CHAPTER 2

Literature review

2.1 Carotenoids

Carotenoids are lipid soluble compounds that occur widely in nature and they can be synthesized by plants and many microorganisms (Pfander, 1992). Carotenoids are responsible for the yellow, orange, and red colors in fruits and vegetables (Yeum and Russell, 2002). However, as a result of different structural characteristics, carotenoids have a variety of different biological functions and chemical behaviors. The applications of carotenoids have not been limited only as the natural pigments, but also as nutraceutrical purpose. Carotenoids have biologic activities associated with antioxidant properties. Thus, they could assist in strengthening the immune system, decreasing the risk of degenerative illnesses, reducing the risk of cardiovascular disease, and preventing macular degeneration and cataracts (Mayne, 1996; Santos and Meireles, 2010; Ziegler *et al.*,1996). In addition, carotenoids are reported to be the precursors of somecompounds responsible for the flavor of foods and the fragrance of flowers (John, 2002).

Despite all of above mentioned benefits, carotenoids have a short shelf life due to the highly unsaturated polyenic chain. Carotenoids are likely to suffer degradation reactions such as oxidation and hydrolysis, which modify their biological actions (Rodriguez and Rodriguez-Amaya, 2007). In addition, carotenoids are lipophilic compounds. Their solubility in water is very low. Thus, their industrial application as colorants in aqueous media has been limited. One of the promising methods to overcome these limitations is to prepare carotenoids as a suspension with a small

particle size to produce a stable suspension. The suggested diameters are in submicron or nanometer range, 100-500 nm. Then, protecting the carotenoids with an encapsulation agent to reduce the degradation rate in the aqueous system (Horn and Rieger, 2001; Paz *et al.*, 2012).

2.1.1 Type of carotenoids

Carotenoids are polyisoprenoid compounds that can be divided into two main groups: (a) carotenes or hydrocarbon carotenoids, which composed of only carbon and hydrogen atoms and (b) xanthophylls that are oxygenated hydrocarbon derivatives that contain at least one oxygen function such as hydroxy, keto, epoxy, methoxy or carboxylic acid groups (EL-Qudah, 2009). Their structural characteristic is a conjugated double bond system, which influences their chemical, biochemical and physical properties.

The most well-known groups are β-carotene and α-carotene (Figure 2.1). Dietary β-carotene is obtained from a number of fruits and vegetables, such as carrots, spinach, peaches, apricots, and sweet potatoes (Mangels *et al.*, 1993). The *α*-carotene generally can be found in carrots, pumpkin, red and yellow peppers. The cryptoxanthin can be found in oranges, tangerines, peaches, nectarines, and papayas. Some of the better known carotenoids that have high antioxidant activity are lutein, lycopene, and zeaxanthin (Figure 2.2). Lycopene (Figure 2.1) is the hydrocarbon carotenoids that impart red color to tomatoes. It is particularly effective at quenching the destructive potential of singlet oxygen. Lutein, zeaxanthin and xanthophylls (Figure 2.3) found in corn and in leafy greens such as kale and spinach and are reported as protective antioxidants in the macular region of the human retina (Snodderly, 1995).

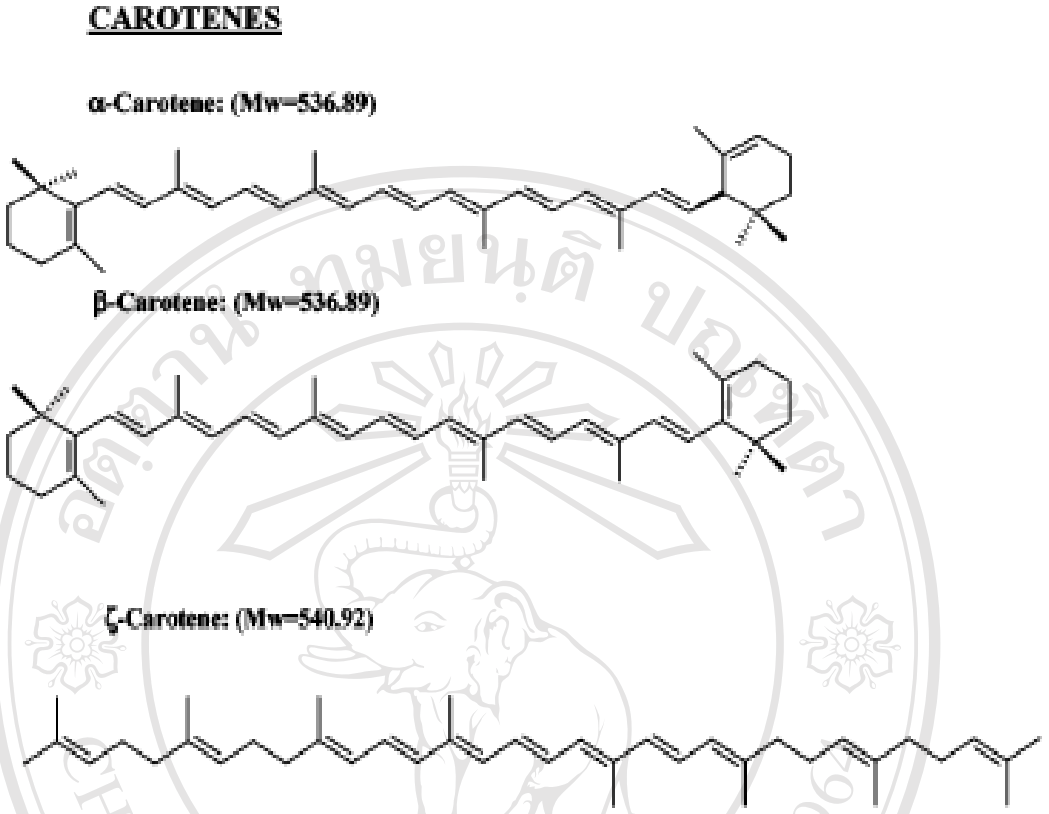

Figure 2.1 Structure of α-carotene, β-caroteneand ζ-carotene (Clara *et al.*, 2004)

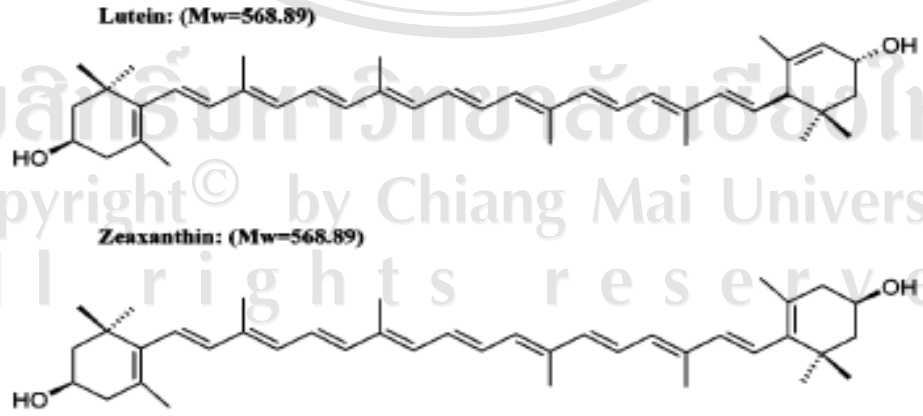

Figure 2.2 Structures of Lutein and Zeaxanthin (Clara *et al.*, 2004)

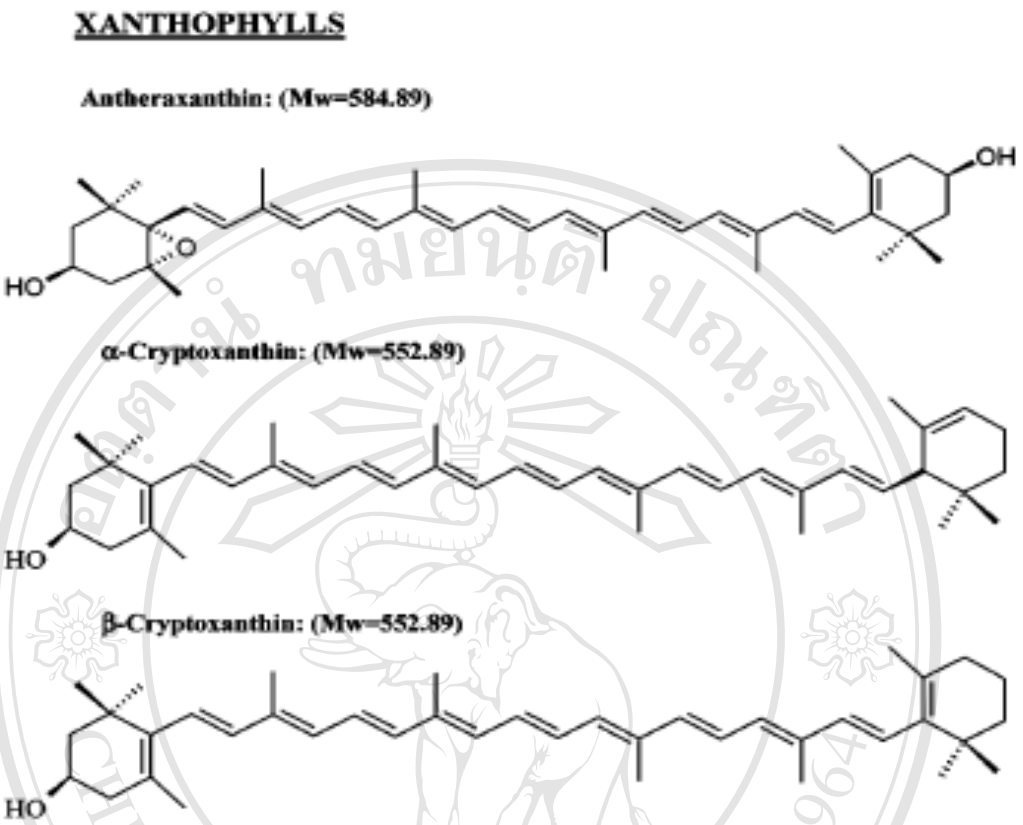

Figure 2.3 Structures of Xanthophlls: Antheraxanthin, α-Crypotoxanthin and β-Crypotoxanthin (Clara *et al.*, 2004)

1) Carotene

Carotene is stored in the liver and converted to vitamin A as needed. The *α*-carotene has the same kind of ring on the left end of the molecule, and a slightly different kind of ring on the right end of the molecule (Krinsky, 2001). The δ-carotene is a precursor of α-carotene. It has the same type of ring as α-carotene on the right end, but the left end is not cyclize. Theγ-carotene is a precursor of β-carotene. The γ-carotene has a ring like β-carotene's rings on one end and no ring on the other. Some food sources of γ-carotene are carrots, sweet potatoes, corn, tomatoes, watermelon, and apricots.The ζ-carotene is different from α and β

carotene because it is acyclic. It is structure similar to that of lycopene (Figure 2.1) but has an additional four hydrogen atoms. β-carotene ($C_{40}H_{56}$) is one of the major carotenoids present in the diet (Johnson, 2002). It along with α-carotene and β-cryptoxanthin, is a source of provitamin A.

2) Lycopene

Lycopene ($C_{40}H_{56}$) is a hydrophobic, acyclic carotenoid containing eleven conjugated double bonds (Wilcox *et al.*, 2003). In uncooked plant products, approximately 95% of lycopene is found in the all-trans form (Nguyen and Schwartz, 1999) and is located in the photosynthetic pigment-protein complex of the thylakoid membrane (Shi and LeMaguer, 2000). In some thermally processed foods and in human serum and tissues, higher quantities of *cis* isomers are found (Clinton, 1998). Several researches have concluded that diets rich in high lycopene foods are associated with a reduced risk of several diseases. In some cell culture and dietary intervention, studies have provided evidence that lycopene may play a positive role in health. Consumption of high lycopene foods has been proposed to reduce the risk of diseases including cardiovascular disease (Wilcox *et al.*, 2003), and cancers of the prostate (Hadley *et al.*, 2002), breast (Shi, 2000), cervix, colon, esophagus, skin, pancreas, bladder, and stomach (Clinton, 1998).

3) Lutein and Zeaxanthin

Lutein and zeaxanthin ($C_{40}H_{56}O_2$) are oxygenated carotenoids, making them members of the xanthophyll group of carotenoids (Alves-Rodrigues and Shao, 2004). Lutein and zeaxanthin are stereo isomers, which complicates analytical techniques for determining the quantities of each stereo isomer present, and likewise, has created difficulties in determining the influence of the individual isomers on human health (Stacewicz-Sapuntzakis *et al.*, 1993). Commercially produced lutein is derived from marigolds and is used in the poultry industry to provide yellow color to the yolks of eggs and the skin of broilers (Sowbhagya *et al.*, 2004). Some studies suggest that lutein and zeaxanthin are associated with a reduced incidence of age-related macular

degeneration and cataracts as determined by epidemiological and intervention studies (Richer *et al.*, 2004). The mechanism by which these carotenoids are thought to decrease macular degeneration and cataract formation, is by increasing the macular pigmentation of ocular tissues, which helps to filter damaging blue light, thus preventing oxidation damage that eventually leads to tissue damage (Berendschot *et al.*, 2000). However, other studies have not shown a relationship between lutein and zeaxanthin and eye health (Mozaffarieh *et al.*, 2003), indicating that more work is needed in this area (Stringham and Hammond, 2005). Although research in these areas is still limited, lutein has been proposed to reduce risk factors for coronary heart disease and stroke, breast cancer, and improving skin health (Johnson, 2002).

2.1.2 Factors influencing the chemical stability of carotenoids in foods

Carotenoids are highly conjugated, and intensely coloured isoprenoid plant compounds, which are unstable when exposed to light and oxygen, therefore should be protected during processing. The basic chemical structure of the carotenoids consists of tetraterpenoids connected by opposite units at the center of the molecule with a polyenic chain ranging from 3 to 15 conjugated double bonds. This structure is susceptible to a number of different modifications such as cyclisation, migration of the double bonds and the addition of oxygenated functions, amongst others, and generates a great diversity of structures (Britton, 1995). The conjugated polyene chain that is characteristic of carotenoids also makes these compounds susceptible to degradation from a number of agents. Depending on the carotenoid, the terminal end groups may also suffer degradation in certain environments. A number of carotenoid oxidizing agents have been identified. Carotenoids degradation pathways are highly influenced by the agent involved in the initiation of degradation. In a functional food fortified with carotenoids, oxidative damage could result in loss of both product quality (color loss, rancidity, etc.) and bioactivity.

1) Autoxidation

Reaction of carotenoids with atmospheric oxygen has been found to occur with relative ease, especially in systems consistingof purified carotenoids in organic solvents. Autoxidation of β-carotene in benzene or tetrachloromethane in the dark at 30°C under one atmosphere of oxygen or by bubbling oxygen through the solvent was found to occur with an induction period of less than one hour, followed by rapid production of oxidation products. Under these conditions, β-carotene was completely consumed within 30 hr. The autoxidation may perhaps begin as β-carotene in solution forms isomers via a biradical process. The twisting of the molecule during the isomerization process may lead to an unpaired spin state, which can react easily with oxygen to form a carbon-peroxyl triplet biradicals. These may go on to form endo-peroxides or to react with a neutral β-carotene molecule, forming an epoxide and a carotene alkoxyl radical. It was concluded that the autoxidation process results first in the production of epoxides, carbonyl compounds, and an uncharacterized oligomers, followed by further oxidation reactions of these compounds to produce secondary short chain carbonyl compounds, carbon dioxide, and carboxylic acids (Mordi *et al.*, 1993). The β-carotene adsorbed to a C_{18} solid phase also exhibited autoxidation when oxygen-saturated water was flowed continuously over the solid support (Henry *et al.*, 2000). Retinoic acid, a compound derived from some provitamin A carotenoids (O-Byrne and Blaner, 2005), has been proposed to react directly with oxygen in the triplet state, at room temperature. This proposed pathway was based on the products formed when retinoic acid was exposed to 5-40 atm oxygen in 90% ethanol at room temperature. These products were formed in the presence of peroxyl radical scavenger (2,6-di-*tert*-butyl-4-methylphenol) even though the formation of all other products was greatly inhibited, suggesting that these are products of direct oxygen addition while the other products produced without basic metabolic panel (BMP) present are due to concurrent autoxidation. The mechanism of oxygenation may involve the collapse of electron donor acceptor complexes forming carbon-peroxylbiradicals followed by intersystem crossing and ring closure (Clark *et al.*, 1997).

2) Thermal Degradation

Thermal treatment to carotenoids in the presence of oxygen results in the formation of volatile compounds and larger non-volatile components (Bonnie and Choo, 1999). Marty and Berset (1990) were found that heating pure β-carotene at 180°C for two hours resulted in the formation of a number of *cis* isomers as well as oxidation products. The level ofair circulation in the sample increased the degradation of β-carotene because of the greater likelihood of β-carotene andoxygen interaction. All of the double bondsof β-carotene could be oxidized and that the breakage of these bonds is likely to occur starting at the terminal end of the molecule and proceeding towards the center of the molecule. A study of β-carotene in toluene (Handelman *et al.,* 1991) conducted at 60°C with oxygen produced a complex mixture of products including, but not limited to epoxides as found by El-Tinal and Chichester (1970). These products led the researchers to conclude that under these oxidation conditions, various radical species are formed and can react with oxygen to form peroxyl radicals, which can undergo propagation reactions with additional carotenoids (Handelman *et al.*, 1991). Even under vacuum, higher temperature treatment (240°C) of crystalline β-carotene resulted in the formation of toluene, m-xylene, pxylene, ionene, and 2, 6-dimethylnaphthalene. At lower temperatures (60°C), with a stream of oxygen being passed through β-carotene in toluene, El-Tinay and Chichester (1970) observed that β-carotene terminal double bonds could be broken, producing various epoxides. In conclusion, there was no lag phase in the decompositionof β-carotene, ruling out autoxidation as a mechanismfor degradation. The reaction of oxygenwith β-carotene might be catalyzed by metals due to the findings that cupric stearate increased the rate of reaction 4.3 fold. The metals may have also been involved in the other autooxidation and thermal oxidation experiments as well.

3) Photodegradation

Light exposure degrades carotenoids, and several mechanisms of action have been proposed. Photooxidation produces species thought to be carotenoids radical cations (Konovalova *et al.*, 2001). Laser flash photolysis studies have produced

evidence to suggest that rapid bleaching of β-carotene in some solvents like chloroform, can occur due to light exciting the β-carotene molecules, which then instantly react with chloroform to form either a carotenoids-solvent free radical adduct or a β-carotene radical due to hydrogen abstraction. The similarstudy has also shown that the β-carotene molecules in the excited state may return to ground state, where they may be attacked by radical by products created during the above reaction and undergo a slower degradation process thought to possibly form β-carotene radical cations (Mortensen and Skibsted, 1996).

4) Acid

Exposure to acids is thought to produce ion-pairs, which can then dissociate to form a carotenoids carbocation. Optical spectra of carotenoidscarbocations have been reported for β-carotene, 8′-apo-caroten-8′-al, and canthaxanthin when exposed to trifluoroacetic acid in benzene, CH_2Cl_2, and acetonitrile solution. Canthaxanthin and 8′-apo-β-caroten-8′-al incorporated into sol-gels could be degraded by sulfuric acid at pH of 3-3.5 (Konovalova *et al.*, 2001).

5) Free Radicals

In foods, free radicals may already be present due to reactions such as lipid oxidation (Choe and Min, 2006). Carotenoids have been found to confer both antioxidative and prooxidantive effects in systems containing pre-formed radicals depending on the type and level of carotenoids used, oxygen concentration present, and the polarity of the solvent (Haila *et al.*, 1997). When radicals are present in a system, several mechanisms of carotenoids interaction are possible. These reactions include electron transfer, hydrogen abstraction, and addition of radical species to form carotenoids radical adducts (Mortensen, 2002).

6) Electron Transfer

Young and Lowe, 2001 reported that neutral carotenoids are capable of participating in electron transfer reactions with radicals as well as with metals like iron. The β-carotene, canthaxanthin, zeaxanthin, astaxanthin, and lycopene have been shown to form radical cations by electron transfer reactions with tryptophan radical cations in a pulse radiolysis study using carotenoids in micelles (Burke *et al.*, 2001). Pulse radiolysis has also shown that radicals of nitrogen dioxide react with carotenoids in this manner in lycopene, lutein, zeaxanthin, astaxanthin, or canthaxanthin in tertbutanol/water mixtures. The same reaction mixtures and pulse radiolysis techniques also found thiyl-sulphonyl radicals to produce carotenoid radical cations, but an ion-pair was detected as well (Mortensen *et al.*, 1997). The electron transfer reactions taking place may depend on the type of carotenoids and radical. For instance, there is evidence from laser flash and steady-state photolysis studies that β-carotene is not likely to interact with peroxyl radical in this manner, but instead are more likely to undergo adduct formation or hydrogen abstraction reactions. If carotenoid radical cations are formed, a number of reactions may occur. One path they may follow is to interact with oxygen to form a carotenoid peroxyl radical cation, which might then be reduced by another carotenoids or ferrous iron (Gao *et al.*, 2003).

7) Hydrogen Abstraction

Reaction of neutral carotenoids with a radical can result in the radical abstracting hydrogen from the carotenoids (Choe and Min, 2006). Liebler and McClure (1996) proposed this as a potential mechanism by which β-carotene oxidation products were formed during oxidation with 2, 4-dimethylvaleonitrile radicals in benzene at 60°C based on atmospheric pressure chemical ionization mass spectrometry results. Woodall *et al.* (1997) shown β-carotene might react with peroxyl radicals, postulating that the allylic C-4 position of the molecule might be the site of attack. This hypothesis was based on electron density calculations and UV-Vis and mass spectrophotometry results produced during the reaction of the beta. Mortensenand Skibsted (1998) also proposed this mechanism as well as adduct formation as possible means by which alkyl, alkoxyl

and alkylperoxyl radicals might react with β-carotene. It has been proposed that the newly formed carotenoids radical might then encounter an additional alkoxyl or peroxyl radicals in the medium and react to form a non-radical product (Choe and Min, 2006; Liebler and McClure, 1996).

8) Adduct Formation

Finally, radicals can also react with carotenoids to form radical adducts. This may occur with alkyl, alkoxyl, and peroxyl radicals (Haila *et al.*, 1997). Laser flash and steadystate photolysis studies of β-carotene indicate that at least for these carotenoids, reaction with peroxyl radicals occurs much more slowly than with alkyl or alkoxyl radicals. β-carotene and lutein can quench peroxyl radicals at low oxygen concentrations usingelectron paramagnetic resonance (EPR) and spin trapping techniques (Iannone *et al.*, 1998). Laser flash photolysis studies of β-carotene and canthaxanthin in aerated solutions of benzene with di-*tert*-butyl peroxide and toluene have provided evidence that the benzylperoxyl radical produced under these conditions reacts with the carotenoids to produce adducts that decay in a first-order reaction (Mortensen, 2002). Acetylperoxyl radicals produced during laser flash photolysis have also been shown to produce adducts with β-carotene in aerated benzene at 20°C. The second-order rate constant of this reaction is $9.2 \times 10^8\ M^{-1}s^{-1}$ (Mortensen, 2001). Pulse radiolysis has also shown that glutathione thiyl radicals and 2-mercaptoethanol thiyl radicals react with carotenoids in a first order reaction to produce adducts followed by bimolecular decay of adducts in solutions of 10 μM lycopene, lutein, zeaxanthin, astaxanthin or canthaxanthin in *tert*-butanol/water mixtures (Mortensen *et al.*, 1997). Liebler and McClure (1996) proposed this as well as hydrogen abstraction as potential mechanisms by which β-carotene oxidation products were formed during oxidation with2,2'-azo6iX2,3-dimethylvaleonitrile (AMVN) radicals in benzene at 60°C based on atmospheric pressure chemical ionization mass spectrometry results. El-Agamey and McGarvey (2005) were the first to observe reversible oxygen addition to neutral 7,7′-dihydro-β-carotene radicals and neutral β-carotene radicals utilizing phenylthiyl radicals produced by laser flash photolysis. The rate constants determined for the addition of

oxygen to these neutral radicals were determined to be 0.64×10^4 $M^{-1}s^{-1}$ for β-carotene radicals and 4.3×10^4 $M^{-1}s^{-1}$ for 7,7′-dihydro-β-carotene radicals. As first described by Burton and Ingold (1984), they were found to decrease when oxygen levels were lowered. Similarly, as oxygen levels were lowered, β-carotene had a greater antioxidant effect in the initiated oxidation of tetralin and methyl linoleate in chlorobenzene at 30°C. Thus, if oxygen is lowered, production of peroxyl radicals is reduced. At higher oxygen pressures, the reaction is driven to the right and autoxidation rates increase due to hydrogen abstraction reactions. Peroxyl radical adducts are also likely to follow other pathways. One path these radical adducts might follow is decay to carotenoids epoxides and new alkoxyl radicals, which may further the extent of oxidative degradation as the newly formed radicals attack other oxidizable substrates in the system (Liebler and McClure, 1996). Diverse factors can be responsible for this quantitative variability of pigments, one of them being exposure to sunlight, since some authors reported an increase of carotenoids with high solar radiation, carotenoids could act as a safety valve venting the excessive visible radiation energy before it can damage the photosynthetic system (Gonzalez *et al.*, 2007).

2.1.3 Maximum level for use of carotenoids as an ingredient in food

The Food and Drug Administration (FDA) responded to the notice on January 2010. The subject of the notice is maximum level use of carotenoids from palm for use as an ingredient in various food categories at the use levels listed in table 2.1 and in medical foods at a level of 180 mg β-carotene per day.

Table 2.1 Food categories and use levels of palm carotenoids

Food category	Maximum use level (mg total carotenoids per kg food)
Fats and oils, margarine	38.9
Canned green peas, salad cream, mayonnaise	155.8

Food category	Maximum use level (mg total carotenoids per kg food)
Meatless bouillons and consommé	311.6
Pickled cucumber	467.4
Baked goods, bread, rolls, cakes	15.58
Meatless soups, soup mixes	15.58
Baking mixes, cookies, crackers, salty snacks	15.58
Grain products, such as pastas or rice dishes	15.58
Ready-to-eat cereals	15.58
Frozen dairy desserts and mixes, ice cream and mixes	15.58
Processed fruits, juices, juice drinks, punches	15.58
Meatless meat products (soy-based imitation meat)	15.58
Processed vegetables, juices	15.58
Sweet sauces, toppings, syrups	15.58
Gelatins, puddings, fillings	15.58
Meatless gravies, sauces	15.58
Egg substitute products (imitation egg products)	15.58
Soft drink-orange drink type only	15.58
Granola bars, cereal bars	15.58
Meal replacement shakes/mixes, functional beverages	15.58

* Generally Recognized as Safe (GRAS) No. GRN 000320

2.2 Chitosan

Chitosan and its derivatives have received a great deal of attention because of their only known naturally occurring polycationic polysaccharide. Main applications include food, agriculture, biochemistry, wastewater treatment, paper, textiles, cosmetics, nanoparticles, hydrogel, liquid crystals, membranes, and microcapsules (Harish-Prashanth and Tharanathan, 2007; Muzzarelli, 2009). Chitosan is a high molecular

weight polysaccharide and is composed by glucosamine and N-acetyl-glucosamine. It is a widely distributed biopolymer since it is readily available via cationic polyelectrolyte in acid solution and because it is non-toxic, biocompatible and biodegradable. Chitosan obtained by alkaline deacetylation of chitin which is widely distributed in shells of crustacean like lobsters, shrimps, and crabs. A natural linear biopolyaminosaccharide of chitsan was obtained by alkaline deacetylation of chitin.

Chitosan enables a high degree of chemical modification. Chitin is a straight chain homopolymer composed of (1,4)-linked N-acetyl glucosamine units, while chitosancomprises of copolymers of glucosamine and N-acetyl glucosamine. Chitosan has one primary amino group and two free hydroxyl groups for each C_6 building unit (Figure 2.4). Due to the availability of free amino groups, it carries a positive charge and reacts with many negatively charged surfaces such as the cell membrane, mucus lining and also with other anionic polymers (Chakraborty *et al.*, 2010; Paliwal *et al.*, 2012).

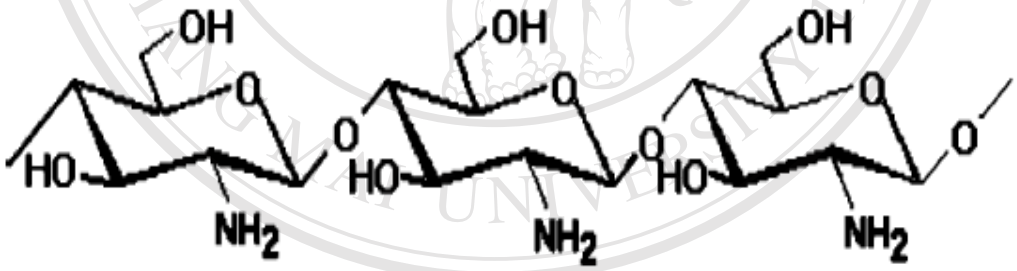

Figure 2.4 Structure of chitosan (Sinha *et al.*, 2004)

Chitosan has been shown to possess mucoadhesive properties (Kockisch *et al.*, 2003; Shimoda *et al.*, 2001) due to molecular attractive forces formed by electrostatic interaction between positively charged chitosan and negatively charged mucosal surfaces. These properties may be attributed to strong hydrogen bonding groups (Schipper *et al.*, 1997), strong charges (Dodane *et al.*, 1999), high molecular weight

(Kotze *et al.*, 1998; Schipper *et al.*, 1996), sufficient chain flexibility (He *et al.*, 1998) and surface energy properties favoring spreading into mucus (Lueben *et al.*, 1994).

The rheological properties of aqueous chitosan dispersion depend on the chitosan molecular weight and degree of deacetylation, as well as the distribution of those acetyl groups along the chain length, pH and ionic strength (Anthonsen *et al.*, 1993; Chandy and Sharma, 1990). Chitosan is a weak base, insoluble in water and organic solvents, however, it is soluble in dilute aqueous acidic solution (pH<6.5), which can convert the glucosamine units into a soluble form of protonated amine ($R\text{-}NH_3^+$) (Lam *et al.*, 2006). As such nano medicine drug delivery system can reduce the drug dosage frequency, treatment time and toxicity (Swai *et al.*, 2008).

Commercially, chitosan is available in the form of dry flakes, solution and fine powder. It has an average molecular weight ranging between 3800 and 2,000,000 and is from 66 to 95% deacetylated (Kas, 1997). Particle size, density, viscosity, degree of deacetylation, and molecular weight are important characteristics of chitosan which influence the properties of pharmaceutical formulations based on chitosan. Properties such as biodegradability, low toxicity and good biocompatibility make it suitable for use in biomedical and pharmaceutical formulations (Illum *et al.*, 2001).

Since chitosan has a capacity of forming film it has been suggested as a biopolymer of choice for the development of contact lens (soft and hard contact lenses). Chitosan has been used for the manufacturing of ocular bandage lenses used as protective devices for acutely or chronically traumatized eyes (Skjak-Braek *et al.*, 1989). Chitosan membranes have also been found useful as artificial kidney membranes because of their suitable permeability and high tensile strength (Amiji, 1995).

2.3 Sodium tripolyphosphate

Sodium Tripolyphosphate (STPP), also known as Pentasodium Triphosphate or Pentasodium Tripolyphosphate ($Na_5P_3O_{10}$), is a straight chain derivative from phosphoric acid. It is classified by the Food and Drug Administration as being a generally recognized as safe substance (FDA, 2006) and is approved to be used as a

food additive, along with other polyphosphates, either in USA and in Europe, in a wide variety of foods including fruits, beverages, meat and seafood sole or in combination with other phosphates (EC, 1995). STPP is identified in labels by the food additive code 451 (E451, in Europe) and is able to act as a buffer, emulsifier, texturizer and sequestrant (Zaika and Kim, 1992).

In pharmaceutical technology, STPP has gained interest as an ingredient of chitosan nano and microparticle systems. In 1989, Bodmeier and the team first reported drug encapsulation by ionotropic gelation due to the formation of inter and intramolecular crosslinks of the positively charged chitosan mediated by the polyanionic tripolyphosphate. This method has the advantage of being simple, non-toxic, developed at room temperature while avoiding the use of organic solvents. The functionality role of STPP in these systems supports its classification as a pharmaceutical excipient having the advantage of being considered safe since it is used in food industry (Pifferi and Restani, 2003).

Chitosan-STPP ionotropic interactions have been prepared by different techniques, to achieve encapsulation of several drugs like vitamins (Desai and Park, 2005), antioxidants (Luo *et al.*, 2010), antimicrobials (Hasanovic *et al.*, 2009), proteins (Pan *et al.*, 2002), nucleic acids (Csaba *et al.*, 2009), and to develop new controlled release drug delivery systems (Buranachai *et al.*, 2010; Srinatha *et al.*, 2008).

2.3.1 Properties and the use of sodium tripolyphosphate

Sodium tripolyphosphate (STPP) is a solid inorganic compound, which belong to the group of condensed inorganic phosphates. STPP have the stable form as the hexahydrated salt, it is widely used in regular and compact laundry detergents, automatic dishwashing detergents, toilet and surface cleaners, where it provides a number of functions including sequestration of water hardness, pH buffering,dirt emulsification and prevention of deposition, hydrolysis of grease and dissolving-dispersing dirt particles. The use of STPP in detergents could be replaced only by a great number of different chemicals, as no one substitute offers all its functions. The amount of STPP which was widely used in household cleaning products in Europe,

since its consumption in household detergents varies considerably between different countries in Europe. In some countries the STPP use in detergents is almost exclusively concentrated on automatic dishwashing products while in other countries the use in laundry detergents is the overwhelming application. Sodium tripolyphosphate exists in two major crystalline forms, known as Phase I and Phase II. Phase I material is formed if the process temperature is maintained above 450°C, while Phase II material is formed at temperatures below 450°C. Typical composition of STPP includes 4% impurity due to presence of sodium pyrophosphate, sodium orthophosphate and sodium metaphosphate. A production plant for STTP, based on wet manufacturing route, essentially consists of neutralization part where phosphoric acid is neutralized to an orthophosphate solution, heating part where, by drying and calcination, the solution is converted into sodium tripolyphosphate (Predrag, 2007). The raw material for sodium tripolyphosphate manufacture is obtained by recovering the phosphate values from phosphoric ore, in the form of phosphoric acid H_3PO_4. In this process, phosphate rock is acidulated with sulfuric acid H_2SO_4, either alone, or in conjunction with phosphoric acid. The result of this operation is formation of precipitate, which contains the calcium values as calcium sulfate, and recovering the impure phosphoric acid, termed wet acid. The resulting wet acid is contaminated (Becker, 1989) with process impurities originate from the reagents used in the production of phosphoric acid, like sulfuric acid and process water. The common impurities in the phosphate rock, such as aluminum, iron, magnesium, calcium, potassium, strontium, chlorides, fluoride, arsenic, cadmium, mercury, uranium, copper, zinc and lead (Predrag, 2007).

2.4 Chitosan crosslink with tripolyphosphate

Although, chitosan as a good biomaterial, it has the main disadvantage of poor solubility in water but the water soluble can be easily synthesized with proper chemical modification (Werle *et al.*, 2009). Nanosizing of drug or incorporation into lipidic and polymeric particles can support deliver drugs with poor aqueous solubility and permeability (Divya *et al.*, 2011). The microspheres, microgels and nanoparticles are

the different forms of chitosan-based drug delivery devices that have been studied.

Chitosan nanoparticles have been extensively investigated because they can control drug release rate, prolong the duration of therapeutic effectiveness, and deliver drugs to their specific sites in the body. Chitosan nanoparticles exhibit superior activity that can be attributed to their small and quantum size effect (Papadimitriou *et al.*, 2008; Qi *et al.*, 2004). Chitosan nanoparticles, which are launched as promising carriers for controlled-release drug delivery, are exploited extensively in the pharmaceutical industry (Gan and Wang, 2007; Yang and Hon, 2009).

Several other methods of synthesis of nano chitosan such as desolvation method, emulsion-droplet coalescence method, reverse micellar method, self-assembly via chemical modification, spray drying (Agnihotri *et al.*, 2004; Patel and Jivani, 2009) and nonaqueous electrochemical method are described in literature (Jingming *et al.*, 2008). By adopting a novel chitosan cross-linked method, i.e. chitosan/gelatin droplet coagulated at low temperature and then cross-linked by anions (sulfate, citrate and TPP), the chitosan beads were prepared. It has been popular because it has the ability to gel quickly, and interacts electrostatically with cationic chitosan. Actually in acidic medium, the amine groups of chitosan molecules are protonated, which interact instantaneously with anionic TPP through electrostatic attraction and are transformed into chitosan-TPP gel (Lee *et al.*, 2001).

Scanning electron microscopy (SEM) observation showed that sulfate/chitosan and citrate/chitosan beads usually had a spherical shape, smooth surface morphology and integral inside structure. Cross-sectional analysis indicated that the cross-linking process of sulfate and citrate to chitosan was much faster than that of TPP due to their smaller molecular size. However, once completely cross-linked, TPP/chitosan beads possessed much better mechanical strength and the force to break the beads was approximately ten times higher than that of sulfate/chitosan or citrate/chitosan beads.

Release media pH and ionic strength seriously influenced the controlled drug release properties of the beads, which related to the strength of electrostatic interaction between anions and chitosan. Sulfate and citrate cross-linked chitosan beads swell and even dissociate in simulated gastric fluidand hence, riboflavin could release completely

in 5 hr; while in simulated intestinal fluid, beads remained in a shrinkage state and drug released slowly. However, swelling and drug release of TPP/chitosan bead was usually insensitive to media pH. Chitosan beads, cross-linked by a combination of TPP and citrate or sulfate together, not only had a good shape, but also improved pH-responsive drug release properties. Salt weakens the interaction of citrate, especially sulfate with chitosan and accelerated beads swelling and hence drug release rate, but it is insensitive to that of TPP/chitosan (Shu and Zhu, 2002).

Several works suggested that ionically cross-linked chitosan beads may be useful in stomach specific drug delivery (Shu and Zhu, 2002). The physically of cross linked chitosan have been used in drug delivery systems due to their enhanced biocompatibility over chemically cross linked. Non-toxicity and quick gelling ability of TPP are the important properties that make it a popular cross linker for ionic gelation of chitosan. Chitosan nanoparticles prepared by TPP as an anionic cross linker are homogeneous, and possess positive surface charge that make them suitable for mucosal adhesion applications (Gan and Wang, 2007; Rayment and Butler, 2008).

Noticeably, the applications of chitosan nanoparticles are well demonstrated as controlled drug delivery systems and it can removal heavy metals in the water treatment (Tamura *et al.*, 2010; Trapani *et al.*, 2009; Zhang *et al.*, 2010). Although, chitosan has high adsorption capacity that can be utilized in water purification systems but chitosan microparticles from crosslinked with tripolyphosphate (TPP) showed the higher significant results in adsorption of ions Copper (II), Lead (II) and Uranium (Lee *et al.*, 2001; Qi and Xu, 2004; Sureshkumar *et al.*, 2010). Hui and Changyou (2009) reduced the size of chitosan to nanoparticles by crosslinked with TPP.

The chitosan nanoparticles were more stable than chitosan and no cytotoxicity. It showed the good storage stability at 48°C, with no apparent agglomeration and severe size increase until 85 days.

2.4.1 The stability of chitosan crosslink with tripolyphosphate

The stability of chitosan-TPP nanoparticles is highly relevant to its potential use as a drug delivery agent as this plays an important role in the function of the

nanoparticle and will determine shelf life. Gordon and his team (2011) studied the physical stability of TPP-chitosan nanoparticles by measured across a range of different temperature conditions: 4, 25 and 40°C using differential sedimentation. After 12 months storage at 4 and 25°C the size of nanoparticles remained similar to those of the freshly prepared samples, whilst after storage at 40°C there were little or no TPP-chitosan nanoparticles remaining after only 6 months. This may be due to the decrease in molar mass of the chitosan possibly due to hydrolysis causing scission of the polymer chains, which resulted in a decrease in nanoparticle size and eventual disintegration. The particle size in itself not enough to determine the stability of nanoparticles, however size is the most important in determining the stability of nanoparticles. Stability could potentially be improved by using freeze-drying although the resulting formulation would require a cryoprotective agent.

Min-Lang *et al.* (2011) studied the storage stability of chitosan/TPP nanoparticles in a phosphate buffer to investigate the effect of initial size and pH of the solution on the changes size of chitosan/ TPP nanoparticles stored in a phosphate buffer at 25°C. The size decreased with increasing pH of the storage phosphate buffer. The initial sizes of the nanoparticles themselves also affected storage stability the larger ones decreased in size. However, the smaller ones increased their size in a phosphate buffer with a pH 7.5 at 25°C for 10 days due to protonation or deprotonation effects on the chitosan molecules. The changes of nanoparticle sizes were classified into instantaneous, aging, and swelling/aggregation stages over the storage time of 97 days.

2.5 Encapsulation

Encapsulation has found numerous applications in food and pharmaceutical industries as vitamin, polyphenol, coating colorants, flavors and other bioactive ingredients in an effort to increase their shelf life, preservation of efficacy and controlled release (Jackson and Lee, 1991; Kyuya *et al.*, 2011; Shahidi and Han, 1993). The encapsulation process is the creation of a barrier to avoid chemical reactions and/or to enable the controlled release of the ingredients. It involves mass transport behavior in

some way between the ingredient and the coating. The entrapped material is usually a liquid but may be a solid or a gas (Vilstrup, 2001).

Food industry has been applying encapsulation for many purposes such as to reduce the reactivity of the core in relation to the light, oxygen and water; to decrease evaporation rate of the ingredient to the outside environment; to promote easier handling of the ingredient to prevent lumping, give a uniform position of the ingredient, convert a liquid to a solid form and promote easy mixing of the ingredient; to control the release of the ingredient to achieve the proper delay for the right stimulus; to mask the core taste and to dilute the ingredient when it is used in only small amount but still achieve uniform distribution.

Figure 2.5 showed a schematic diagram of microcapsules. In general, a hydrophobic core is usually protected by a hydrophilic shell, and hydrophilic material is protected by a hydrophobic shell. The shell can consist of one or more materials. The shell of the capsule is designed to prevent diffusion of the core material into the food until the desired time. Its functions involve protecting sensitive food components such as flavours, vitamins or salts from water, oxygen or light, converting liquids that are difficult to handle into free flowing powders, and isolating specific food components from other food components during storage (Wilson and Shah, 2007).

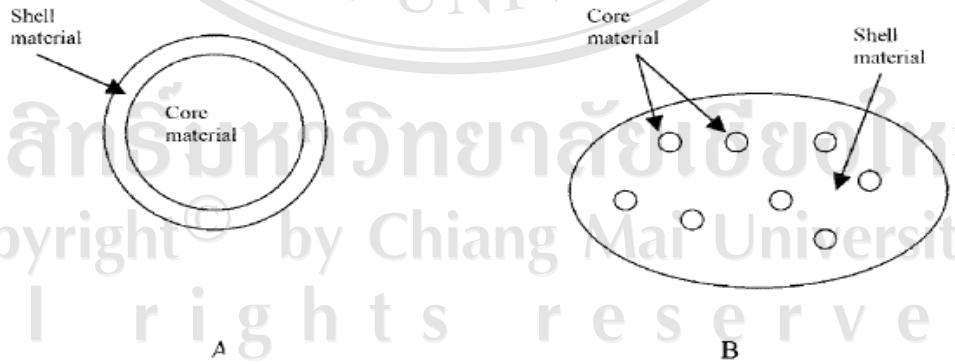

Figure 2.5 Diagrams of two representations of microcapsules:
(A) continuous core surrounded by continuous shell; (B) core material dispersed in a matrix of shell material (Wilson and Shah, 2007)

The natural water soluble polymers have extensively been investigated recently as a matrix to entrap compounds of biological significance. The loaded beads are then used as immobilized biocatalysts or for controlled release of the entrapped compounds (Ouwerx *et al.*, 1998). 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 and Barbosa-Canovas, 1995). This technology is used in foods and beverages to control the release of active ingredients, protect ingredients from the environment, lower flavor loss during the product shelf life, extend the flavor perception and mouth feel over longer period of time, and enhance the ingredient bioavailability and efficacy (Berry, 2004).

The encapsulation of active components in powders has become a very attractive process in the last decades, being adequate for food ingredients as well as for chemicals, drugs or cosmetics. The main objective is to build a barrier between the component in the particle and the environment. This barrier may protect against oxygen, water, light; avoid contact with other ingredients, in e.g. a ready meal; or control diffusion. The efficiency of protection or controlled release mainly depends on the composition and structure of the established wall but also on the operating conditions during the production and use of these particles.

The barrier is generally made of compounds with chains to create a network, with hydrophilic and/or hydrophobic properties. The final powder has a specific composition regarding the active component, but it must also have good handling properties, mixing ability with water or other powders. Flow properties of a powder may also depend on the properties of the wall material, and e.g. on the relative amount of lipids remaining unencapsulated on the surface of particles. Such a powder may require specific stability properties during heating or freezing processes, and for an optimal release of the encapsulated active component (Funchs *et al.*, 2006).

The usual encapsulating agents are proteins, gums, carbohydrates, lipids, fats, waxes, lecithins and fibers. Important parameters are temperature and humidity during processing and during the storage, on the one hand, and the end-use properties on the other. The initial formulation leading finally to powders, will be chosen to predict and

to control physical phenomena like drying, melting, glass transition, crystallisation, caking. Blending two or more agents may provide the desired characteristics. Commercially available microencapsulated flavors and ingredients include carrier materials which have been designed to suit the end foodstuff into which they will be added (Sinton, 1998).

2.5.1 Encapsulation of carotenoids

Encapsulation could assist the application of carotenoids in food easier and increasing the shelf life (Burton, 1989). Microencapsulation of carotenoids is a method that increases bioaccessibility by controlled release during digestion. Bioaccessibility is calculated as the fraction of carotenoids that is released from a food matrix and incorporated into mixed micelles that can be absorbed in the small intestine (Garrett *et al.*, 1999). Several research groups have successfully microencapsulated carotenoids in polymer matrixes and evaluated the shelf stability (Elizalde *et al.*, 2002; Loksuwan, 2007; Nunes and Mercadante, 2007; Rascon *et al.*, 2011). However, this research has yet to assess the release of carotenoids that have been microencapsulated from a food matrix during digestion.

Matalanis and Mc-Clements (2012) assessed the rate of lipid digestion of hydrogel (pectin−sodium caseinate) encapsulated and sodium caseinate stabilized oil in water emulsions using an in vitro pH static digestion model. Encapsulation did not cause a significant difference in the rate of lipid digestion. However, that study did not measure the amount of lipid that had been incorporated into mixed micelles. Carotenoids must be incorporated into mixed micelles to be bioaccessible. In vitro digestion models are becoming increasingly popular for predicting the bioaccessibility of carotenoids, because their results provide qualitative insights into factors that influence carotenoids bioavailability. In vitro digestion models can be classified as static or dynamic models. Static models do not attempt to mimic the mechanical and physiological processes that occur in vivo, such as pH change and peristalsis, whereas dynamic models attempt to mimic these processes (Oomen *et al.*, 2002).

The most widely used model to evaluate bioaccessibility of carotenoids is a static in vitro digestion coupled with a $Caco^{-2}$ cell absorption model developed by Garrett *et al.* (1999). Similar to the Garrett in vitro digestion model, most models used in carotenoids bioaccessibility studies are static models that do not examine the release of carotenoids during gastric digestion and do not mimic the mixing patterns and forces that occur in the stomach due to peristalsis. The Human Gastric Simulator developed by Kong and Singh (2010) is a dynamic gastric digestion model that is designed to mimic the peristaltic movements that occur in the stomach. This model may provide a more accurate simulation of the food disintegration and carotenoids release that occurs in vivo than previously tested static digestion models.

In 1997, Desobry, Netto and Labuza compared three different drying processes including spray dry, freeze dry and drum dry for β-carotene encapsulated in maltodextrin. The losses of β-carotene during drying in encapsulation process were 8, 11 and 14% with freeze drying, spray drying and drum drying, respectively. Some morphological and dimensional differences resulted from the different encapsulation processes. For example, the spray dried capsules were spherical shape. The high ratio of surface to volume for a sphere and the large amount of small spheres would favor oxidation of β-carotene. The drum and freeze-dried powders had complex forms because they were ground after dehydration. Although they had the same wall/carotene ratio, the larger particle provided a larger barrier against diffusion of oxygen into the inner carotene and also had less surface carotene per volume. However, with freeze drying, the large pores extending into the interior would allow for easy diffusion. The drum-dried powder was very compact and formed into large pieces. The freeze-dried maltodextrin particles were more regular, thinner and smoother. The smaller diameter of the spray dried capsules should increase the exchange between oxygen and surface β-carotene as noted. The bulk density differences would favor faster oxidation of freeze dried since it is the smallest, indicating the least collapse. The different encapsulation processes also affected the amount of surface carotene which decreased as the particle size increased. The drum-dried product provided the best encapsulation with only 24% of surface carotene as compared to the spray dried at 38% and freeze dried at 35%.

Although the same grinding process was used for the freeze dried and drum dried products, they did provide for a different particle sizes, possibly due to different cohesion forces set up when the dried matrix formed.

The retention of carotene during storage was compared for the three processes. About 80% of the spray dried β-carotene degraded in 7 wk at 45°C or in 12 wk at 35°C. After 12 wk at 25°C, the carotene retention was 30±2%. Adding the 11% loss during drying, only 27% of the original carotene was retained. Drum drying gave the best β-carotene preservation for all temperatures. The drum dried powder had retention of about 38% after 15 wk, freeze dried powder had retention of about 50% and spray dried powder had retention of about 66%. This better retention was expected, based on particle size and surface carotenoids content. Based on the smallest particle size and highest surface carotenoids content, the spray dried powder showed the fastest degradation kinetics.

Sonia *et al.* (2007) studied β-Carotene encapsulated in a mannitol matrix as affected by divalent cations and phosphate anion. The effects of addition of divalent cations and phosphate buffer on the degree of β-carotene encapsulation in a mannitol matrix during freeze drying were analyzed. This effect was related to phosphate buffer concentration and also confirmed that the maintenance of amorphous mannitol is necessary to allow hydrogen bond interactions between mannitol and proteins or other biomolecules and then contribute to stabilize freeze drying formulations.

Yoshinari *et al*. (2003) investigated the interactions with anionic molecules and discovered that mannitol hydroxyl groups of C6 and C1 form hydrogen bond with the boron central atom of sodium tetraborate and boric acid. Therefore, when solid mannitol is produced from aqueous solution in presence of such anions the formation of hydrogen bonds necessary to form a crystalline mannitol network, can be prevented by the mannitol/borateboric acid or mannitol/divalent cation molecular interactions. Phosphate anions, being strong electron acceptors could interact in the same way with hydroxyl mannitol electron donors at C1 and C6 and form a complex, thus inhibiting mannitol crystallization. Collapse phenomena may affect diffusion of oxygen from the surface to the inside of the matrix and increase retention of β-carotene. Surface color was not an

appropriate indicator for β-carotene degradation, because it was mostly dependent on the optical properties of the matrix, which changed with the degree of matrix hydration and collapse. The results of this research could be useful to obtain formulations for preserving and stabilizing pharmaceuticals, nutraceuticals and food additives of high cost during freeze drying and storage.

Laos, *et al.* 2007 prepared and evaluated furcellaran beads as an encapsulation material for β-carotene from sea buckthorn (*Hippophaerhamnoides* L.) juice. Beads were prepared by ionotropic gelation. The influence of bead formulation factors on the particle size and firmness was investigated and the encapsulation efficiency of β-carotene in beads was studied. The nature of the cation, the polymer and cation concentration, and the proportion of volumes of the outer to the inner phase influenced the size and firmness of furcellaran beads. With increasing proportion of sea buckthorn juice in the formulae, firmness of furcellaran beads decreased. The encapsulation efficiency of β-carotene from sea buckthorn juice in furcellaran capsules was 97%. It suggested that furcellaran beads may be applied for β-carotene encapsulation.

The application of supercritical carbon dioxide as anti-solvent for the encapsulation of β-carotene in poly-hydroxybutirate-co-hydroxyvalerate(PHBV) with dichloromethane as organic solvent is using the Solution Enhanced Dispersion by Supercritical fluids technique. For the precipitation experiments with pure compounds, the parameters were investigated at 4 and 8 $mgml^{-1}$of β-carotene, 30 $mgml^{-1}$PHBV in the organic solution, 80 to 200 bar pressure, 1 $mlmin^{-1}$solution flow rate, 40 $mlmin^{-1}$ anti-solvent flow rate and constant temperature of 313°K. Pure β-carotene precipitation indicated that an increase in pressure led in most cases to particles with larger sizes, while the opposite trend was verified for pure PHBV precipitation. The morphology of precipitated PHBV particles was spherical and was not influenced by increasing pressure. The morphology of β-carotene microparticles changed from plate-like to leaf-like particles when raising operational pressure, but was not influenced by its concentration in the organic solution as verified by micrographs of scanning electronic microscopy. For the co-precipitation experiments it was evaluated the effect of β-carotene concentration at 2-30 $mgml^{-1}$ in the organic solution, at fixed parameters:

PHBV concentration at 30 $mgml^{-1}$ in organic solution, temperature at 313 K, pressure at 80 Bar, solution flow rate at 1 $mlmin^{-1}$ and anti-solvent flow rate at 40 $mlmin^{-1}$. The encapsulation data showed that increasing the concentration of β-carotene, keeping fixed the PHBV content, resulted in increasing the encapsulation efficiency (Wagner *et al.*, 2010).

In 2011, Divya*et al.* prepared the β-Carotene encapsulated chitosan, oleic acid coated Fe_3O_4 nanoparticles in modifying solvent displacement technique. Nanoparticles containing β-carotene were produced by interfacial deposition of the metal oxide/polymer nanocomposite, due to the displacement of acetone from the dispersed phase. Tween 20 is used as the stabilizing agent. The increase in the particle size indicates the encapsulation of β-carotene in Chitosan, oleic acid coated Fe_3O_4 nanoparticles. The entrapment of β-carotene is verified by optical microscope image and also achieved better entrapment efficiency. From the prepared formulation, the in vitro release profile shows optimum release.

1) The stability of encapsulated carotenoids

In 2000, Selim*et al.* studied the degradation kinetic of saffron water soluble carotenoids, mainly crocins, encapsulated in three different amorphous matrices including pullulan, 40,000 Da polyvinylpyrrolidone (PVP40) and 360,000 Da polyvinylpyrrolidone (PVP360) at 0.43, 0.53, 0.64 and 0.75 a_w in the dark at 35°C. They found that PVP 40 was the most effective carrier under all storage conditions. The lower degradation rates were observed for PVP40 under conditions where this matrix was fully plasticized and collapsed. This study implied that the degradation kinetics is not governed by factors related to the physical state and molecular mobility of the inert matrix. Carotenoids losses have been also observed at temperatures below the T_g of the polymeric matrices. Encapsulation of saffron carotenoids greatly improved their stability to oxidation. However, the estimation of the T_g of the polymer used as wall material was not a useful predictor of colorant stability because the molecular mobility of the reactants was not rate limiting even when the encapsulating amorphous matrix

existed in the glassy state. Other factors such as microstructure and porosity of the polymeric matrix were more important as modifiers of reaction kinetics.

In 2003 Robert *et al.* studied stability of the spray dried encapsulated carotenoids from Rosa Mosqueta (*Rosa rubiginosa*) Oleoresin that were encapsulated in starch or gelatin. The stability was studied at 25, 40 and 55°C in the dark. Degradation of *trans*-rubixanthin, *trans*-lycopene, and *trans*-β-carotene followed a pseudo first order kinetic model for both encapsulating agents. The gelatin matrix provided a greater protective effect over the main carotenoids pigments, as shown by the lower degradation rate constants and the longer half life values at 21°C. In contrast, the carotenoids pigments showed the same degradation rate in starch, but *trans*-β-carotene was more stable in gelatin. The kinetic compensation effect obtained according to the calculated thermodynamic parameters suggested that the carotenoids are degraded by the same mechanism.

Izutsu *et al.* (1994) and Pyne *et al.* (2003) reported that mannitol crystalline fraction decreased with an increase on phosphate buffer concentration. The degradation rate of encapsulated β-carotene as a function of % RH and its relationship with the physical state of the matrix during storage at 25°Cwas also studied. The presence of phosphate salts significantly delayed mannitol crystallization at a highly satisfactory degree during freeze drying, the degree of β-carotene encapsulation increased. This effect was maintained over quite long time during storage of the freeze-dried samples at 25°C. Unavoidable local variations in water content during 3 years storage caused the decrease of T_g values and made the crystallization degree to increase. The divalent cations showed a synergistic effect and also modified the kinetics of β-carotene degradation during storage, increasing its stability. The mechanism of crystallization inhibition likely includes a change in hydrogen bond network or/and change in molecular mobility in the presence of divalent cations and phosphate anions.

In 2010, Zhu *et al.* studied stabilized drug of flash nanoprecipitation using β-carotene as a model. A flash nanoprecipitation (FNP) was presented for preparation of suspensions of nanoparticles. The FNP technique is a highly hydrophobic drug is dissolved along with a block copolymer in a water miscible organic solvent. Spherical

β-carotene nanoparticles with an average diameter was less than 100nm and very high loading, 80-90%(w/w), were produced using either branched polyethyleneimine or chitosan. Higher molecular weight polyelectrolytes had a better stabilizing effect than the lower ones, yielding the nanoparticles as small as 60 nm. The polyelectrolytes in an acidic or neutral pH were more effective than those in a basic solution. Zeta potential measurements demonstrated that the polyelectrolytes provided both electrostatic and steric stabilization. The positively charged of amino functional in polyelectrolytes adsorbed onto the surface of the β-carotene nanoparticles due to their hydrophobic surface and slight negative charge. Smaller size particles were produced when the polyelectrolyte in water solutions were mixed simultaneously with β-carotene in tetrahydrofuran rather than mixed sequentially indicates that especially chitosan adsorbs extremely rapidly, at a rate comparable to the precipitation of β-carotene. The β-carotene nanoparticles precipitated very fast that they were in the amorphous state, independent on the nature of the stabilization. Amorphous drug nanoparticles had a higher bioavailability in vivo.

In 2011, Martha and her team were stored the spray dried encapsulated paprika oleoresin by using gum arabic and soy protein as wall materials. Microencapsulation proved to be a convenient strategy to prevent carotenoids degradation avoiding oxygen-mediated auto-oxidation reactions, but its good results depend on wall material selected. The degradation rate constant of carotenoids pigments in paprika oleoresin encapsulated in a wall material was a valid parameter for revealing the quantitative changes involved. Gum arabic showed to be stable at low water activities and soy protein as shown a very good protection against oxidation to paprika oleoresin microcapsules at high water activities. The use of certain wall materials as soy protein isolated may represent an improvement in the nutritional value of this study.

2.5.2 Encapsulation using chitosan crosslink tripolyphosphate

Alsarra*et al.* (2004) determined the effects of preparative parameters on the properties of chitosan hydrogel beads containing *Candida rugosa* lipase. A solution of *Candida rugosa* lipase was prepared in a 1.5% (w/v) chitosan and 1.0% (v/v) acetic acid

medium, and dropped into a TPP solution. The pH and the TPP concentration each had an effect on the entrapment, retention, and activity of lipase. The effect of TPP concentration at a fixed pH of the gelling medium at 7.0 shown more lipase was entrapped with an increase in the TPP concentration until that concentration was above 0.5% (w/v). An increase in the concentration of crosslinking agent would further support the formation of the hydrogel matrix. However, an increase in the TPP concentration requires more acid to bring the pH to 7.0 and this result in a higher concentration of sodium and chloride ions in the gelling medium from the pentabasic TPP and the hydrochloric acid, respectively. The ionic strengths for 0.4%, 0.5%, 0.6%, 0.7%, and 0.8% (w/v) TPP media were 0.252, 0.381, 0.461, 0.533, and 0.615, respectively. The higher ionic strength was interfered with the ionic interaction between chitosan and TPP, whereas this ionic interaction was an essential requirement for crosslinking success. This study confirmed that the concentration of the counter ion agent played a vital role in maximizing the entrapment of the enzyme.

The chitosan-TPP nanoparticles have been previously developed to encapsulated proteins and nucleic acids (Alves and Mano, 2008). Then, the metronidazole with a coat consisting of alginate was encapsulated with chitosan-TPP by Akanksha and Navneet in 2010. The drug entrapment efficiency was found to be in the range 75.2±1.31% and 82.1±0.75%.

Chitosan microcapsules displayed a limited amount of swelling which is related to the degree of cross-linking with the TPP. The microcapsules showed better mucoadhesive property at the intestinal pH of 7.4 than at the gastric pH of 1.2 in the in-vitro wash-off test. The drug release was found to be slow and extended over long duration of time.

In 2012, Sofia, Dimitris and Dimitrios synthesized chitosan grafted copolymers with polyethylene glycol by crosslinked ionic with polyglutamic acid and TPP to encapsulated bovine serum albumin. The result showed TPP had given higher encapsulation efficiency, smaller particles size and more stable than thepolyglutamic acid.

Konecsni, Low and Nickerson (2012) developed the chitosan-TPP submicron particles as carriers for rutin, and determined the release property characterized using the simulated gastric juices and fluids of the small intestine. Particle size, charge and entrapment efficiencies were investigated as a function of the chitosan to TPP molar ratio (2.0:1.0-5.0:1.0). Size was found to decrease from 814 nm for the 2.0:1:0 mass ratio to 528 nm for the ratios between 2.5:1.0 and 4.0:1.0, and then again to 322 nm for the 5:0:1.0 mass ratio, whereas all particles carried a positive surface charge, increasing from +21 to +59 mV as the ratio increased from 2.0:1.0 to 5.0:1.0. The percent entrapment was found to rise from 3.68% to 57.6% as the ratios increased from 2.0:1:0 to 4.0:1:0, before reaching a plateau. Submicron particles were found to retain rutin in simulated gastric fluids, whereas in conditions which simulated fluids from the small intestine, only 20% of the entrapped rutin was released and 80% remained absorbed to the chitosan-TPP carriers. The chitosan-TPP submicron particle was suggested for the delivery of phenolics in food and natural health products.

Tripathy *et al.* (2012) developed chitosan–TPP nanoparticles (NPs) conjugated chloroquine in application for attenuation of the Plasmodium berghei infection in Swiss mice. NPs were prepared by ionotropic gelation between CS and sodium TPP. Cationic CS could react with multivalent counter ions to form the intermolecular and/or intramolecular network structure by ionic interaction between NH_3^+ protonated groups and negatively charged counter ions of TPP. Due to hydrolysis, the small molecule polyelectrolyte, sodium TPP, dissociate in water and released out OH^- ions, so both OH^- and $P_3O_{10}^{5-}$ ions coexisted in the TPP solution and could ionically react with NH_3^+ of CS (Figure 2.6).

FTIR spectroscopy was used to compare the spectra of the CS-TPP, CS-TPP loaded chloroquine and chloroquine. The chloroquine loaded chitosan NPs reveals one most important peak at 1565 cm^{-1} that assigned to N=O stretching of amine presence in drug. The peak at 2927 cm^{-1} was attributing C-H stretching of methylene group. The skeleton bands of heterocyclic aromatic ring appears in the region 1565 cm^{-1} and 1406 cm^{-1} stretching vibrations of C=C, C=N which indicated the presence of chloroquine on chitosan NPs. It was found that chitosan–TPP nanoparticle size was in the range of

150-225 nm and the drug loaded nanoparticles size was in the range of 150-300 nm. The highest fraction of chloroquine NP present in the solution was of less than 250 nm. Chitosan-TPP nanoparticles are mainly characterized by a positive zeta potential. Interaction is therefore strong towards any negative surface charge. The positive charge of Nch (+32.9 mV) was slightly higher than CS–TPP NPs (+30 mV). This is due to presence of positively charge amine group present in drug molecule. Nanoconjugated chloroquine was successfully prepared in polymer: drug ratio of 1:1. Actual drug content was approximately 28% and the encapsulation efficiency was over 54%.

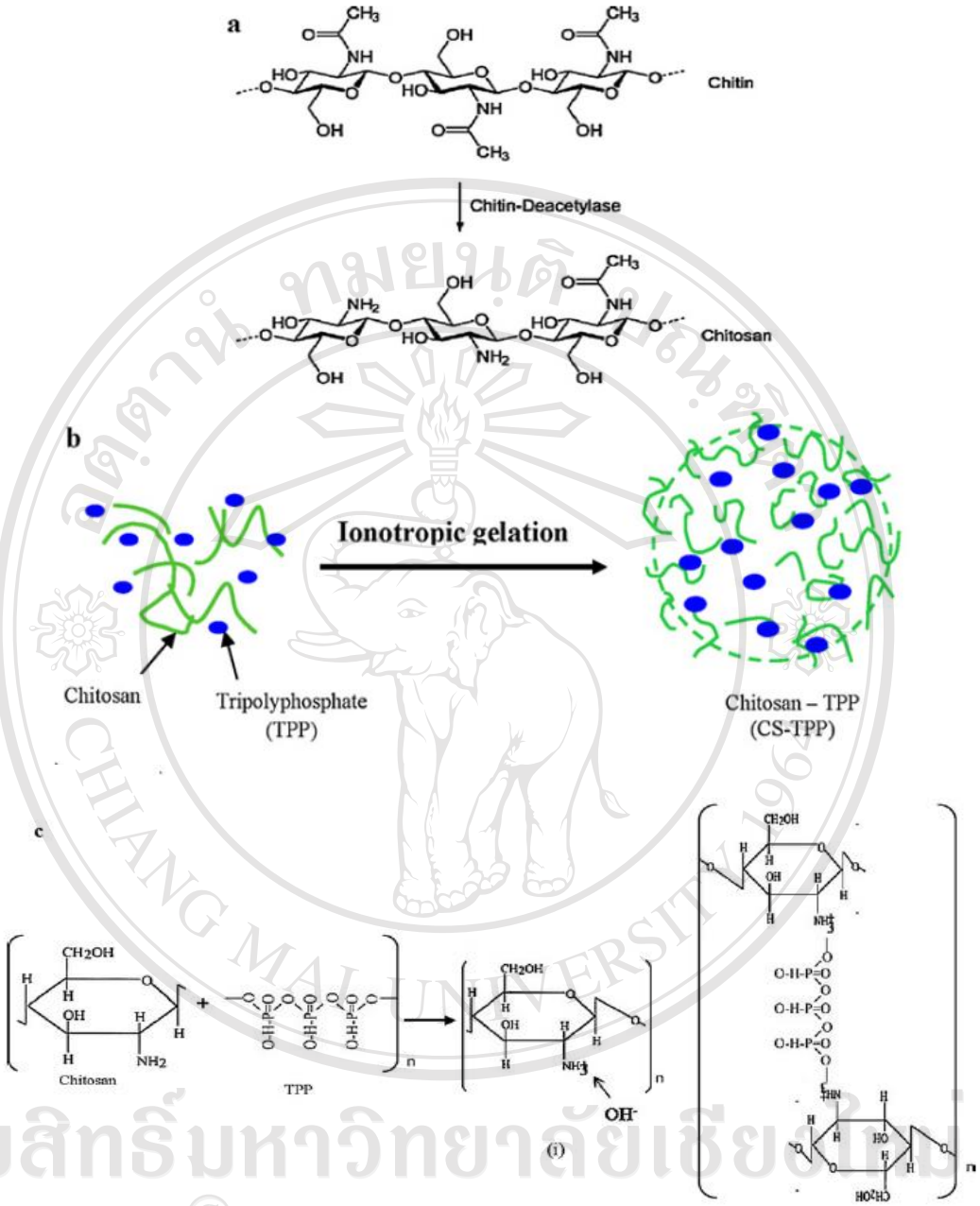

Figure 2.6 Schematic representation of a) chemical structure of chitosan, the polymer is obtained by the partial deacetylation of naturally occurring polymer, chitin; b) the preparation of CS–TPP nanoparticle; c) interaction mechanism between chitosan and tripolyphosphate (Tripathy *et al.*, 2012)

2.6 $ABTS^{\bullet+}$ Assay

A number of assays have been introduced for the measurement of the total antioxidant activity of body fluids, (Cao *et al.*, 1995; Lonnrot *et al.*, 1996) food extracts (Salah *et al.*, 1995; Whitehead *et al.*, 1995) and pure compounds (Kono *et al.*, 1995; Rice-Evans *et al.*,1996; Salah *et al.*, 1995). Each method relates to the generation of a different radical, acting through a variety of mechanisms and the measurement of a range of end points at a fixed time point or over a range (Miller and Rice-Evans, 1994).

Two types of approach have been taken, namely, the inhibition assays in that the extent of the scavenging by hydrogen-or electron-donation of a pre-formed free radical is the marker of antioxidant activity, as well as assays involving the presence of antioxidant system during the generation of the radical. The antioxidant activity determination of carotenoids through the method of DPPH was not performed due to the interference that occurs at the wavelength used to measure the depletion of the free radical DPPH (Jimenez-Escrig *et al.*, 2000).

Generation of the ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of solutions of pure substances aqueous mixtures and beverages (Rice-Evans and Miller, 1995). The original $ABTS^{\bullet+}$ assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation, in the presence or absence of antioxidants. This has been criticized on the basis that the faster reacting antioxidants might also contribute to the reduction of the ferrylmyoglobin radical. A more appropriate format for the assay is a decolorization technique in that the radical is generated directly in a stable form prior to reaction with putative antioxidants (Jimenez-Escrig *et al.*, 2000).

The modified technique for the generation of $ABTS^{\bullet+}$ involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate. This has absorption maxima at wavelengths 645 nm, 734 nm and 815 nm, as well as the more commonly used maximum at 415 nm. Addition of

antioxidants to the pre-formed radical cation reduces it ABTS, to an extent and on a time-scale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction. The extent of decolorization as percentage inhibition of the $ABTS^{\bullet+}$ radical cation is determined as a function of concentration and time and calculated relative to the reactivity of Trolox as a standard, under the same conditions (Miller *et al.,* 1993; Miller and Rice-Evans, 1996).

The method is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts. The major improvement in the assay for lipophilic compounds such as carotenoids is the design improvement incorporating the radical cation and the antioxidant both in the lipophilic phase. The reaction between the carotenoids and $ABTS^{\bullet+}$ is essentially complete after 1 min, little further reaction taking place thereafter. The antioxidant activity of lycopene was of the same order as obtained using previous methodology that produced the radical cation using manganese dioxide as oxidant (Miller *et al.,* 1996). The value for β-carotene was significantly higher. This method improves the assay because the application of manganese dioxide as oxidizing agent can involve molecular chemistry with the potential to produce a two electron oxidation of ABTS to the radical dication that limits its definition and applicability. Then, it gives a measure of the antioxidant activity of the range of carotenoids, phenolics, and some plasma antioxidants, determined by the decolorization of the $ABTS^{\bullet+}$, through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm.