (1 % w/v) that solidified the CS. The microsphere suspension

obtained was then centrifuged, filtered, washed with distilled water, frozen at -80 °C overnight, and dried in a freeze drier for 24-48 hr. The encapsulation process using the ultrasonic atomizer system is described in Figure. 3.1. When the freeze-drying process was completed, the encapsulated powder was kept in amber-colored bottles and stored at 4 °C for further characterization of its properties.

Characteristics of encapsulated tuna oil

Fatty acid composition

Fatty acid composition in the encapsulated tuna oil was determined by using the AOAC Official Method 991.39 (AOAC, 2000) with the same GC analysis condition.

Particle size diameter

The particle size of the encapsulated powder was determined by using a light scattering instrument (Mastersizer Model MSS, Malvern Instruments Limited).

Color

The L, a, b values of powders were measured using a colorimeter (Minolta Data Processor DP-301, CR-300 series, Minolta Co., Ltd., Japan). L is the lightness, while a and b represent the colors where -a is greenness, +a is redness, -b is blueness, and +b is yellowness.

Moisture content

The powder was dried in a vacuum oven at 105 °C until constant weight was reached. Percent loss in weight was reported as water content.

Water activity (a_w)

Water activity (a_w) was measured at 25 ± 0.50 °C with an Aqua Lab water activity meter (Model CXZ, Decagon Devices, Inc., Pulman, WA).

Encapsulation Efficiency (EE)

The equation below was used to derive the EE.

$$EE = \frac{[\text{Total oil content (g/100g powder)} - \text{Free oil content (g/100g powder)}] \times 100}{\text{Starting oil content (wt\% DM)}}$$

Free oil content

The free oil was extracted using the same method as describe by Millqvist-Fureby (2003) for free fat extraction.

Total oil content

The method of Klinkesorn, Sophanodora, Chinachoti, Decker, & McClements (2006) was used to determine the total oil content with a slight modification in the sample size from 0.5 g to 1.0 g.

Data analysis

The data were analyzed by the analysis of variance (ANOVA) using SAS version 9.1 (SAS Institute, Cary, NC). Duncan's multiple range test was used to compare the means with a significance level of $P< 0.05$.

Results and Discussion

Emulsion stability

The stability of tuna oil in water emulsions stabilized by various concentrations of CS is shown in Figure 3.2 (a-c). Creaming index of emulsions containing only CS at all concentrations and at 0.5 % (w/w) mixed with either MD or WPI showed the highest value as compared to the others. However, there was no creaming in emulsions containing CS 1.0 % (w/w) mixed with MD at 10 and 20 % (w/w) tuna oil. On the other hand, a small but significantly higher ($P<0.05$) creaming rate was observed in emulsions made from CS 1.0 and 1.5 % (w/w) mixed with WPI than those containing MD at both concentrations. Dickinson (2003) stated the most initial

manifestation of instability of oil in water emulsion is creaming which can lead to phase separation with a distinctive clear or semi-transparent lower serum phase and cream. This may then be followed by droplets coalescence within the cream and oiling off at the top of the sample. The results of our study clearly indicated that CS alone is unable to produce a stable emulsion. The emulsion is most stable when mixed with MD or WPI in proper ratios. These results are in agreement with Laplante, Turgeon, & Paquin (2005b) who have also observed the inability of CS or WPI alone to produce stable emulsions. Rodriguez, Albertengo, & Agullo (2002) have also shown the emulsification capacity of CS that combines both electrosteric and viscosifying stabilization mechanisms. In addition, CS is a unique polysaccharide of a cationic nature with hydrophilic zones rich in glucosamine and hydrophobic zones rich in N-acethyl-glucosamine which enable it to adsorb at the oil/water interfaces. Interaction between CS and WPI can co-adsorb at the oil/water interface resulting in higher emulsion stability caused by increased interfacial electrostatic stability (Laplante, Turgeon, & Paquin, 2005a). The minimal concentration of 1.0 % (w/w) CS combined with MD showed the highest emulsion stability at every oil concentration used. MD is used for oil/water emulsion as texture modifiers to modify viscosity or gelation of the aqueous continuous phase surrounding the oil droplets. Moreover, for this work we used MD with low DE (DE=5) which has a higher percentage of long oligosaccharide chains that can form strong network structures within the continuous phase to hold the droplets in place and can combine with CS which effects viscosity to result in higher emulsion stability.

Particle size analysis of emulsions

Average particle diameter (D3,2) of the oil/water emulsions ranged from 0.8 to 14.1 μm and are listed in Table 3.1. There was no significant changes ($P<0.05$) in average droplet

diameter between day 0 and day 30 when CS was prepared at 1.0 and 1.5 % (w/w) mixed with 10 % (w/w) MD containing 10 or 20 % (w/w) tuna oil. Yet, these emulsions had the lowest range of average particle size ranging between 0.8 and 1.4 μm. Our results are in agreement with the generally accepted principle indicating the smaller emulsion droplets are more physically stable than the larger emulsion droplets. At all concentration of tuna oil, CS at 0.5 % (w/w) mixed with 1 % (w/w) WPI, or with 10 %(w/w) MD produced significantly larger ($P<0.05$) droplets after 30 days of storage. The results could suggest that CS concentration at 0.5 % (w/w) was inadequate to maintain emulsion stability. Generally, the coalescence rate is fundamentally related to the particle size distribution with the rate of coalescence being slow when the droplet size is small (Damodaran, 1997). These results confirm that physical stability and droplet size of the emulsions depend significantly on the proper ratio of wall materials and percent oil load. The examples of droplet size distribution from a light microscope are shown in Figure 3.3.

Influence of droplet charge

The droplets in most food emulsions have an appreciable electrical charge and the ability of a charged polyelectrolyte to adsorb to the surface of an oppositely charged droplet should depend on the electrical characteristics on the droplet, as well as on the molecular characteristics in the continuous or dispersed phase, especially on pH and ionic strength (McClements, 2005). In this section, we therefore investigated the effect of droplet charge (ζ-potential) on the adsorption of CS and CS mixed with MD or WPI to oil droplet surfaces. In the absence of biopolymers (CS and CS mixed with MD or WPI), a negative ζ-potential was found with Tween-stabilized droplets in agreement with Hsu & Nacu (2003) who found that oil droplets stabilized by non-ionic surfactants tend to have a negative charge. However, the addition of biopolymers caused the ζ-potential to go from negative to positive (Figure 3.4). This result suggests that

biopolymers did adsorb to the surface of the Tween-coated droplets. When using only CS at all concentrations, higher CS concentrations produced greater ζ-potential that the net positive charge might be due to from the electrostatic attraction between the cationic CS and the anionic droplet surface, thereby reducing the negative charge on the oil droplets. However, the concentration of CS might not be enough to stabilize the emulsion causing the droplets to frequently collide with each other and over an extended period, oil droplets coalescence resulted in creaming of the emulsion. CS mixed with MD or WPI increased a positive ζ-potential similarity (no significant difference, $P<0.05$) and resulted in stable emulsions (Figure 3.2). As mentioned above that interaction between CS and WPI can co-adsorb at the oil/water interface and caused an increase in the interfacial electrostatic interaction to prevent the droplets from coming close together to form aggregates. More than that, the high positive ζ-potential was probably due to the positive charge derived from both CS and WPI. Basically, WPI have acidic and basic groups whose degree of ionization depends on the pH and ionic strength of the surrounding aqueous phase. For these emulsions, WPI exhibit the positive charge because the pH of the emulsions was below its isoelectric point (4.8 ± 0.2). CS mixed with MD also showed a high positive charge and gave the highest emulsion stability (Figure 3.2). It is possible that the MD molecules bound anionic surface active lipids that were originally associated with the tuna oil droplets, thereby reducing the negative charge on the droplets. MD also increases the viscosity of the aqueous phase, which retards the movement of oil droplets, thereby inhibiting their coalescence. We noticed that CS concentrations at 1.0 and 1.5 % (w/w) mixed with WPI or MD containing 10 or 20 % (w/w) tuna oil showed no significant difference ($P<0.05$) in ζ-potential. The possible reason might be that the charge density of the continuous phase is sufficiently high then when the surface charge of droplets reaches a critical value, strong

electrostatic repulsion between the droplet surface and charged polyelectrolytes in the continuous phase will limit further adsorption of the polyelectrolytes. Thus, the final ζ-potential of the droplets is determined by this critical value (Mun, Decker, & McClements, 2006). However, partial desorption of biopolymer molecules from the droplet surfaces may occur and can lead to some bridging flocculation resulting in emulsion instability with extended storage periods.

Characterization of encapsulated tuna oil powder

Fatty acid composition

The fatty acid composition of encapsulated tuna oil powder was analyzed and the results are shown in Table 3.2. The fatty acid composition of tuna oil before encapsulation had a slightly higher percent of total saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) but was lower in monounsaturated fatty acid (MUFA) composition. The major fatty acids present in all of them were C16:0, C18:1, C20:5 and C22:6 with EPA and DHA compositions reduced from 28.3 % to 24.9 % and 24.3 % for encapsulated powder made from CS 1.0 % (w/w) mixed with MD 10 % (w/w) containing 20 % (w/w) tuna oil (A) and encapsulated powder made from CS 1.0 % (w/w) mixed with WPI 1.0 % (w/w) containing 20 % (w/w) tuna oil (B), respectively. The results showed that the encapsulation process using an atomizer can still preserve the important fatty acids, EPA and DHA content. The content in our product was slightly higher than the commercial specification of 100 mg/g. There was a large increase in eicosenoic acid (C20:1n-9) after the encapsulation process for both A and B formulations.

Moisture content and a_w

The moisture content of both encapsulated A and B were 2.89 % and 3.02 %, respectively (Table 3.3). The results were under the maximum moisture specification (3 % and 4 %) for most dried powders in the food industry (Klinkesorn & et al., 2006) with a_w about 0.3.

Color

The lightness of encapsulated powder made from CS and MD was higher than that made from CS and WPI (Table 3.3). This was probably due to the fact WPI has a yellow color. The entire encapsulation process, however, had little effect on the color of the finished product.

Encapsulation efficiency (EE)

The encapsulated powder made from CS and WPI (B) has a lower EE than the other one (A) (Table 3.3). This was consistent with the results of emulsion stability and particle size analysis. The wall material interaction between CS and MD has the best capacity to prevent emulsion instability and has smaller particle size.

Conclusions

Using an ultrasonic atomizer for tuna oil encapsulation is a promising alternative method. The encapsulated powder, made from stable emulsion formulations, contains high EPA and DHA and low moisture content (~3 %). The powder has acceptable color and acceptable EE. However, the effects of ingredients used for encapsulation on the oxidation of tuna oil need to be further studied.

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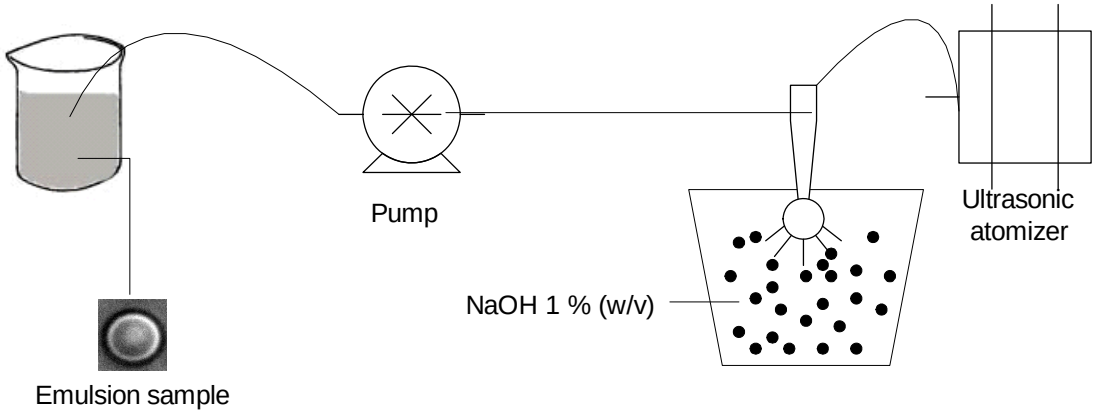
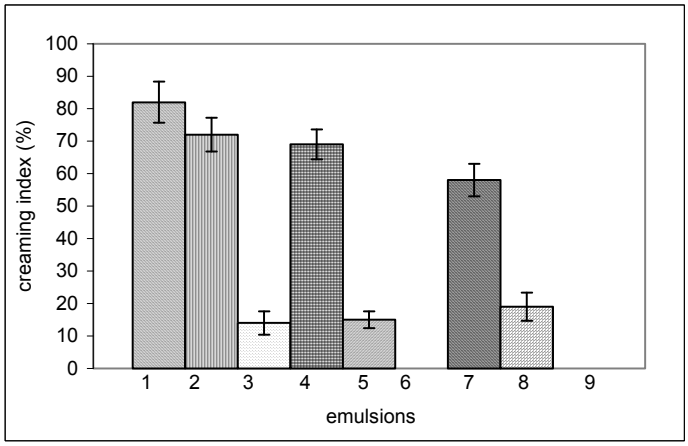
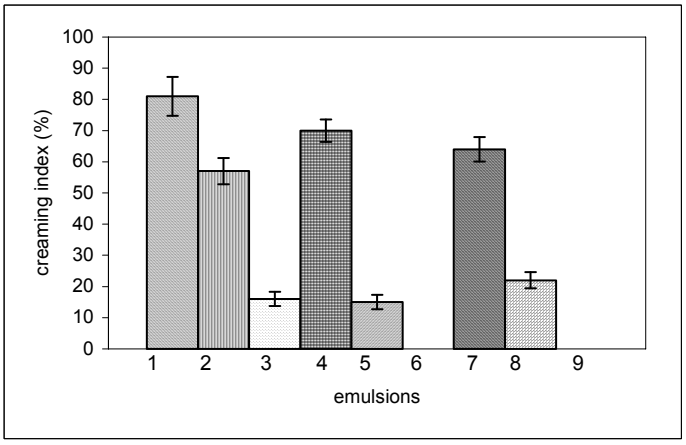


Figure 3.1. Schematic description of the encapsulaion system using an ultrasonic atomizer

(a)



(b)



(c)

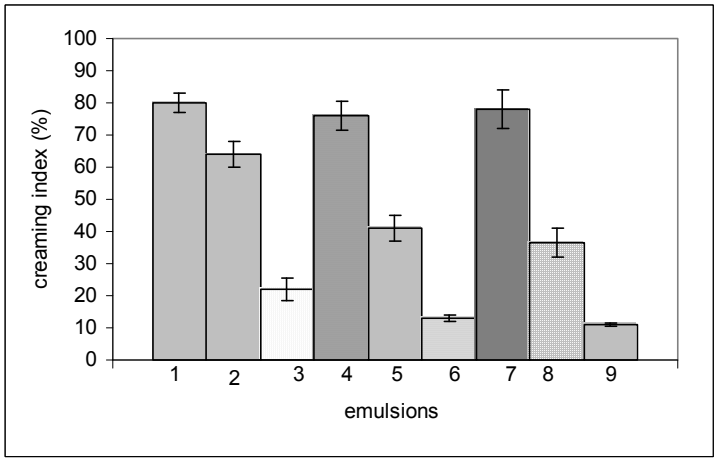
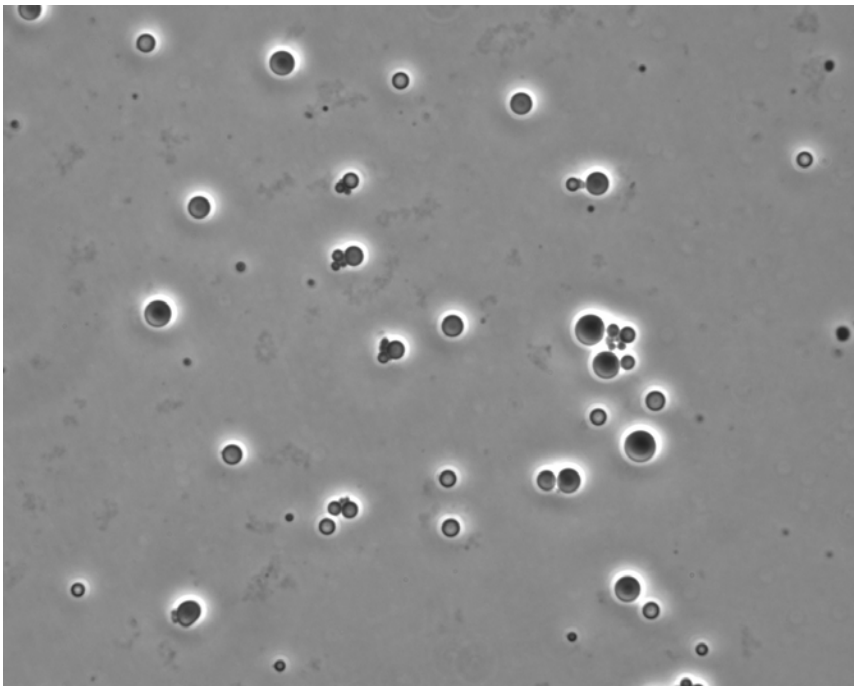


Figure 3.2. Effect of CS concentration mixed with 1 % (w/w) WPI and 10 % (w/w) MD (DE=5) on the creaming rate of (a) tuna oil 10 % (w/w), (b) tuna oil 20 % (w/w) and (c) tuna oil 30 %(w/w) emulsion after 30 days storage at room temperature.

Where 1 = CS 0.5 % (w/w) 2 = CS 0.5 % (w/w) + WPI 1 % (w/w)
3 = CS 0.5 % (w/w) + MD 10 % (w/w) 4 = CS 1.0 % (w/w)
5 = CS 1.0 % (w/w) + WPI 1% (w/w) 6 = CS 1.0 % (w/w) + MD 10 % (w/w)
7 = CS 1.5 % (w/w) 8 = CS 1.5 % (w/w) + WPI 1 % (w/w)
9 = CS 1.5 % (w/w) + MD 10 % (w/w)

(a)



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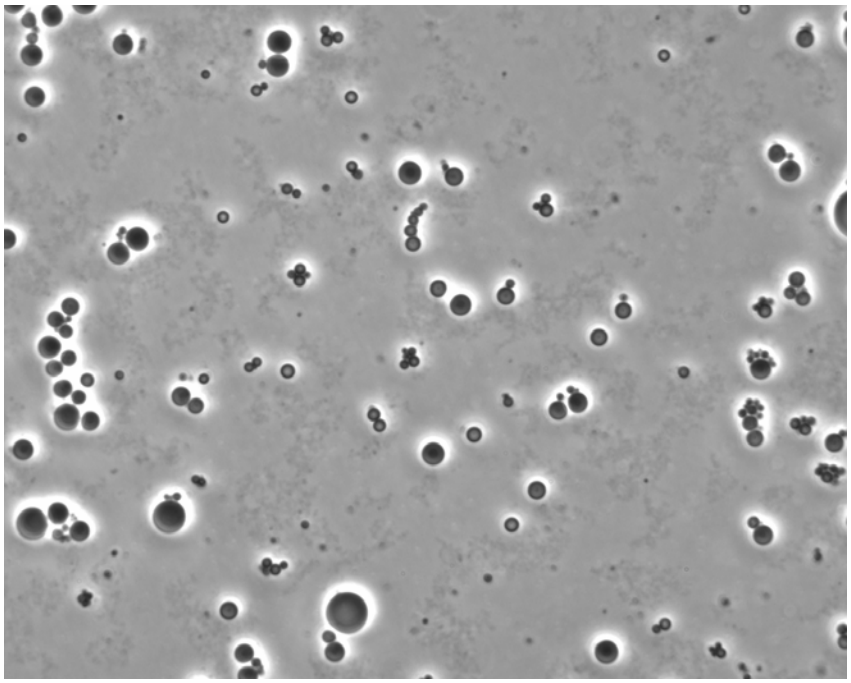
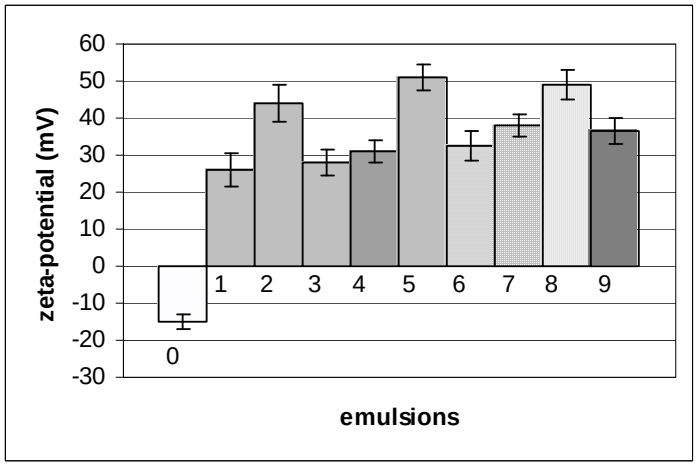
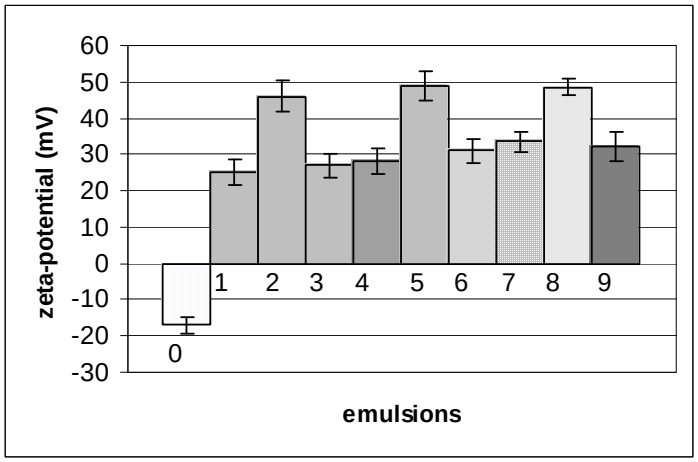


Figure 3.3. Image of samples of tuna oil at 20 % (w/w) emulsions: (a) CS 1% (w/w) mixed with MD 10 % (w/w); (b) CS 1 % (w/w) mixed with WPI 1 % (w/w) using light microscope.

(a)



(b)



(c)

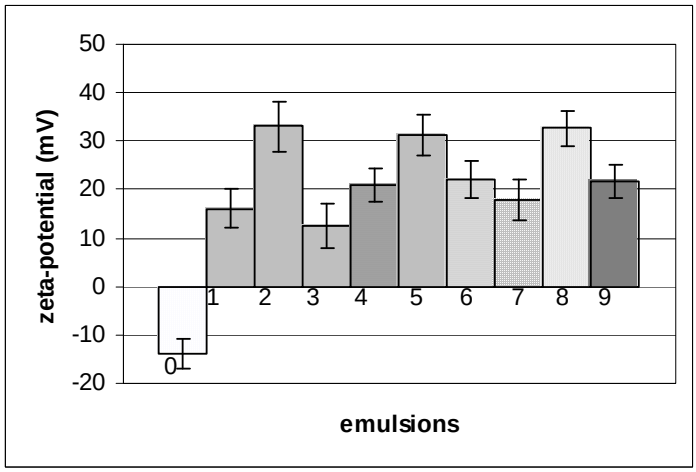


Figure 3.4. Influence of CS concentration mixed with 1 %(w/w) WPI and 10 % (w/w) MD (DE=5) on electrical charge of emulsion droplets (zeta-potential) of (a) tuna oil 10 % (w/w), (b) tuna oil 20 % (w/w) and (c) tuna oil 30 %(w/w)

Where 0 = no CS
1 = CS 0.5 % (w/w)
2 = CS 0.5 % (w/w) + WPI 1 % (w/w)
3 = CS 0.5 % (w/w) + MD 10 % (w/w)
4 = CS 1.0 % (w/w)
5 = CS 1.0 % (w/w) + WPI 1 % (w/w)
6 = CS 1.0 % (w/w) + MD 10 % (w/w)
7 = CS 1.5 % (w/w)
8 = CS 1.5 % (w/w) + WPI 1 % (w/w)
9 = CS 1.5 % (w/w) + MD 10 % (w/w)

Table 3.1. Average particle diameter (D3,2) of CS at various concentrations (0.5, 1.0 or 1.5 %(w/w)) mixed with 1 %(w/w) WPI or 10 %(w/w) MD (DE=5) containing 10, 20 or 30 %(w/w) tuna oil (TO) emulsions at day 0 and day 30

Emulsions	Average particle size diameter (D3,2) (μm)					
	day 0			day 30		
	10%(w/w)TO	20%(w/w)TO	30%(w/w)TO	10%(w/w)TO	20%(w/w)TO	30%(w/w)TO
CS 0.5 %(w/w)	1.56±0.12b	2.26±0.09b	2.64±0.15b	11.89±1.36a	10.87±2.09a	12.12±1.98a
CS0.5%(w/w)+WPI1%(w/w)	1.23±0.06b	2.17±0.11b	2.24±0.09b	9.88±1.56a	11.36±1.78a	11.29±0.99a
CS0.5%(w/w)+MD10%(w/w)	1.11±0.08b	1.80±0.07b	1.91±0.14b	8.89±1.06a	9.25±1.56a	9.37±1.68a
CS 1 %(w/w)	1.19±0.07b	2.01±0.08b	2.06±0.11b	10.05±1.89a	10.89±1.56a	11.16±1.92a
CS1%(w/w)+WPI1%(w/w)	0.92±0.03d	0.96±0.03d	1.35±0.03c	2.19±0.15b	2.25±0.21b	4.75±0.88a
CS1%(w/w)+MD10%(w/w)	0.85±0.06b	0.88±0.05b	0.89±0.04b	1.22±0.08b	1.26±0.14b	3.96±0.08a
CS 1.5 %(w/w)	0.98±0.08b	2.15±0.07b	2.18±0.06b	10.08±1.32a	10.85±1.87a	9.89±1.02a
CS1.5%(w/w)+WPI1%(w/w)	0.92±0.06d	0.91±0.11d	1.37±0.02c	2.97±0.13b	3.08±0.20b	4.56±0.12a
CS1.5%(w/w)+MD10%(w/w)	0.84±0.04d	0.87±0.06d	0.92±0.02d	1.05±0.04c	1.29±0.08b	3.45±0.05a

Values are average of triplicate analyses ± standard deviation

Within rows, means followed by different letters differ significantly ($P<0.05$)

Table 3.2. Fatty acid composition in tuna oil (g/100g)[a]

fatty acids	tuna oil	encapsulated tuna oil powder	
		A	B
C14:0	3.80 ± 0.20	3.20 ± 0.40	2.90 ± 0.40
C16:0	21.20 ± 0.95	19.30 ± 1.10	18.54 ± 1.09
C16:1	5.90 ± 0.36	5.80 ± 0.60	5.20 ± 0.52
C18:0	6.50 ± 0.70	5.40 ± 0.09	5.23 ± 0.60
C18:1	14.60 ± 1.06	13.60 ± 0.89	11.91 ± 0.87
C18:2n-6	1.60 ± 0.09	1.35 ± 0.05	1.47 ± 0.03
C18:3n-3	1.10 ± 0.05	0.93 ± 0.00	0.97 ± 0.03
C20:0	0.60 ± 0.03	1.30 ± 0.12	1.32 ± 0.05
C20:1n-9	6.10 ± 0.30	12.40 ± 1.06	11.7 ± 0.95
C20:2n-6	2.10 ± 0.12	1.20 ± 0.15	1.00 ± 0.00
C20:4n-6	2.20 ± 0.10	1.30 ± 0.26	2.98 ± 0.36
C20:5n-3	6.70 ± 0.40	5.20 ± 0.80	5.40 ± 0.70
C22:0	1.00 ± 0.02	2.40 ± 0.32	2.72 ± 0.20
C22:6n-3	21.60 ± 1.54	19.70 ± 1.26	18.90 ± 1.21
C24:1	1.50 ± 0.10	1.60 ± 0.06	1.70 ± 0.12
unidentified	3.50	5.32	8.06
total	96.50	94.68	91.94
EPA+DHA	28.30	24.90	24.30
Saturated fatty acid (SFA) = 33.10%		31.60	30.71
Monounsaturated fatty acid (MUFA) = 28.10%		33.40	30.51
Polyunsaturated fatty acid (PUFA) = 35.30%		29.68	30.72

[a]Average ± standard deviation (n=4)
A = encapsulated powder made from CS 1 %(w/w) mixed with MD 10 %(w/w) containing 20 %(w/w) tuna oil
B =encapsulated powder made from CS 1 %(w/w) mixed with WPI 1 %(w/w) containing 20 %(w/w) tuna oil

Table 3.3. Properties of the encapsulated powder A and B after freeze drying

Properties	Encapsulated tuna powder	
	A	B
Shape	Spherical	Spherical
particle size (μm)	33.4 ± 3.2	38.7 ± 2.9
Color	*L*=94.6, *a*=0.25, *b*=4.29 White to off-white	*L*=86.99, *a*=-0.3, *b*=15.23 off-white
encapsulation efficiency (%)	83.5 ± 1.25	79.3 ± 1.56
moisture content (%)	2.89 ± 0.04	3.02 ± 0.03
a_w	0.30 ± 0.02	0.32 ± 0.01

A = encapsulated powder made from CS 1 %(w/w) mixed with MD 10 %(w/w) containing 20 %(w/w) tuna oil
B =encapsulated powder made from CS 1 %(w/w) mixed with WPI 1 %(w/w) containing 20 %(w/w) tuna oil

CHAPTER 4

OXIDATIVE STABILITY AND STRUCTURE OF CHITOSAN-BASED TUNA OIL EMULSION AND ITS ENCAPSULATED POWDER FORMED BY ULTRASONIC ATOMIZER

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Abstract

The ability of chitosan mixed with maltodextrin or whey protein isolate to improve oxidative stability with and without α-tocopherol both in oil-in-water emulsions and tuna oil encapsulated powder derived by ultrasonic atomization technique was investigated. Peroxide and Anisidine values were used to measure the primary and secondary oxidation changes, respectively. Zeta-potential and structural changes of emulsions during storage at 37 °C were also investigated. There was significantly improved in oxidative stability of all emulsions with or without α-topherol compared to bulk tuna-oil. However, the zeta-potential was not solely related to their ability to produce the emulsions with high oxidative stability. The structure of emulsion droplets changed as Peroxide or Anisidine values increased, they came closer together and became bigger. This study could lead to the application of the tuna oil encapsulated powder with the high oxidative stability to improve the ω-3 content in some food products.

Keywords: encapsulation, emulsion, oxidative stability, chitosan, tuna oil

Introduction

The health benefits of fish oil which might help in improve brain and eye function, preventing cardiovascular disease have been well known for decades (Lands, 2005). However, fish oil is easily oxidized that can alter the flavor and nutritional quality and might further produce toxic compounds. These problems have been major challenges to utilize oils that contain high concentrations of polyunsaturated fatty acids in foods. The encapsulation technique has been developed to turn reactive, sensitive or volatile additives into stable ingredients (Gouin, 2004).

In the encapsulation process, emulsion is an important step that must be considered to prevent oxidation. The greater the oxidative stability of the emulsion, the higher the quality of encapsulated fish oil powder.

In recent years, studies have been concentrated on oxidation in oil-in-water emulsions systems. Studies on oxidative stability have examined corn oil (Frankel, Huang, Kanner, & German, 1994; Hu, McClements, & Decker, 2003b; Surh, Decker, & McClements, 2006), menhaden oil (Donnelly, Decker, & McClements, 1998), salmon oil (Mancuso, McClements, & Decker, 1999; Silvestre, Chaiyasit, Brannan, McClements, & Decker, 2000; Hu, McClements, & Decker, 2003a), and algae oil (Hu, Mc Clements, & Decker, 2004). The oxidation of bulk lipids has been studied extensively, not only to identify the products of lipid oxidation and the conditions that influence their production, but also to study the mechanisms involved (Nawar, 1996).

Additives used to prevent oxidation of oils that have been studied, Xie, Xu, & Liu (2001) indicated that the NH_2 groups of chitosan support antioxidant activity by reacting with an OH radical to form stable macromolecule radicals. Kamil, Jeon, & Shahidi (2002); Klinkeson,

Sophanodora, Chinachoti, McClements, & Decker (2005); Kim & Thomas (2006) further indicated the potential of chitosan to be used to produce oxidative stability in the high lipid food systems. Kagami, Sugimura, Fujishima, Matsuda, Kometani, & Matsumura (2003) have shown the ability in antioxidant of maltodextrin as a wall material for fish oil encapsulation. The possible mechanism for whey proteins as an antioxidant was suggested by Tong, Sasaki, McClememt, & Decker (2000b). However, few reports on the antioxidant activity of those wall materials for preventing oxidation of tuna oil are available in the literature.

The available methods to monitor lipid oxidation in foods and biological systems may be divided into two groups. The first group measures primary oxidative changes (hydroperoxides) and the second determines secondary changes (aldehydes) that occur in each system (Shahidai & Wanasundara, 2002). In this work, peroxide value (PV) and *p*-anisidine value (*p*-AnV) were used to determine primary and secondary changes, respectively. Measuring secondary products is important in the determination of lipid oxidation in food products for human consumption because they are generally odour-active, whereas primary oxidation products are colorless and flavorless. In addition, aldehydes do not decompose rapidly like hydroperoxides, thus allowing the past history of an oil to be determined with the AnV analysis (Osborn & Akoh, 2004). The objectives of this study were to examine the potential of chitosan mixed with either maltodextrin or whey protein isolate, which were used as the wall materials for the encapsulation of tuna oil to prevention oxidation in both emulsions and encapsulated powder.

Materials and Methods

Materials

Chitosan (CS) with the degree of deacethylation (DD) = 80 and tuna oil were obtained (T. C. Union Company, Samutsakorn, Thailand). Maltodextrin (MD), M040 with an average

dextrose equivalent (DE = 5) was provided by GRAIN Processing Corporation (Muscatine, IA). Whey protein isolate (WPI) was supplied by AMPC, Inc., (Ames, IA). The emulsifier used was Tween 80 (Fisher Scientific, FairLawn, NJ). Acetic acid, hydrochloric acid, chloroform, methanol, isooctane, and sodium hydroxide were purchased from Mallinckrodt Baker, Inc., (Phillipsburg, NJ). Acetate buffer (pH 4.6), barium chloride dehydrate, iron sulfate, ammonium thiocyanate, P-anisidine, and α-tocopherol were purchased from Sigma-Aldrich Company (St. Louis, MO).

Emulsion preparation

One percent (w/w) CS was first dispersed in 0.25% (v/v) aqueous acetic acid and continuously stirred at 25 °C until the mixture was completely dissolved by visual examination. The 10 % (w/w) MD or 1 % (w/w) WPI was then slowly added. When total dissolution was observed visually, tuna oil 20 % (w/w) previously mixed with Tween 80 (2.5 % (w/w)) was added to the solution. The mixture was then emulsified using a homogenizer (OMNI International, Waterbury, CT) at 5,000 rpm for 30 min.

For oxidation evaluation, the following samples were prepared;

A = control #1 (tuna oil without α-tocopherol)

B = control #2 (tuna oil mixed with α-tocopherol)

C = emulsion made from CS 1 % (w/w) with WPI 1 % (w/w) (without α-tocopherol)

D = emulsion made from CS 1 % (w/w) with WPI 1 % (w/w) (with α-tocopherol)

E = emulsion made from CS 1 % (w/w) with MD 10 % (w/w) (without α-tocopherol)

F = emulsion made from CS 1 % (w/w) with MD 10 % (w/w) (with α-tocopherol)

For samples mixed with α-tocopherol, 0.01 % (w/w) α-tocopherol (based on fat content) was added to the tuna oil prior to the preparation of emulsion.

Characteristics of emulsions

Oxidation determination

The emulsion samples (5 ml) were placed in screw-cap test tubes and allowed to oxidize at 25 °C and 37 °C in an oven for 14 and 30 days, respectively. Samples were collected periodically to determine any oxidative changes. Before an oxidation level determination, oil in the emulsion was extracted by mixing with isooctane/2-propanol (3:1, v/v), vortexing, and centrifugation at 1,000 rpm for 5 min to isolate the organic phase. The solvent was then removed by evaporation. Oil samples were used at levels of 0.1 and 0.5 g for PV and AnV measurements, respectively. Peroxide values (PV) were determined to assess the primary oxidation changes using the International Dairy Foundation (IDF) method (Shantha & Decker, 1994), while the Anisidine values (AnV) were determined according to the AOCS Official Method Cd 18-90 (AOCS, 1998) to evaluate secondary changes.

Zeta-Potential measurement

Each emulsion was collected and diluted by 50 fold using acetate buffer (pH 4.6) prior to the measurement. The diluted emulsion was poured into a cuvette and then put into a chamber of a particle size analyzer (BTC Brookhaven Instruments Corporation, Holtsville, NY). The zeta-Potential measurement was reported as the average and standard deviation of measurements with five readings made per sample.

Microstructure

The microstructure of emulsions was determined using a light microscope (Nikon Eclipse E600, Southern MicroInstrumens, Marietta, GA). The emulsion was diluted 1:5 from the original concentration to avoid the particles clumping together. A drop of emulsion was observed at an objective magnification of 100X. Images of the structure for all emulsions were acquired using

image processing software (MagnaFire software, Optronic Laboratories, Inc., Goleta, CA) with CCD camera and stored on a computer.

Encapsulation preparation

An ultrasonic atomizer (Model CPX134PB; Cole-Parmer instruments, Vernon Hills, IL), equipped with a 1/32 inch diameter ultrasonic probe was used in this study. It was operated at 40 kHz with 130 W high-intensity, and at 120 V. The amplitude of probe oscillation was set at 55 in order to effectively optimize the solution. Each emulsion sample was pumped into the ultrasonic atomizer through the ultrasonic probe using a peristaltic pump (Model 7523-50, Cole-Parmer Instrument, Barrington, IL). The liquids were dispersed as droplets from the vibrating tip into NaOH solution (1 %(w/w) that solidified the CS. The microsphere suspension obtained was then centrifuged, filtered, washed with distilled water, frozen at -80 °C overnight, and dried in a freeze drier for 24-48 hr.

Oxidation determination

The encapsulated powder was kept in screw-cap bottles and stored at 25 °C and at 37 °C for 30 and 14 days, respectively. PV and AnV were determined with the same methods as oxidative stability for the emulsions.

Data analysis

The data were analyzed by the analysis of variance (ANOVA) using SAS version 9.1 (SAS Institute, Cary, NC). Duncan's multiple range test was used to compare the means with a significance level of $P< 0.05$.

Results and Discussion

Emulsion oxidation

Primary and secondary oxidation

Figure 4.1 and 4.2 compare oxidation rates of bulk tuna oil and tuna-oil-in- water emulsions with and without α-tocopherol at 25 °C and 37 °C, respectively. PV and AnV were measured to determine the primary and the secondary rate of oxidation, respectively. Bulk tuna oil without α-tocopherol (A) obviously showed the highest PV and AnV compared to the others. Emulsion F showed the highest oxidative stability during storage at 25 °C. However, it was slightly higher than emulsion D and E. Storage temperature had an impact on the oxidative stability of bulk tuna oil and tuna oil-in-water emulsions stabilized with WPI or MD with increasing storage temperature (25 °C to 37 °C). The effect of temperature on oxidative stability became quite obvious at 37 °C with lipid hydroperoxide and Anisidine values being about 3- and 2-fold greater at 37 °C than at 25 °C for bulk tuna oil and emulsions, after 3 and 8 days of storage, respectively. Normally, most food emulsions can be considered to consist of three distinct regions that have different physicochemical properties: the interior of a droplet, the continuous phase, and the interfacial membrane. The interfacial region is potentially very important in lipid oxidation since it represents the region where lipid- and water-soluble prooxidants can interact and it is where surface-active compounds such as lipid peroxide and chain breaking antioxidants concentrate (Mancuso, McClements, & Decker, 1999). The combination of CS and MD play an important role as texture modifiers in the continuous phase as mentioned in chapter 3 (section emulsion stability). The observed increased oxidative stability of emulsions E and F could therefore be due to its thicker interfacial membrane and higher viscosity in continuous phase that may protect lipids from oxidation by acting as a barrier to the

penetration and diffusion of molecular species that promote lipid oxidation into the droplets. More than that, CS itself has antioxidant property through the reaction of NH_2 groups of CS and hydroxyl free radicals to form stable macromolecule radicals (Xie, Xu & Liu, 2001). The oxidative stability was increased when incorporated with α-tocopherol. The ability of α-tocopherol to increase lipid peroxides is likely due to the primary antioxidant mechanism of α-tocopherol which involves the donation of hydrogen to a peroxyl radical to form lipid peroxides which are more stable and less readily available to further promote autoxidation (Eitenmiller & Lee, 2004). These results are in agreement with Klinkeson, Sophanodora, Chinachoti, McClements, & Decker (2005) who have also indicated that in oil-in-water emulsions, lipophilic antioxidants would concentrate in the oil droplets or at the oil-water interfaces and inhibit lipid oxidation more effectively than hydrophilic antioxidants that can partition into the water phase. The combination of CS and WPI in the ability to increase oxidative stability is discussed in the following zeta-potential section.

Zeta-potential

The zeta-potential of emulsion C significantly decreased ($P<0.05$) after 5 days of storage at 37 °C compared to emulsion D but the potential were not significantly changed between emulsion E and F (Figure 4.3). As we mentioned that CS mixed with WPI increased the electrical charge and gave the highest positive ζ-potential, when oil-in-water emulsion droplets are surrounded by cationic emulsifiers, prooxidant metals might be repelled and lipid oxidation rates decrease. Generally, most common prooxidant transition metals formed in oils are iron and copper. Even with low reactivity and solubility, ferric iron could be an important lipid oxidation catalyst during the long-term storage of emulsified lipids, especially if it is able to interact with the interfacial membrane of emulsion droplets (Hu, Decker, McClements, 2005). This study

showed results similar to Tong, Sasaki, McClements, & Decker (2000a) findings, who determined that α-tocopherol and whey proteins are effective antioxidants in salmon oil-in-water emulsions and that they could provide an additional antioxidant defense system in food emulsions. Hu, McClements, & Decker (2003 a,b) also showed that when salmon or corn oil emulsions are stabilized with proteins, oxidation rates are dramatically slower when the pH is below the pI of the protein and thus the emulsion droplet is cationic.

From the results, we could suggest that the ability of the CS-WPI emulsion to alter oxidation rates was not solely related to their ability to produce cationic emulsion droplets because the positive charge of the emulsions droplets was in the order of emulsion D > C > F ≈ E whereas emulsion F had the highest in oxidative stability. This suggests that other factors are also influencing the ability of the adsorbed protein or carbohydrate to inhibit lipid oxidation. Donnelly, Decker, & McClements (1998) have proposed that whey protein may inhibit oxidation through their physical accumulation at lipid-water interfaces thus forming physical barriers between water-soluble prooxidants and lipids.

Microstructure

In this study, we wanted to investigate the relationship between structural change and lipid oxidation of emulsions. The droplet structures of emulsion C and E during storage at 25 °C and 37 °C for the period of 30 and 14 days are presented in Figure 4.4 and Figure 4.5 – 4.6, respectively. Figure 4.4 showed the droplets of emulsions C and E didn't come close together after being freshly prepared on the first day because of strong repulsive force between them. A slightly changes was observed following 30 days of storage showing the droplets came closer together because the attractive forces became stronger so the formed flocs tended to have more open structures in which each droplets is only linked to two or three of its neighbors and still

retain their individual integrity. The results are related with the results of emulsion stability and particle size study in chapter 3 that showed CS mixed with MD or WPI higher emulsion stability and the smaller particle sizes. From the structural work we might suggest that the oxidative stability is possibly related to the structure of the emulsion. If the droplet structures don't tend to promote flocculation or coalescence after an extended storage period then the oxidative values might not become too high and remain at an acceptable level. Figures 4.5 and 4.6 showed the structural changes after 5, 10 and 14 days of storage at 37 °C. The droplets obviously became bigger after 5 days of storage because the attraction between the droplets was relatively weak compared to the thermal energy, so the droplets do not always stick together after a collision and they may be able to roll around each other after sticking together. Thus, a droplet that encounters a floc is able to penetrate closer to its center and flocs are able to undergo structural rearrangements, which means that the droplets can pack more closely together (McClements, 2005).We noticed that the interface of some droplet surfaces were ruptured after 10 days of storage which was related to the higher PV and AnV determined during the study. The higher values are presumably because the membrane of emulsion droplets are no longer acting as a barrier to protect the interactions between lipids and aqueous prooxidants permitting easier lipid oxidation. More than that lipid oxidation can lead to the development of potentially reactive products which are more surface-active than triacylglycerols and therefore accumulate preferentially at an oil-water interface, which increase their susceptibility to oxidation and may increase the tendency for emulsion droplets to flocculate or coalesce (McClements, 2005).

Encapsulated powder oxidation

The oxidative stability of tuna oil in encapsulated powder is presented in Figure 4.7 and 4.8 for storage at 25 °C and at 37 °C, respectively. PV and AnV showed significant increase in

bulk oil after 3 days and 1 day of storage at 25 °C and 37 °C, respectively. However, oxidation in all encapsulated samples increased gradually compared to bulk oil for both 25 °C and 37 °C of storage. Microencapsulation significantly protected tuna oil against oxidation in comparison with the bulk oil samples. These results are in agreement with Heinzelmann & Franke (1999); Heinzelmann, Franke, Valesco, &Marquez-Ruiz (2000) who have shown that encapsulation of fish oil by freeze-drying technique can improve oxidative stability. Kolanowski, Laufenberg, & Kunz (2004) also indicated that the oxidative stability of fish oil was significantly improved by microencapsulation with modified cellulose in comparison with the bulk fish oil. In contrast, Marquez-Ruiz, Valesco, & Dobraganes (2000) have found that the oxidative stability in bulk oil was better than microencapsulated oil produced with a freeze-drier. It should be considered that freeze-drying products always have a porous, sponge-like matrix, which could allow oxygen to access to the oil component. Further study is needed to determine the surface structure of the encapsulated powder and to clarify possible differences between this study and the finds of Marquez-Ruiz, Valesco, & Dobraganes (2000).

Conclusions

The results showed that CS mixed with WPI or MD was successfully used as the wall materials for tuna oil microencapsulation. The lipid oxidation in emulsions was related to the lipid oxidation in encapsulated oil powder. Oxidative stability of emulsion was improved as well as the encapsulated powder, especially when adding more α-tocopherol compared to bulk tuna oil. However, the surface structure of the encapsulated powder needs to be further studies to determine if it is porous, allowing oxygen to access the encapsulated oil.

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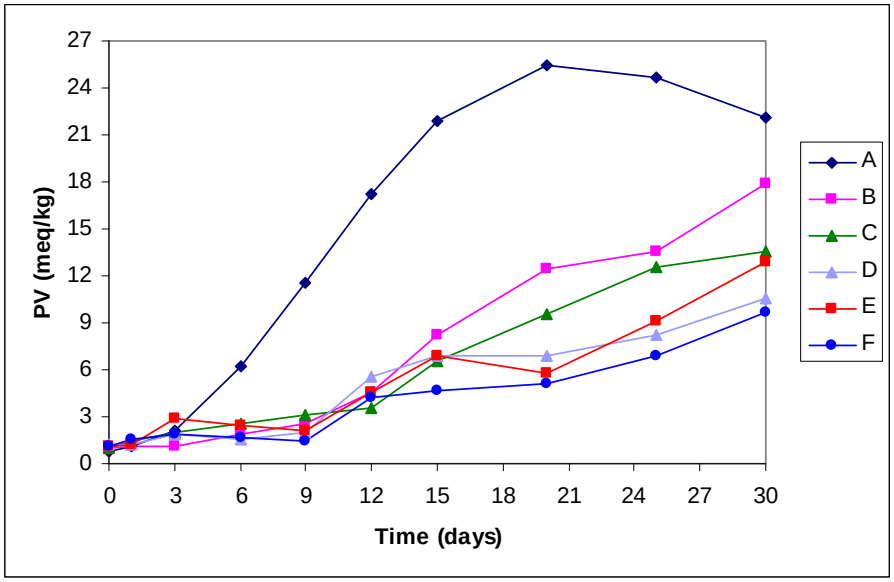
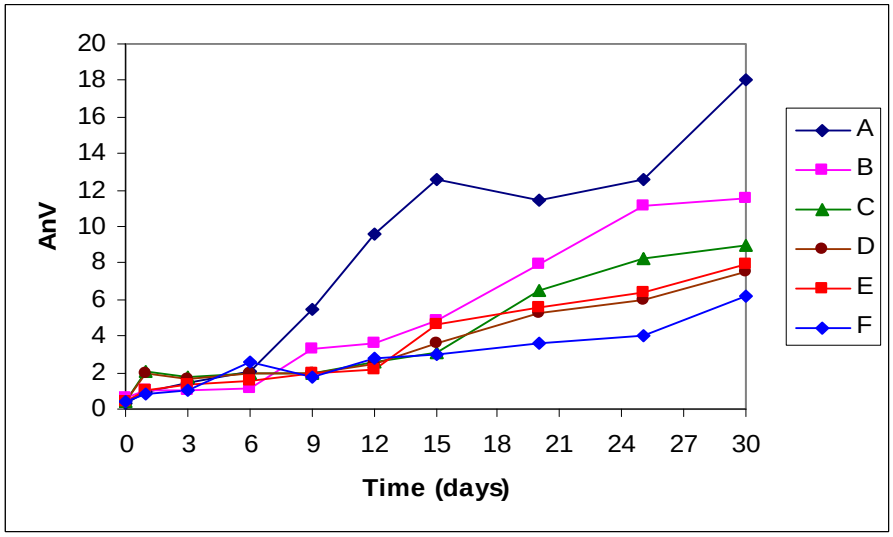


(a)



(b)

Figure 4. 1. Formation of lipid hydroperoxides (a) and Anisidine value (b) of bulk oil and oil-in-water emulsions in the absence and presence of α-tocopherol during storage at 25 °C
Data shown are the average of duplicates

Where A = bulk tuna oil

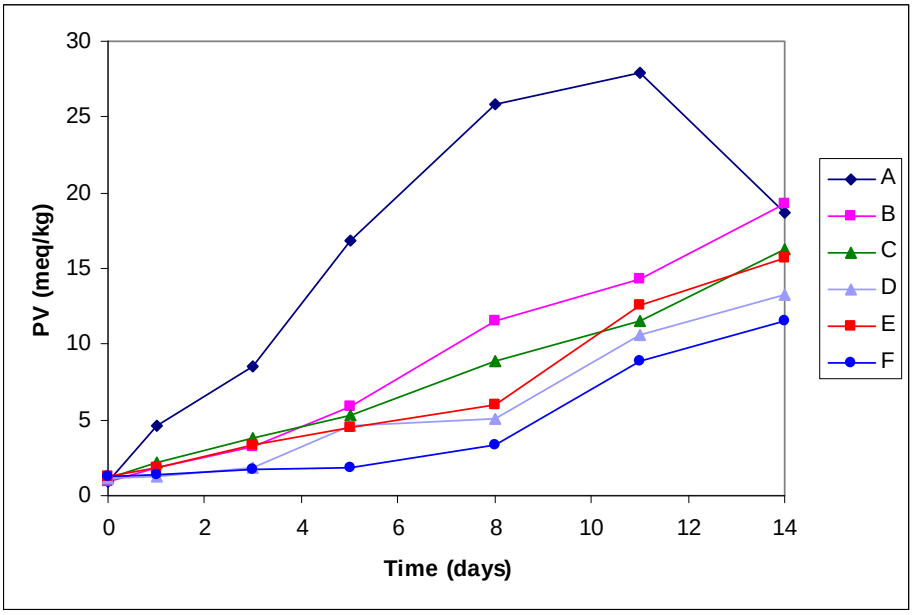
B = bulk tuna oil with α-tocopherol

C = emulsion made from CS1 % (w/w) mixed with WPI 1 % (w/w)

D = emulsion made from CS1 % (w/w) mixed with WPI 1 % (w/w) and α-tocopherol

E = emulsion made from CS1 % (w/w) mixed with MD 10 % (w/w)

F = emulsion made from CS1 % (w/w) mixed with MD 10 % (w/w) and α-tocophero



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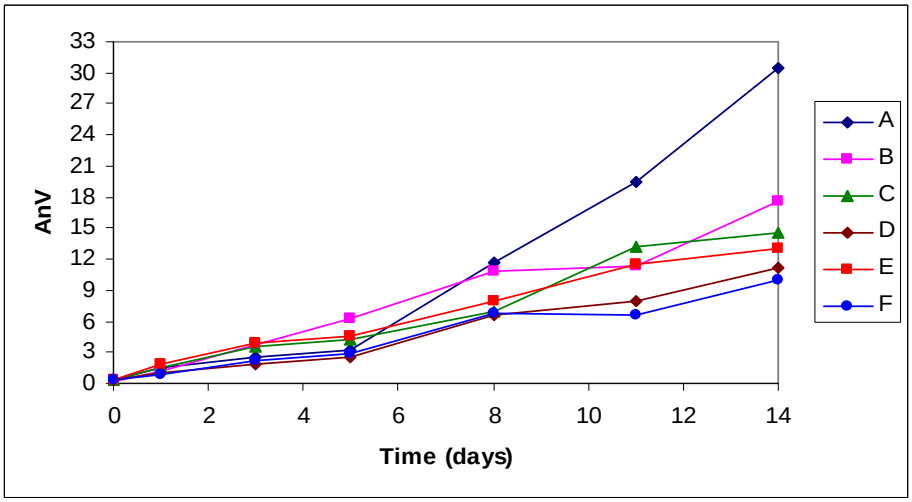


(b)

Figure 4.2. Formation of lipid hydroperoxides (a) and Anisidine value (b) of bulk oil and oil-in-water emulsions in the absence and presence of α-tocopherol during storage at 37 °C
Data shown are the average of duplicates

Where A = bulk tuna oil

B = bulk tuna oil with α-tocopherol

C = emulsion made from CS1 % (w/w) mixed with WPI 1 % (w/w)

D = emulsion made from CS1 % (w/w) mixed with WPI 1 % (w/w) and α-tocopherol

E = emulsion made from CS1 % (w/w) mixed with MD 10 % (w/w)

F = emulsion made from CS1 % (w/w) mixed with MD 10 % (w/w) and α-tocopherol

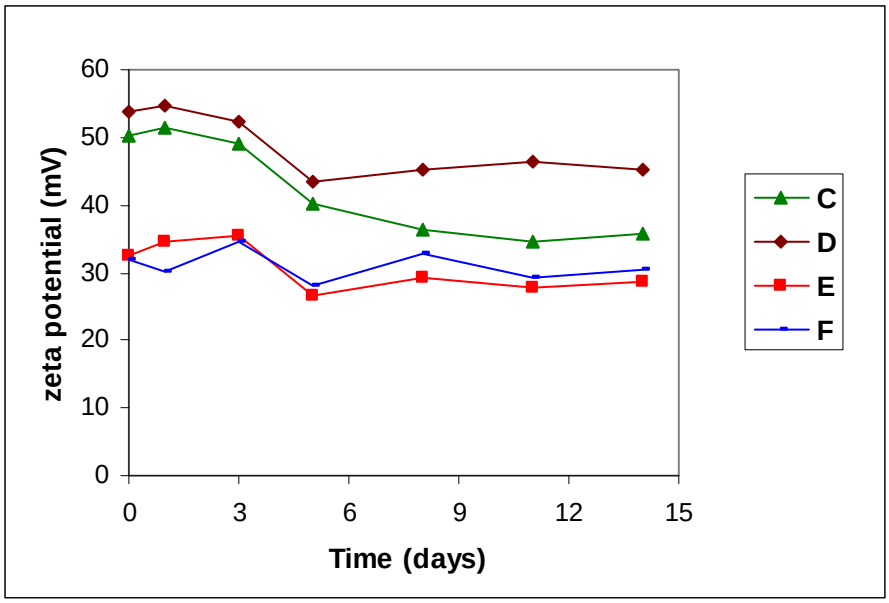


Figure 4.3. Zeta-potential of tuna oil-in-water emulsions consisting of 20 % (w/w) tuna oil, 1 % (w/w) CS mixed with 1 % (w/w) WPI or 10 % (w/w) MD with and without antioxidant during storage at 37 °C. Data shown are the average of means (n=5)

Where C = emulsion made from CS1 % (w/w) mixed with WPI 1 % (w/w)

D = emulsion made from CS1 % (w/w) mixed with WPI 1 % (w/w) and α-tocopherol

E = emulsion made from CS1 % (w/w) mixed with MD 10 % (w/w)

F = emulsion made from CS1 % (w/w) mixed with MD 10 % (w/w) and α-tocopherol

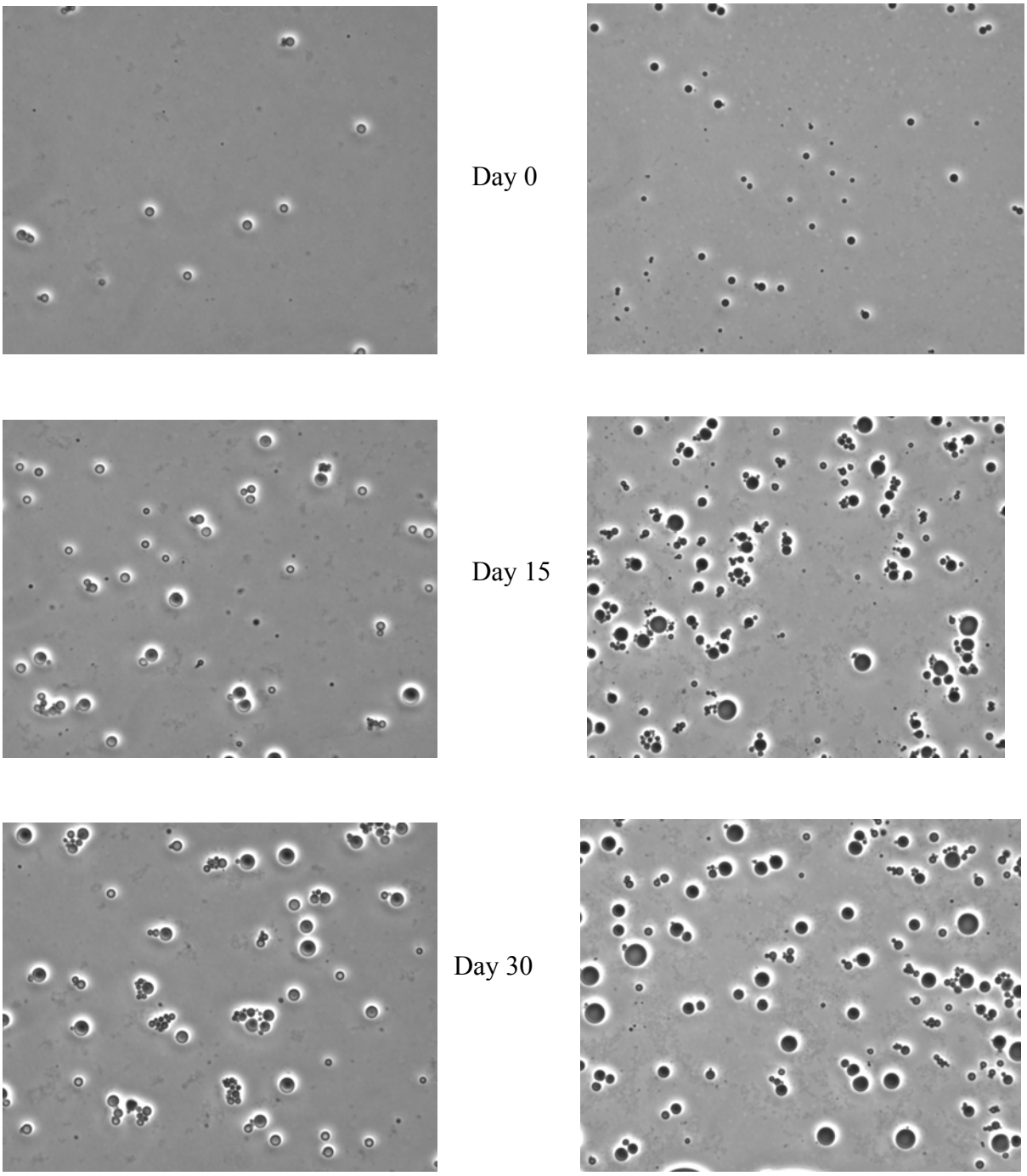


Figure 4.4. Structural changes of emulsion droplets made from CS 1 % (w/w) mixed with WPI 1 % (w/w) (a) or MD 10 % (w/w) (b) storage at 25 °C of day 0, 15, and 30

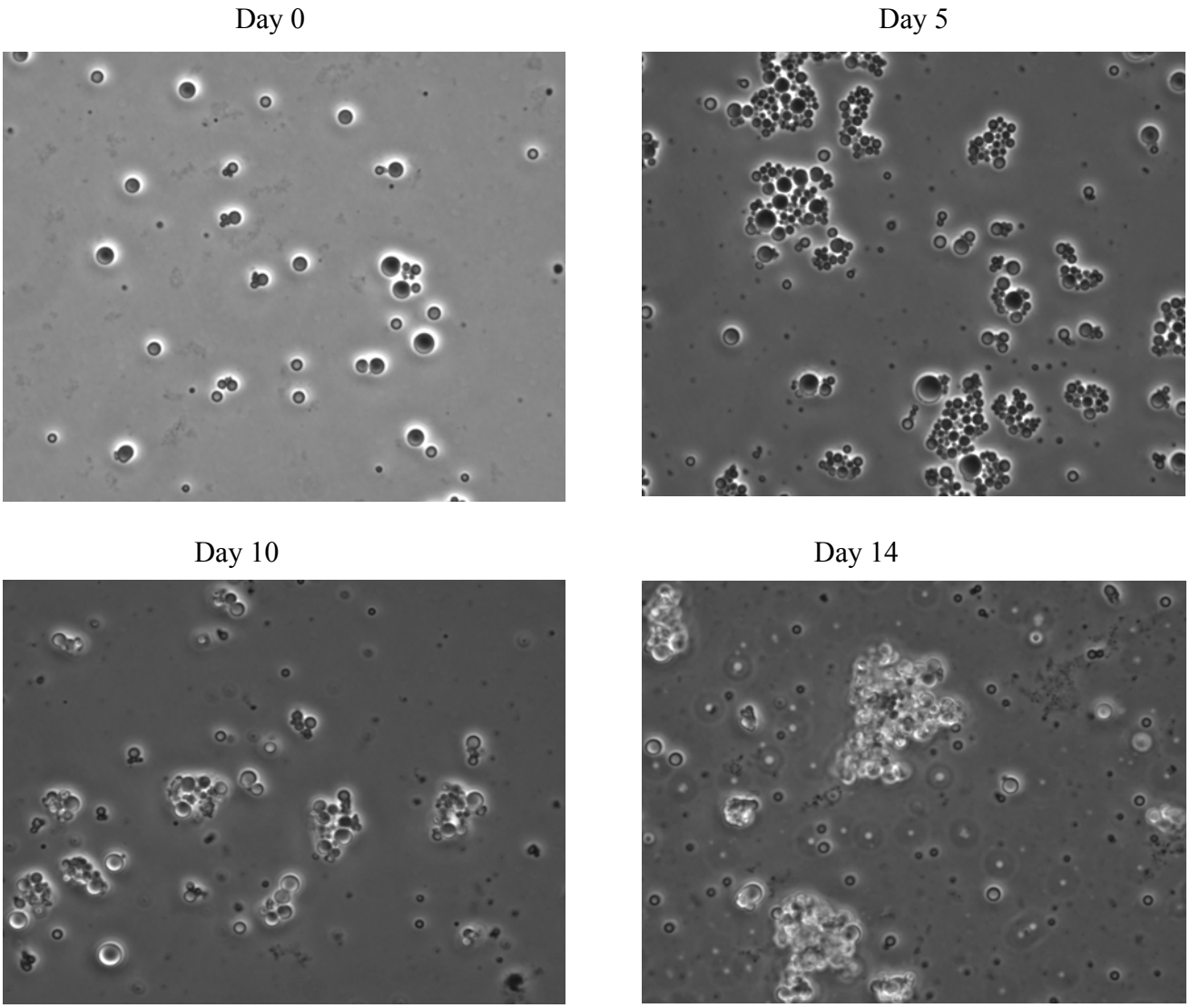


Figure 4.5. Structural changes of emulsion droplets made from CS 1 %(w/w) mixed with WPI 1 % (w/w) at 37 °C storage of day 0, 5, 10, and 14

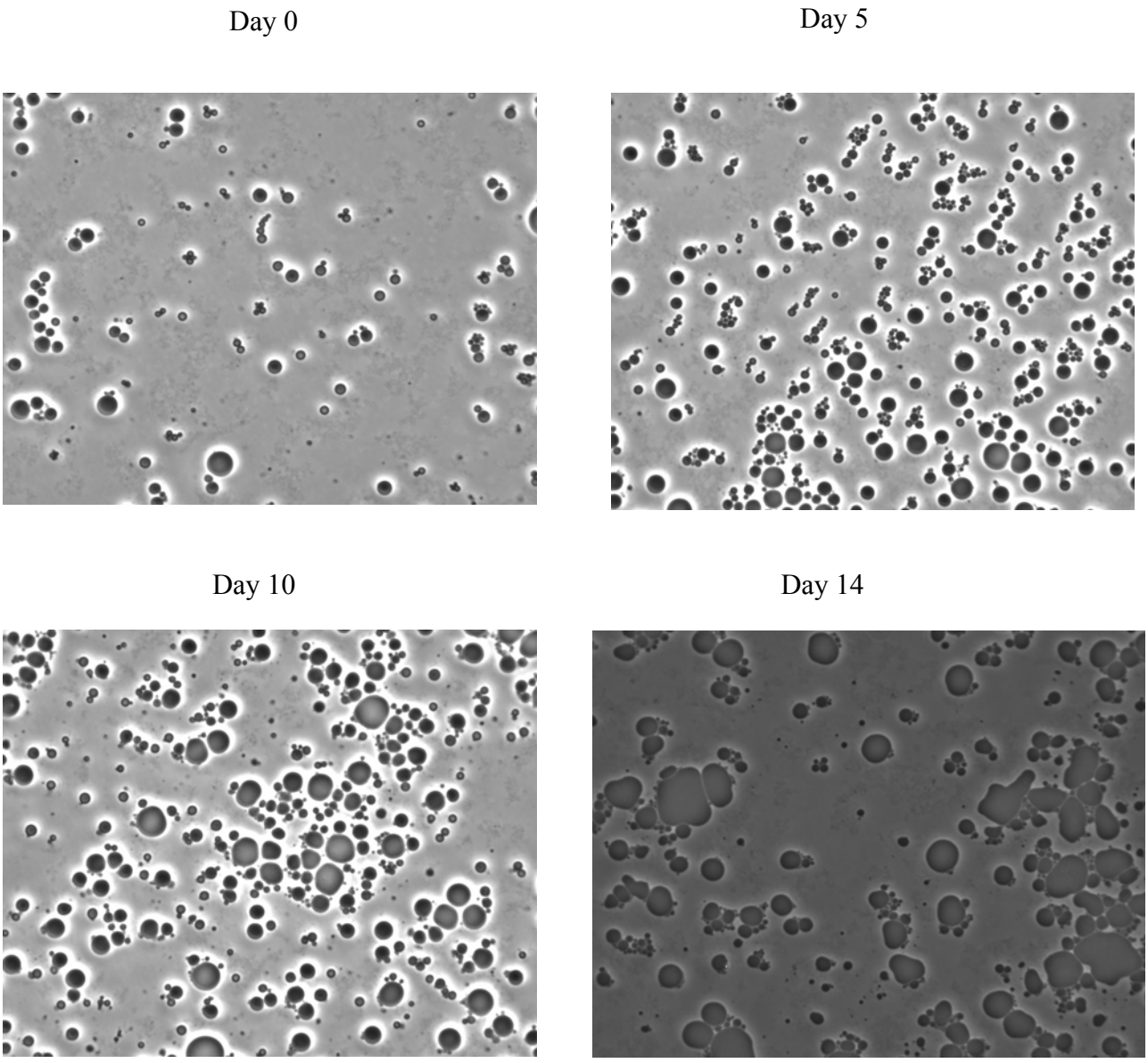
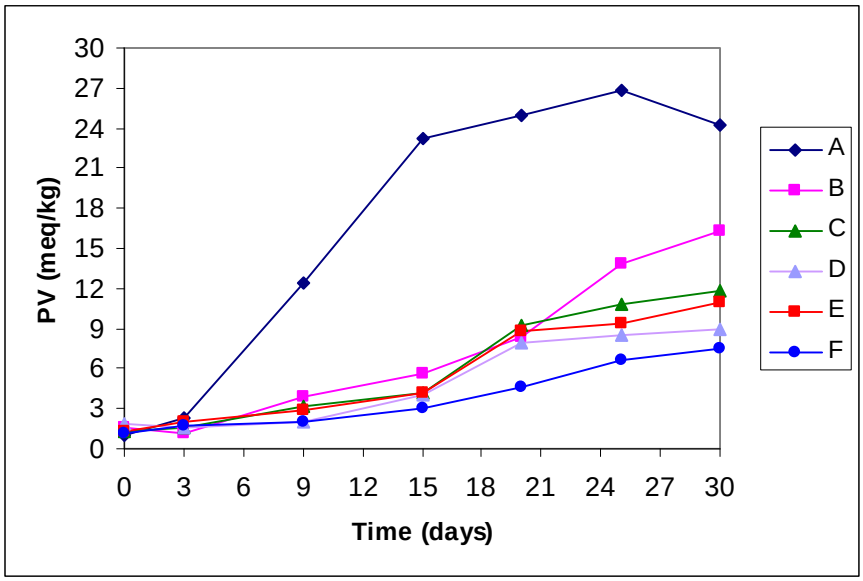
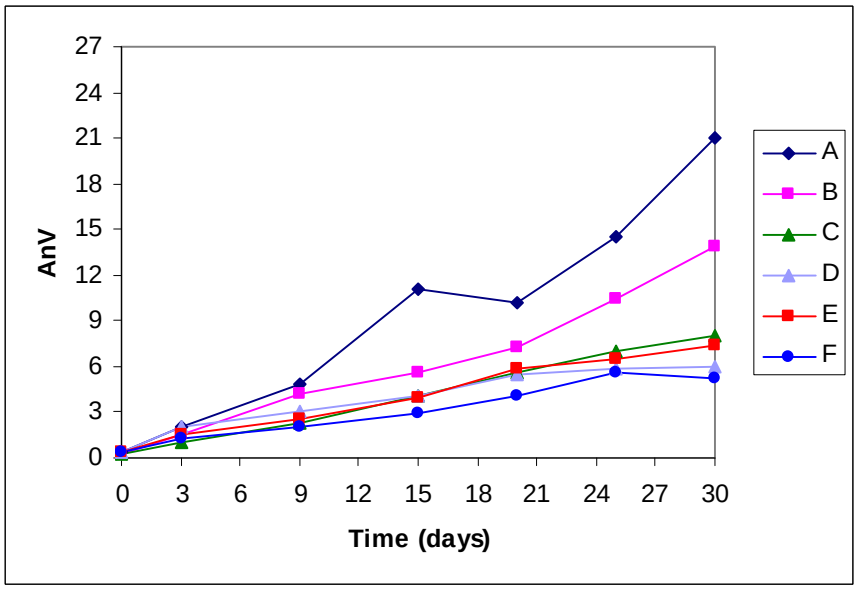


Figure 4.6. Structural changes of emulsion droplets made from CS 1 %(w/w) mixed with MD 10 % (w/w) at 37 °C storage of day 0, 5, 10, and 14



(a)



(b)

Figure 4.7. Formation of lipid hydroperoxides (a) and Anisidine values (b) of bulk tuna oil and tuna-oil-encapsulated powder in the absence and presence of α-tocopherol during storage at 25 °C. Data shown are the average of duplicates

Where A = bulk tuna oil

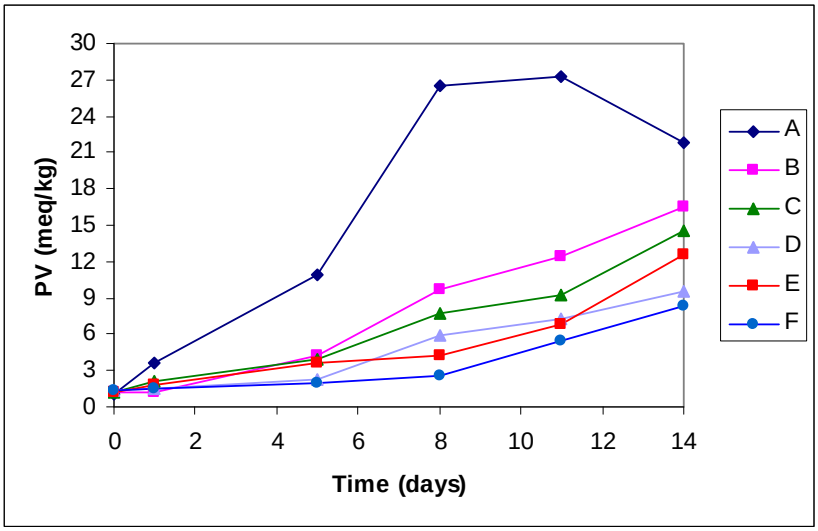
B = bulk tuna oil with α-tocopherol

C = encapsulated powder made from CS1 % (w/w) mixed with WPI 1 % (w/w)

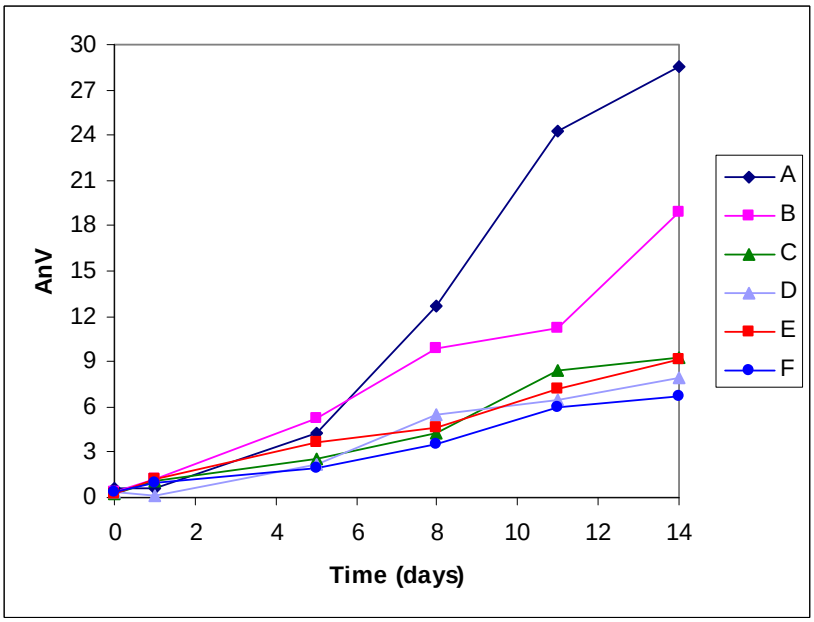
D = encapsulated powder made from CS1 % (w/w) mixed with WPI 1 % (w/w) and α -tocopherol

E = encapsulated powder made from CS1 % (w/w) mixed with MD 10 % (w/w)

F = encapsulated powder made from CS1 % (w/w) mixed with MD 10 % (w/w) and α-tocopherol



(a)



(b)

Figure 4.8. Formation of lipid hydroperoxides (a) and Anisidine values (b) of bulk tuna oil and tuna-oil-encapsulated powder in the absence and presence of α-tocopherol during storage at 37 °C

Data shown are the average of duplicates

Where A = bulk tuna oil

B = bulk tuna oil with α-tocopherol

C = encapsulated powder made from CS1 % (w/w) mixed with WPI 1 % (w/w)

D = encapsulated powder made from CS1 % (w/w) mixed with WPI 1 % (w/w) and α -tocopherol

E = encapsulated powder made from CS1 % (w/w) mixed with MD 10 % (w/w)

F = encapsulated powder made from CS1 % (w/w) mixed with MD10 % (w/w) and α-tocopherol

CHAPTER 5

MICROSTRUCTURAL EXAMINATION OF TUNA-OIL ENCAPUSULATION BY CONFOCAL SCANNING MICROSCOPY, SCANNING ELECTRON MICROSCOPY AND FOURIER TRANSFORM INFRARED SPECTROSCOPY

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[1]Klaypradit W, Huang Y-W. To be submitted to *Lebensmittel-Wissenschaft und-Technologie.*

Abstract

The confocal scanning microscope (CLSM), scanning electron microscope (SEM), and fourier transform infrared spectroscopy (FTIR) have been used to localize tuna-oil in oil-in-water emulsions, examine the outer-microstructure of encapsulated powder, and assess tuna-oil encapsulation, respectively. Nile red was used as the fluorescent lipid probe and enabling us to distinguish between encapsulated oil and the continuous phase which was labeled with Alexa 633 for emulsions made from chitosan 1 % (w/w) mixed with maltodextrin 10 % (w/w) or whey protein isolate 1 % (w/w). The outer-surface exhibited no pore or fewer oil droplets, which was in agreement with the previous oxidative stability study. FTIR could be used to explain that the tuna-oil in the microspheres is physically encapsulated in the wall matrix.

Keywords: confocal scanning microscope, scanning electron microscope, fourier transform infrared spectroscopy, microencapsulation, chitosan

Introduction

The ultrasonic atomizer and freeze drying are successfully used encapsulate tuna oil. Previous studies by Marquez-Ruiz, Valesco, & Dobraganes (2000) found that the oxidative stability in bulk oil was better than in microencapsulating oil by freeze-drying. In addition, Kolanowski, Laufenberg, & Kunz (2004) mentioned that microencapsulated oils might have a porous, sponge-like matrix, which could lead the oxygen accessibility contact with the oil component. To resolve the issue, it is of interest to analyze and visualize the structure of emulsion droplets and encapsulated powder after freeze drying to determine the characterization of the encapsulated particles.

In general, foods are complex multicomponent systems and microstructural elements that are difficult to observe in their natural or transformed states. Fortunately, advances in biology and materials science with powerful microscopes can help in the study of food microstructure and its impact on processing and product properties from the atomic level to the micron range. Examples are the laser confocal scanning microscope (CLSM) and the recently developed scanning electron microscopy (SEM) (Aguileria, 2005). SEM is a technique for visualization of surfaces and is not suitable to observe the inner structures of objects. The depth of focus allows imaging of wide areas and large, irregular-shaped objects (Hoppert, 2003). SEM images have a great depth of focus and are relatively easy to understand. However, the main drawback is dimensional changes and shrinkage of soft biological specimens (Aguilera & Stanley, 1999) because the most complex samples require chemical fixation and treatment with organic solvents or rapid freezing (Hoppert, 2003). Recently, studies examining the outer-structure of fish oil microencapsulated powder have been extensively reported in the literature (Heinzelmann, Franke, Jensen, & Haahr, 2000; Kagami, Sugimura, Fujishima, Matsuda, Kometani, &

Matsumura, 2003; Kolanowski, Laufenberg, & Kunz, 2004; Klinkeson, Sophanodora, Chinachoti, Decker, & McClements, 2006). Confocal laser scanning microscopy (CLSM) is now a widely used technique for optical image slicing and is almost exclusively used for imaging of fluorescent specimens (Wilson, 2002). CLSM provides only an image of the in-focus plane with the out-of-focus parts appearing as a black background so that not only the resolution is improved but also elimination of out-of-focus light allows observation of thick samples by enabling scanning in the Z-axis without interference from light above and below the focal plane (Hoppert, 2003; Sheppard & Shotton, 1997). Some related work has been reported about using CLSM to examine and characterize encapsulated oil (Lamprecht, Schafer, & Lehr, 2000; Macierzanha & Szelag, 2006), emulsifier adsorption on the interface between water and triacylglycerol (Heertje, Van Aalst, Blonk, Don, Nederlof, & Lucassen-Reynders, 1996). Fourier Transform Infrared Spectroscopy (FTIR) can be used to detect the orientation in polymer specimens and also to quantify it (Hendra & Maddams, 1996). There are two kinds of information that are typically obtained from the IR spectrum. These are qualitative and quantitative results. Qualitative analysis is defined as the molecular identification of unknown samples. Quantitative analysis is defined as the determination of component concentrations for known sample material. FTIR spectra of chitosan and chitosan derivatives have been investigated (Osman, & Arof, 2003; Xing, Lui, Yu, Guo, Li, 2005; Kim, & Thomas, 2006). Zaleska, Ring, & Tomasik (2001) showed the IR spectra of whey protein isolate (WPI) and WPI-starch. Kagami, Sugimura, Fujishima, Matsuda, Kometani, & Matsumura (2003) used FTIR to measure the oil surface of microcapsules. Loret, Schumm, Puden, Frith, & Fryer (2005) have shown the pure spectra of maltodextrin. Kosaraju, D'ath, & Lawrence (2006) studied the structural interactions of polyphenolic compounds in olive leaf extract in chitosan encapsulation.

The objectives of this study were as follows; (1) to explore oil localization in emulsion with CLSM, (2) to examine the outer-surface of the encapsulated particles by means of the SEM technique, and (3) to investigate the relationship between FTIR and the ability to form effective encapsulation.

Materials and Methods

Materials

Chitosan (CS) with the degree of deacethylation (DD) = 80 and tuna oil were purchased (T. C. Union Company, Samutsakorn, Thailand). Maltodextrin (MD), M040 with an average dextrose equivalent (DE = 5) was provided by GRAIN Processing Corporation (Muscatine, IA). Whey protein isolate (WPI) was supplied by AMPC, Inc., (Ames, IA). The emulsifier used was Tween 80 (Fisher Scientific, FairLawn, NJ). Acetic acid was purchased from Mallinckrodt Baker, Inc., Phillipsburg, NJ. Acetate buffer (pH 4.6) was purchased from Sigma-Aldrich company (St. Louis, MO). Concanavalin A conjugates or Con A (Alexa Fluoro 633) and Nile red were purchased from Molecular Probes Company, Carlsba, CA.

Methods

Confocal Laser Scanning Microscope (CLSM)

Sample preparation

CS 1.0 % (w/w) was first dispersed in 0.25 % (v/v) aqueous acetic acid and continuously stirred at room temperature until the mixture was complete dissolved by visual examination. Then 10 % (w/w) MD or 1 % (w/w) WPI was slowly added. When total visual dissolution was obtained, 400 µl Alexa 633 (stock solution of 100 µg/ml PBS) was added and continuously stirred for 1 hour. Nile red (stock solution of 1 mg/ml) 4 µl was added to tuna oil (20 % w/w) previously mixed with Tween 80 (2.5 % w/w) and stirred for 1 hour. The oil/ Tween/ Nile red

mixture and CS solution were mixed and then emulsified using a homogenizer (OMNI International, Waterbury, CT) at 5,000 rpm for 30 min. The emulsion was centrifuged at 8,000 rpm for 30 min, the cream layer was collected then re-dispersed in acetate buffer solution at the ratio 1: 2 (emulsion : acetate buffer) and transferred to a glass slide for viewing.

A Leica TCS SP2 spectral confocal microscope (Leica Microsystems, Heidelnerg Gmblt, Germany) equipped with 100x oil immersion objective (numerical aperture = 1.3, Leica, Germany) was used for microscopic observation. An HeNeon laser with excitation wavelength (λ) = 543 and a green Neon laser exciting at 633 were used to excite Nile red and Alexa 633, respectively. Emission spectra was collected at 645-655 and 630-640 nm for wall components and tuna-oil, respectively.

Scanning Electron Microscope (SEM)

Tuna-oil encapsulated powder samples made from CS 1 % (w/w) (A) and CS 1 % (w/w) mixed with 1 % (w/w) WPI (B) or 10 % (w/w) MD (C) (procedure was described in section covering the ultrasonication process, chapter 3) were prepared by vapor fixation for one hour using 4 % osmium solution (OsO_4) to stabilize the oil. The samples were adhered to sample holders, and sputter coated with gold (SPI-Module, Structure Probe, Inc., West Chester, PA) at 15 milliamps for 80 seconds. The samples were examined to determine the outer topography of the microcapsules with a Zeiss 1450EP variable pressure SEM (Carl Zeiss MicroImaging, Inc., Thornwood, NY) at 10 kV.

Fourier Transform Infrared Spectroscopy (FTIR)

A FTIR (model Nicolet 6700, OMNIC Thermo Electron Corporation, Madison, WI) coupled with an attenuated total reflectance (ATR) accessory was used to investigate the ability of tuna-oil encapsulation. Each microcapsule sample was mounted in the flat crystal chamber of

the ATR accessory. The crystal was washed with ethanol between each sampling. The background spectrum was obtained by measuring the empty chamber. A 4 cm^{-1} resolution was used and the ATR spectra were averaged on 32 scans with wavenumber range 4,000-500 cm^{-1}.

Results and Discussion

Confocal Laser Scanning Microscope

To examine the oil droplets localization with the CLSM, Nile red and Alexa 633 were applied as the fluorescent vital stain of the lipid droplets and continuous phase, respectively, in oil-in-water emulsion. Generally, Nile red serves as an excellent fluorescent lipid probe and has been used for the qualitative detection of fat but there is no indication in the literature of potential quantitative application (Greenspan, & Fowler, 1985; Barnes, & Fulcher, 1989). Alexa 633 is one type of ConA which is one of the most widely used and well characterized lectins. Con A has a broad applicability primarily because it recognizes a commonly occurring sugar structure. Therefore, Alexa 633 was used to recognize CS or MD which are also one type of carbohydrate group. Figures 5.1 (a) and 5.2 (a) showed the red particles on the black background only when the Nile red wavelength was excited. Staining the oil phase with Nile red enabled us to distinguish between encapsulated oil and the continuous phase of emulsion because CLSM provides only an image of the in-focus plane with the out-of-focus parts appearing as black background (Hoppert, 2003; Sheppard & Shotton, 1997). When both wavelength of Nile red and Alexa 633 were excited, the red oil particles distributed on the green continuous phase were observed (Figures 5.1 (b) and 5.2 (b)). However, we noticed that the continuous phase of Figure 5.1 (b) has a green color covering all the background area, but Figure 5.2 (b) shows the green spots scattered on the black area. This is possibly due to the fact that Alexa 633 can recognize only CS mixed with MD (Figure 5.1 (b)) because they are both carbohydrates but can not

recognize WPI when mixed with CS as the continuous phase (Figure 5.2 (b)). For that reason, WPI partially is out-of-focus and could be seen as the black background. Based on the results, we concluded that the tuna-oil phase was encapsulated and distributed within the continuous phase.

Outer Microstructure-Scanning Electron Microscope (SEM)

The structure of the microencapsulated particles (A-C) after freeze drying as determined by SEM is presented in Figures 5.3 - 5.5. All powder samples have spherical or oval shapes. Some surface wrinkles were found in a fashion similar to the results of other previous carbohydrate-based encapsulation reports. Sheu, & Rosenberg (1998) indicated that microcapsules made from WPI/MD (DE=5) had surface dents and large capsules exhibited "caps" within dents. Kagami, Sugimura, Fujishima, Matsuda, Kometani, & Matsumura (2003) have found the formation of dents on the outer surfaces of fish oil microcapsules formed by maltodextrin (DE=18) / sodium caseinate as wall materials. Klinkeson, Sophanodora, Chinachoti, Decker, & McClements (2006) also showed the outer morphology of tuna oil microcapsules made from CS mixed with corn syrup solid (DE=36) by spray drying with some wrinkles and pores on the surface. Figure 5.3 shows the high oil content covered on the surface of the particle as compared to those made from CS mixed with WPI or MD (Figure 5.4 and Figure 5.5, respectively). This finding agrees with the results of the emulsion stability study described in chapter 3. It is presumably because the oil droplets were not completely saturated with CS causing partial contact between the oil and oxygen on the droplet surface leading to oxidation. It should be considered that freeze-drying products always have a porous, sponge-like matrix, which could allow oxygen access to the oil component producing the increased oxidation reaction (Kolanowski, Laufenberg, & Kunz, 2004; Dalgleish, 1990). The report done by

Heinzelmann and his co-workers (2000) reported that the outer structure of freeze-dried microencapsulated fish oil has many pores of different sizes for both in the ungrounded pellet and grounded powder. Therefore, they flushed the freeze-drying chamber with nitrogen after finishing the drying process to fill the micropores of the dried particles with nitrogen gas, reducing oxygen contact which led to improved oxidation stability. However, Figures 5.4-5.5 at 6000x and 5000x, respectively, shows very little oil on the droplet surface and we also found hardly any pores. It is possible to conclude that the combination of CS with MD or WPI can act as the good barrier wall material to keep the oil inside the droplet and prevent oxygen from coming in contact with the oil which could improve the product's oxidative stability. The results agree with oxidative stability test of encapsulated powder discussed in chapter 4 that showed the ability of the wall materials to reduce the oxidation rate compared to bulk oil.

Fourier Transform Infrared Spectroscopy

In this section, we attempted to apply the FTIR technique to determine if the tuna-oil in the microspheres is physically encapsulated in the wall matrix. The microspheres made from the combination of 1 % (w/w) CS and 10 % (w/w) MD without tuna-oil (D) were prepared as the standard and used to compare to the encapsulated tuna-oil powder using CS/MD as a wall matrix (E). The FTIR spectrum of D and E are shown in Figure 5.6 and 5.7, respectively. The spectra within the range of 500-4000 cm^{-1} was determined. Absorption peaks derived from the tuna-oil/CS-MD microspheres were quite similar to that of the CS-MD molecule. This might indicate that the majority of oil in the microsphere is physically encapsulated in the CS-MD matrix, although the interaction of the oil with functional groups of wall materials cannot be completely ruled out.

Conclusions

CLSM, SEM, and FTIR are useful tools to explain the characteristics of tuna-oil encapsulated powder for this study. Clearly, the fluorescence of lipid-Nile red mixtures is very sensitive to the oil phase and can be localized by CLSM. Compared to an ordinary light microscope, CLSM can provide additional information such as the three-dimensional localization and quantification of the encapsulated phase. The results are interesting and should be further studied. The outer-surface determined by SEM has not shown any pore or oil droplets, offering reasonable evidence to support the ability of the wall materials to help in reduce the oxidation rate compared to bulk oil. The new concept, applying FTIR to find if the encapsulated phase is physically encapsulated in the wall matrix has been suggested. However, the effect of the interaction between the materials used for the microcapsules need further study.

Acknowledgements

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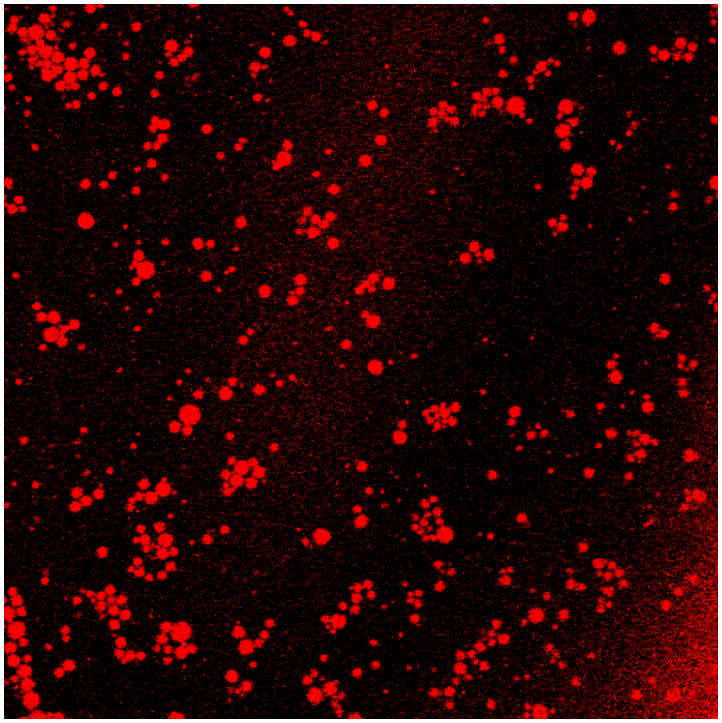
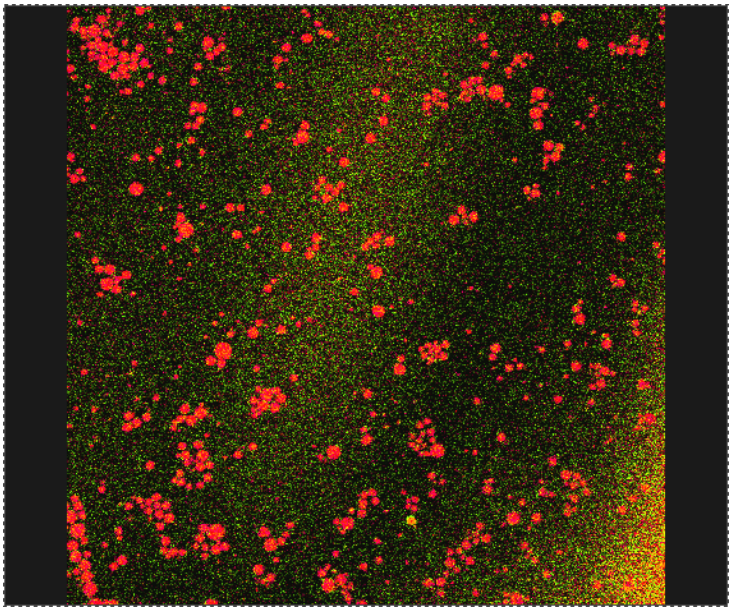
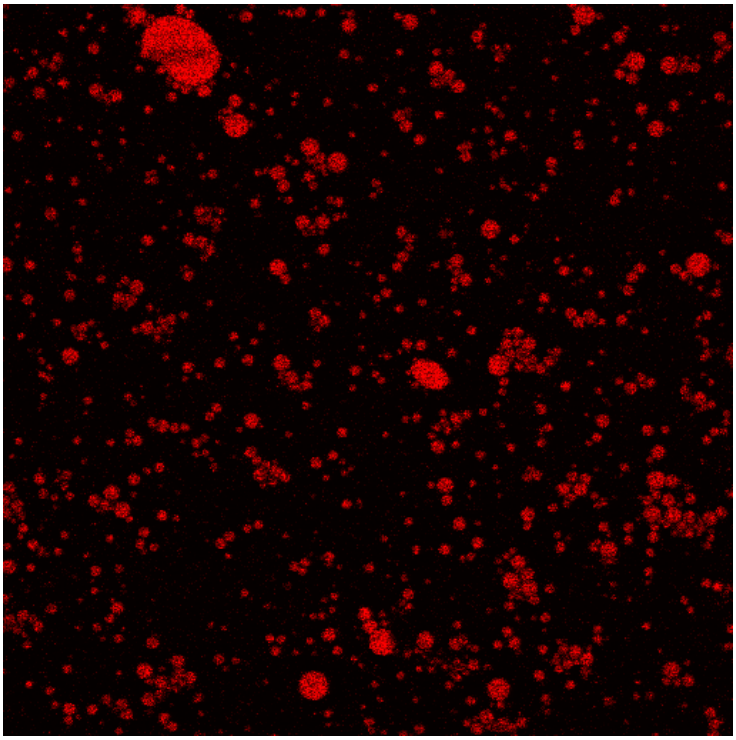


(a)

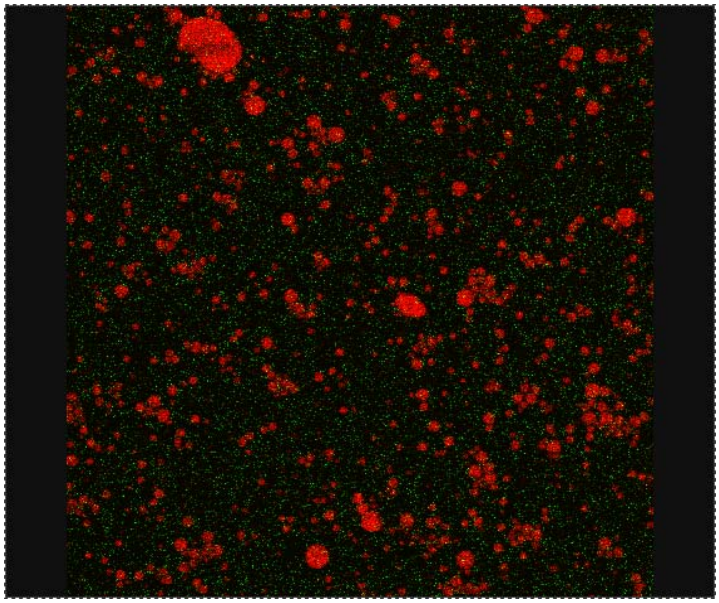


(b)

Figure 5.1. Confocal images of tuna-oil-in-water emulsion made from CS 1 % (w/w) mixed with 10 % (w/w) MD; (a) only Nile red wavelength was excited, (b) Nile red and Alexa 633 wavelength were excited.

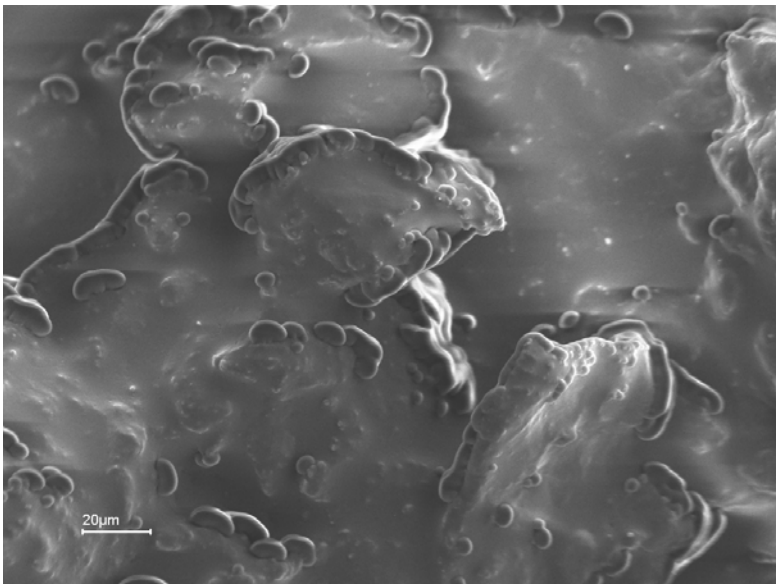


(a)

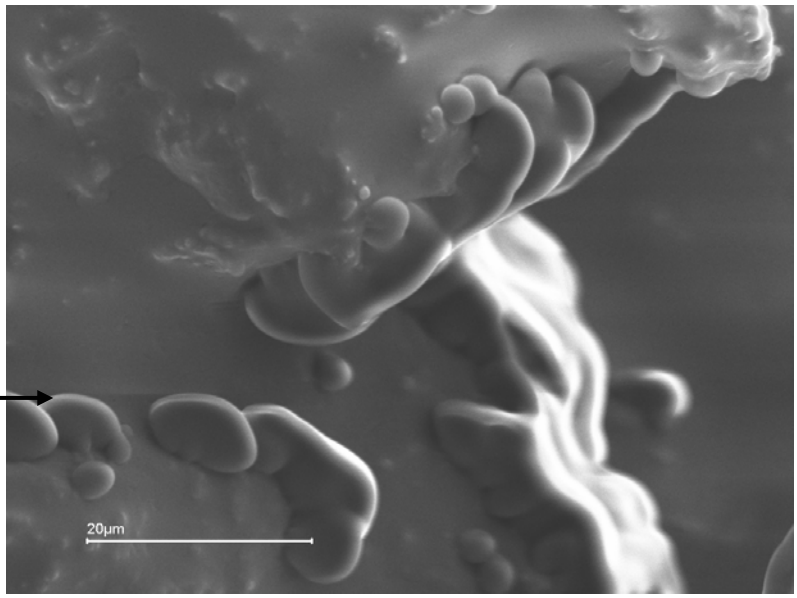


(b)

Figure 5.2. Confocal images of tuna-oil-in-water emulsion made from CS 1 % (w/w) mixed with 1 % (w/w) WPI; (a) only Nile red wavelength was excited, (b) Nile red and Alexa 633 wavelength were excited.



500x



6000x

oil

Figure 5.3. Scanning electron micrographs of tuna-oil encapsulated powder (A) made from CS 1 % (w/w) as a wall material

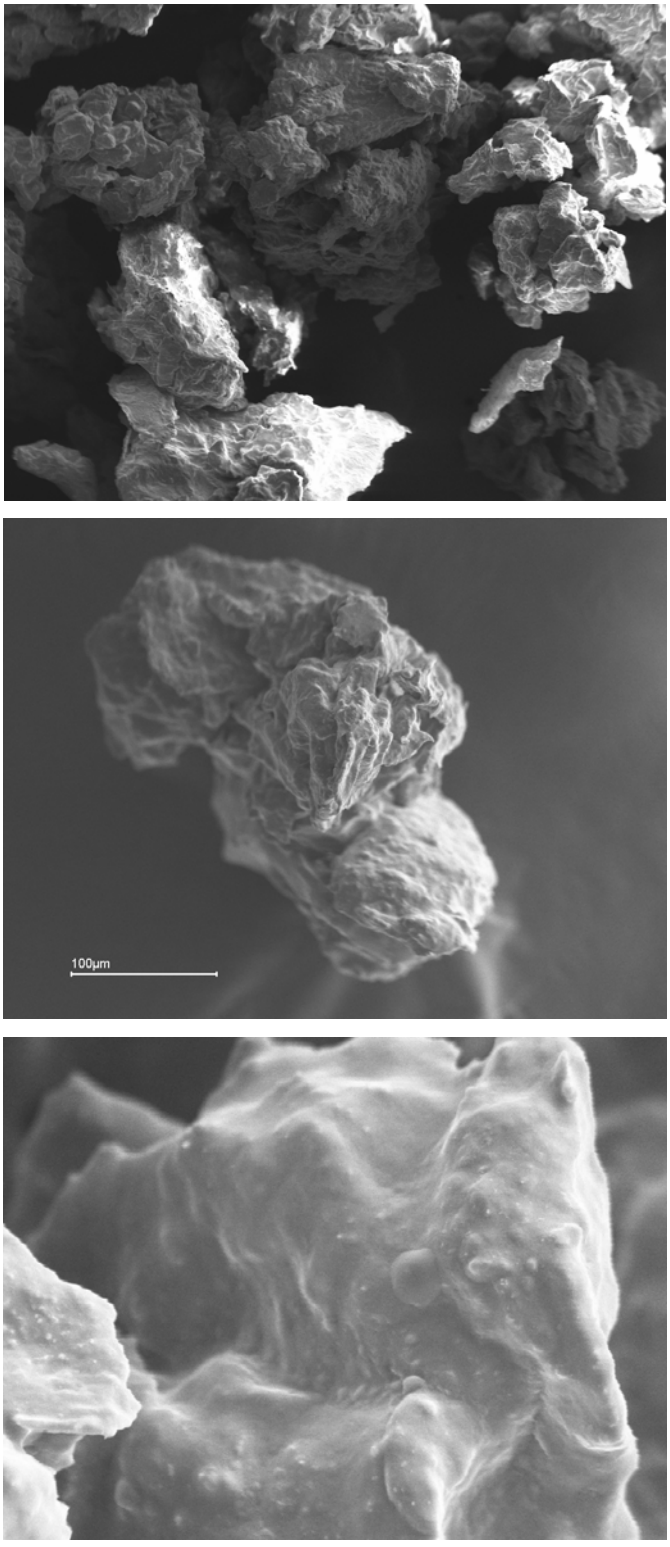
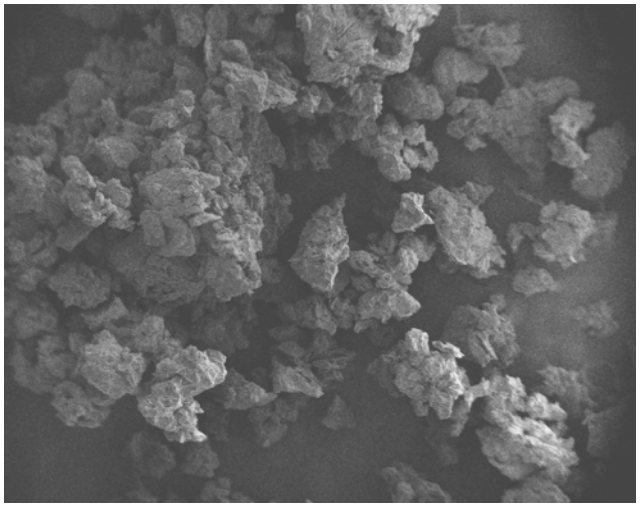
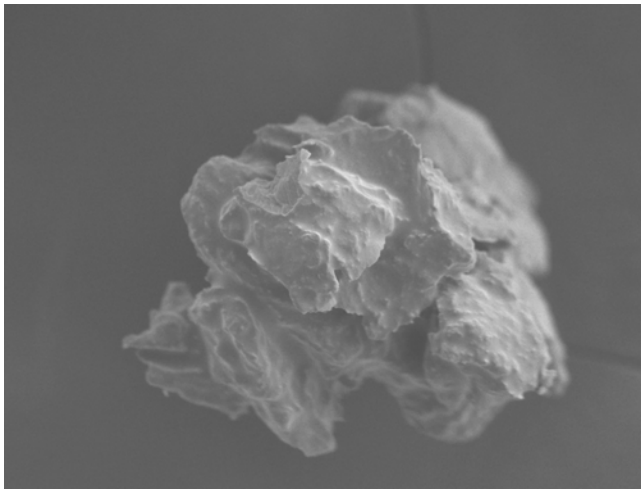


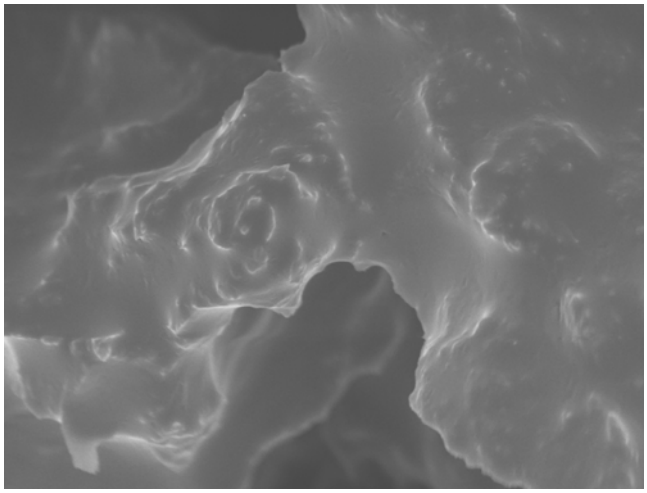
Figure 5.4**.** Scanning electron micrographs of tuna-oil encapsulated powder made from CS 1 % (w/w) mixed with WPI 1 % (w/w) (B) as the wall materials



25x



350x



5000x

Figure 5.5. Scanning electron micrographs of tuna-oil encapsulated powder made from CS 1 % (w/w) mixed with MD 10 % (w/w) (C) as the wall materials

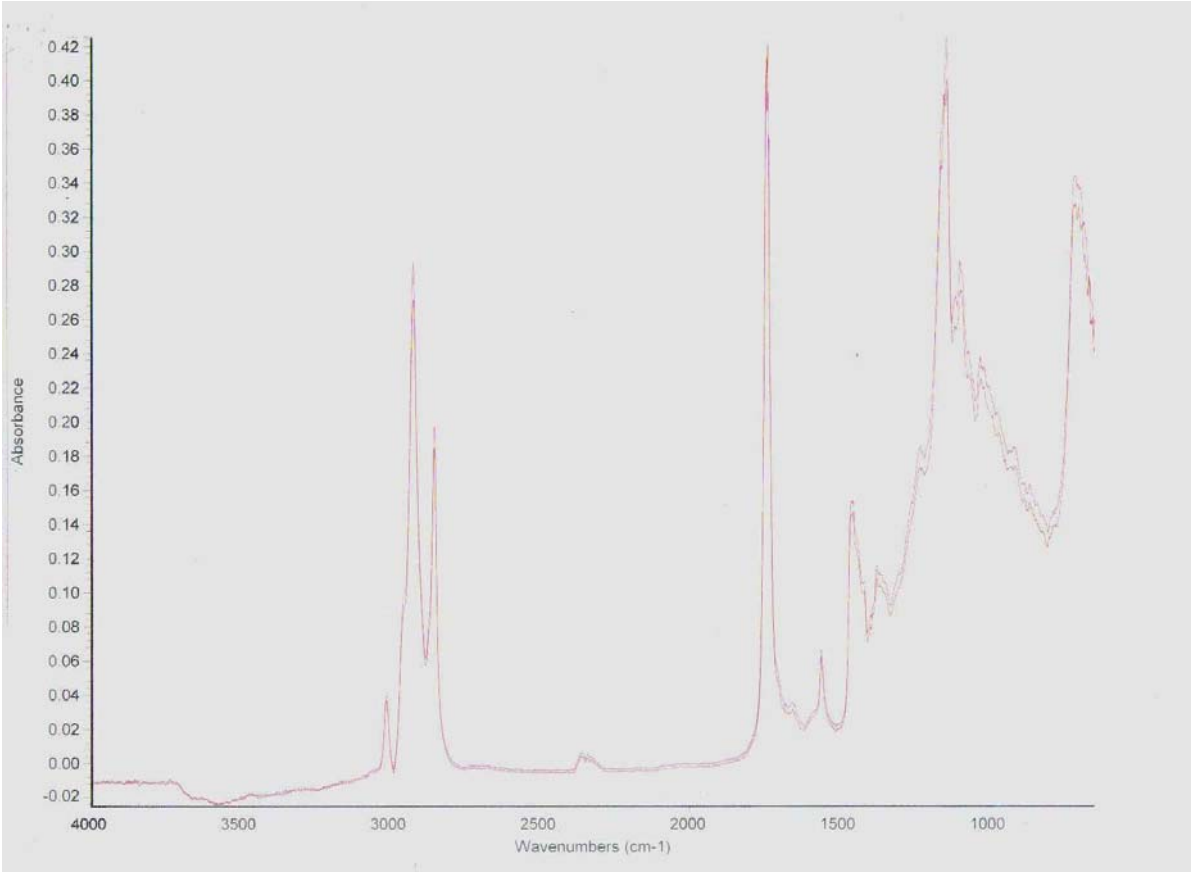


Figure 5.6. FTIR spectrum of microparticle gained from combination of 1 % (w/w) CS and 10 % (w/w) MD without tuna-oil

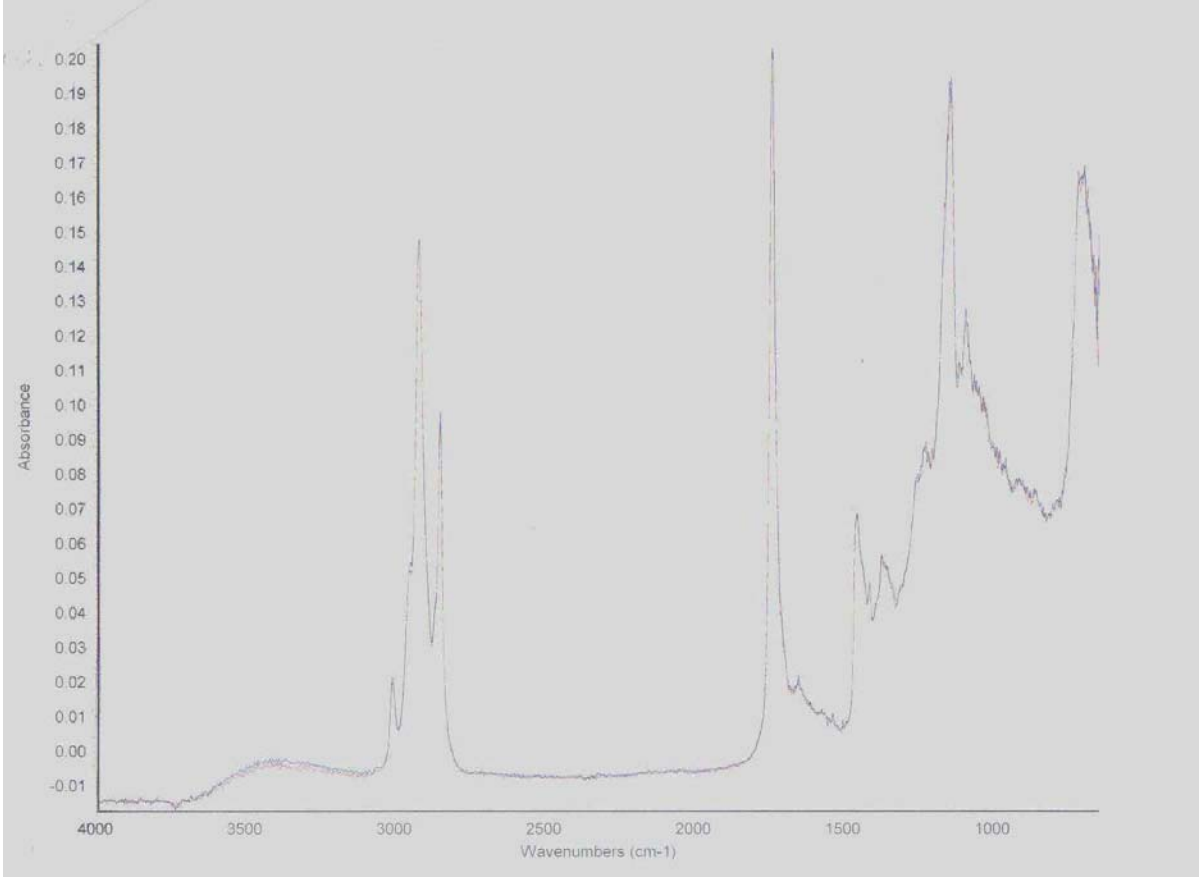


Figure 5.7. FTIR spectrum of tuna-oil encapsulated powder made from combination of 1 % (w/w) CS and 10 % (w/w) MD

CHAPTER 6

CONCLUSIONS

The first part of the study investigated the feasibility of CS-based encapsulation of tuna-oil by a new encapsulation technique employing an ultrasonic atomizer. Emulsion preparation indicated that the optimal ratio at 20 % (w/w) tuna-oil of CS to MD was 1:10, while CS to WPI was 1:1. There was a significant difference ($P<0.05$) in particle sizes in the emulsions when the preparation conditions were varied with the combination of CS and MD giving the smallest particle size with the highest emulsion stability. The encapsulated powder exhibited remarkable characteristics including EPA and DHA content which were slightly higher than commercial specifications (100 mg/g), low moisture content and water activity, acceptable appearance and encapsulation efficiency.

In the second part, the oxidative stability of tuna oil in emulsions and after encapsulation was investigated. In addition, the relationship between emulsion structure and lipid oxidation was elucidated. According to the results, there was significantly improvement in oxidative stability of all emulsions with or without α-topherol compared to bulk tuna-oil. However, the zeta-potential was not solely related to their ability to produce emulsions with high oxidative stability. The structure of emulsion droplets came close together and became bigger as the Peroxide or Anisidine value increased.

Finally, The CLSM enabled us to distinguish between encapsulated oil and the continuous phase. SEM was used to examine the outer-surface, which exhibited no pore and less

oil droplets. FTIR could be used to conclude that the tuna-oil in the microspheres is physically encapsulated in the wall matrix.

Based on this study, the combination of CS 1 % (w/w) with MD 10 % (w/w) or WPI 1 % (w/w) could effectively function as the capsule wall materials for not only tuna-oil but also other oils encapsulated by means of ultrasonic atomization in conjunction with freeze-drying to achieve acceptable storage and oxygen barrier characteristics. However, several factors that increase the rate of oxidation need to be prevented during the production process.