MICROSTRUCTURAL EXAMINATION OF ENCAPSULATED TUNA OILS

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Abstract: Three steps of encapsulation consisting of; (1) dissolution of chitosan 1.0 g/100g with maltodextrin 10.0 g/100g or whey protein isolate 1.0 g/100g, mixing with tuna oils 20 g/100g and emulsification; (2) atomization into a reactive solution using ultrasonic atomizer; and (3) freeze drying were conducted. Oxidative stability of encapsulated powder with or without α-tocopherol compared to bulk tuna oils was improved. The confocal scanning microscopy enabled us to distinguish between encapsulated oil and the continuous phase. Scanning electron microscope demonstrated the encapsulated particle with no pores or surface oil droplets. Fourier transform infrared spectroscopy applied to microencapsulated tuna oils indicated that tuna oils in the microspheres are physically encapsulated in the wall matrix. The results suggest that chitosan mixed with maltodextrin or whey protein isolate have the potential to be used as the wall materials for encapsulating tuna oils or other oils using ultrasonic atomizer.

Introduction: Fish oils are susceptible to oxidation, causing flavor and nutritional quality changes or possibly further producing toxic compounds. Encapsulation is a potential technique developed to transform reactive, sensitive or volatile compounds into more stable ingredients by means of packing materials, sealing in capsules and releasing them when appropriately triggered[1]. Several additives have been used to prevent oils from oxidation including chitosan (CS), maltodextrin (MD) and whey protein isolate (WPI).[2-7] Recently, ultrasonic atomizer together with freeze drying has been reported to successfully encapsulate tuna oils with chitosan (CS) and maltodextrin (MD) or whey protein isolate (WPI) as the wall materials.[8] However, little is known for the antioxidant activity of the combination of these wall materials for preventing oxidation of tuna oils.

At present, Scanning Electron Microscopy (SEM), Confocal Laser Scanning Microscope (CLSM), and Fourier Transform Infrared Spectroscopy (FT-IR) play important roles in the food microstructure investigation.[9] Several examinations of the outer structure of fish oil microencapsulated powder have been documented.[7,10] Recently, Klaypradit *et al.* have examined fish oil oxidation by FT-IR analysis.[21] As a consequence, the microstructure study of emulsion or encapsulated powder will provide us a better information on their properties from atomic level to micron range and might support the oxidative data on how oxidation can be prevented.

The objectives of this study were as follows; (1) to examine the potential of CS mixed with either MD or WPI for oxidation prevention in encapsulated powder, (2) to explore oil localization in emulsion with CLSM, (3) to examine the outer-surface of the encapsulated particles by means of the SEM technique, and (4) to investigate the relationship between FT-IR and the ability to form the effective encapsulation.

Methodology:

Materials:

Chitosan (CS) with a degree of deacethylation (DD) = 80 and tuna oils were used. Maltodextrin (MD) with average dextrose equivalent (DE) 5 and whey protein isolate (WPI) were provided. Acetic acid, hydrochloric acid, chloroform, methanol, isooctane, and sodium

hydroxide were of analytical grade. Acetate buffer (pH 4.6), barium chloride dehydrate, iron sulfate, ammonium thiocyanate, P-anisidine, and α-tocopherol were purchased. The emulsifier used was Tween 80. Concanavalin A conjugates or Con A (Alexa Fluoro 633) and Nile red were purchased.

Emulsion preparation

The optimal formulation for emulsion preparation was prepared according to Klaypradit and Huang. [22]

Confocal Laser Scanning Microscope (CLSM)

Chitosan 1.0 g/100g was firstly dispersed in 0.25 mL/100 mL aqueous acetic acid and continuously stirred at room temperature until the mixture was complete dissolved as indicated by visual examination. Then 10.0 g/100g MD or 1.0 g/100g WPI was slowly added. When total visual dissolution was obtained, 400 µl Alexa 633 was added and continuously stirred for 1 hour. Nile red 4.0 µl was added to tuna oils previously mixed with Tween 80 and stirred for 1 hour. The oil/ Tween/ Nile red mixture and CS solution were mixed and then emulsified using a homogenizer 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 equipped with 100x oil immersion objective was used for microscopic observation. A 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 were collected at 645-655 and 630-640 nm for wall components and tuna oils, respectively.

Encapsulation preparation

The encapsulated samples were prepared according to Klaypradit and Huang using an ultrasonic atomizer. [22]

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. Samples were collected periodically to determine any oxidative changes. Peroxide values (PV) were determined to assess the primary oxidation changes using the International Dairy Foundation (IDF) method while the Anisidine values (AnV) were determined according to the AOCS Official Method Cd 18-90 [23] to evaluate the secondary changes.

Scanning Electron Microscope (SEM)

After freeze –drying, encapsulated tuna oils powder samples made from only CS 1.0 g/100g and CS 1.0 g/100g mixed with 1.0 g/100g WPI or 10.0 g/100g MD were prepared by vapor fixation for one hour using 4.0 g/100g osmium solution (OsO_4) to stabilize the oil. The samples were adhered to sample holders, and sputter coated with gold 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 at 10 kV.

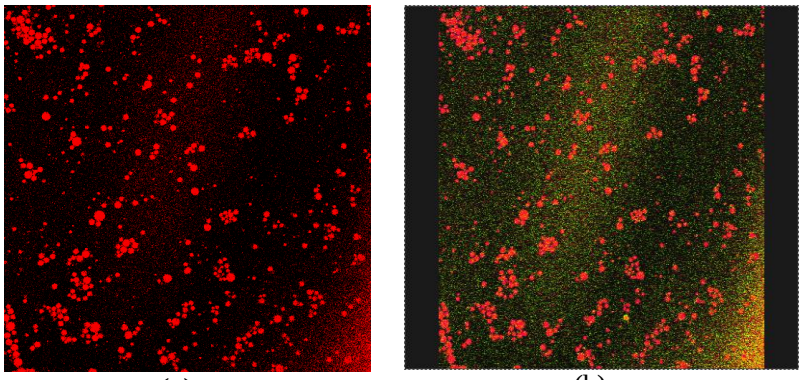
Fourier Transform Infrared Spectroscopy (FT-IR)

A FT-IR coupled with an attenuated total reflectance (ATR) accessory was used to investigate the ability of tuna oils 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 wave number range 4,000-500 cm^{-1}.

Results, Discussion and Conclusion:

Confocal Laser Scanning Microscope (CLSM)

Fig. 1 presents the examination of 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 o/w 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. [24] Alexa 633 is one type of Con A 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. Fig. 1(a) exhibits 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 the 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. When both wavelength of Nile red and Alexa 633 were excited, the red oil particles distributed on the green continuous phase were observed as shown in Fig.1 (b). We noticed that the continuous phase of Fig. 1 (b) has a green color covering all the background area. It has to be emphasized that Alexa 633 can recognize CS mixed with MD (Fig. 1 (b)) since they are both carbohydrates but it can recognize only CS not WPI as the continuous phase for the o/w emulsion made from CS mixed with WPI (images not shown) in that WPI partially is out-of-focus and could be seen as the black background. From the results, we could suggest that the tuna oils phase was encapsulated and distributed within the continuous phase.



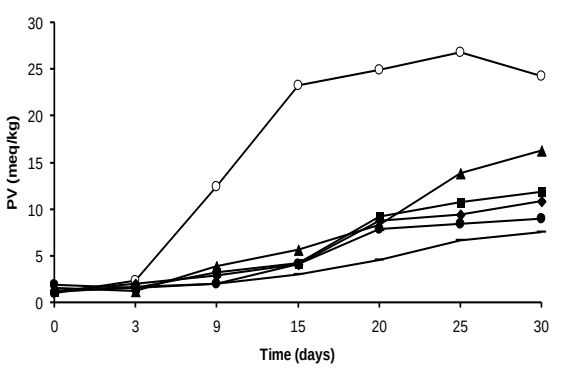
(a) (b)

Fig. 1 Confocal images of tuna oils-in-water emulsion made from CS 1.0 g/100 g mixed with 10.0 g/100g MD; (a) only Nile red wavelength was excited, (b) Nile red and Alexa 633 wavelength were excited.

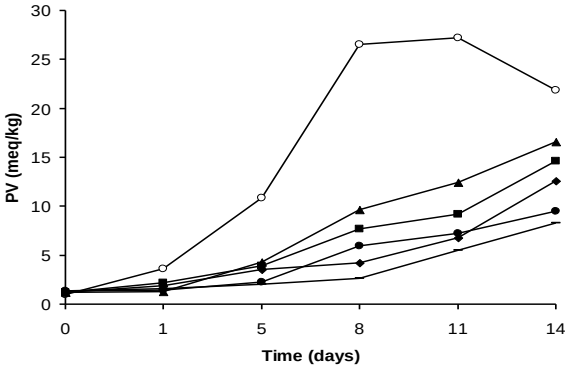
Oxidation of encapsulated powder

The oxidative stability of tuna oils in encapsulated powder is presented in Fig. 2 (a, b) and (c, d) 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 oils against oxidation in comparison with the bulk oil samples. Our results are in agreement with previous works that encapsulation of fish oil by freeze-drying technique can improve oxidative stability. [12, 25] Chitosan has been documented to pose antioxidant properties and potential to be used to improve oxidative stability in high lipids food systems [3-4, 26] Kim and Thomas reported that incorporation of CS with various molecular weights can reduce lipid oxidation in ground Salmon [17]. However, the precise antioxidation mechanism of CS is still not clear. Xie *et al.* have proposed that the NH_2 groups of CS act as antioxidant by

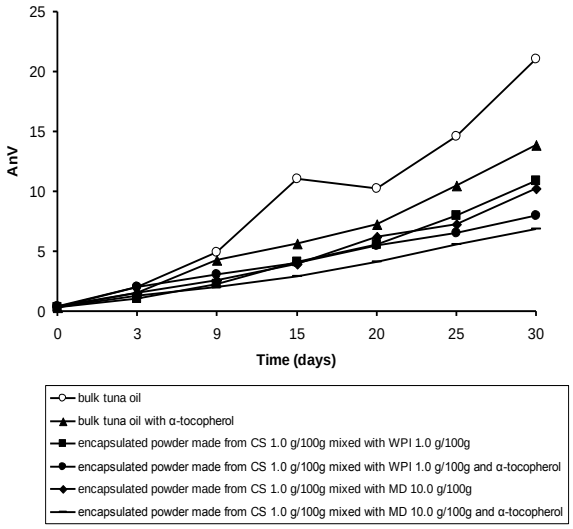
scavenging OH radical to form stable macromolecule radicals. [2] Kim and Rajapaske also suggested that amino and hydroxyl groups attached to C-2, C-3 and C-6 positions of the pyranose ring react with unstable free radicals to form more stable macromolecule radicals. [27] The combination of CS and MD play an important role as texture modifiers to modify viscosity of the aqueous continuous phase surrounding the oil droplets. The increased oxidative stability of the encapsulated powder made from CS mixed with MD observed in this study could therefore be attributed to its thicker interfacial membrane and higher viscosity in continuous phase during the emulsion preparation step that might protect lipids from oxidation by acting as a barrier to the penetration and diffusion of molecular species that promote lipid oxidation into the droplets. The interaction between CS and WPI provided good wall materials for tuna oils encapsulation because of their positive emulsifying properties in o/w emulsion [8]. They also showed a good oxidation protection property for the encapsulated powder which presents in Fig. 2. The antioxidant mechanism of WPI may be attributed to its ability to inactivate free radical through their sulfhydryl groups and other amino acids. [28] The oxidative stability was increased when incorporated with α-tocopherol. The ability of α-tocopherol to decrease 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. The results indicated that the combinations of CS and MD or WPI have the potential to prevent oxidation of the tuna oils encapsulation.



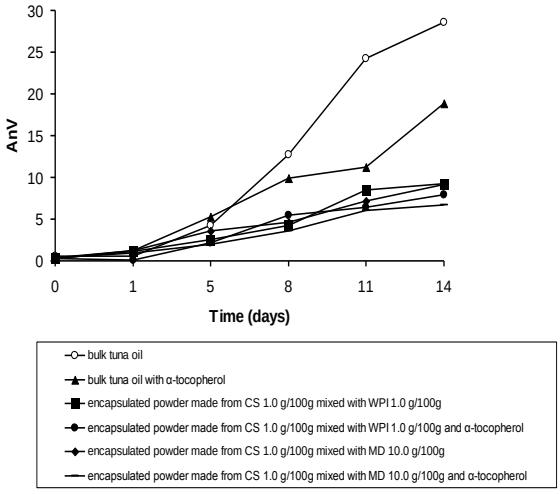
(a)



(c)



(b)



(d)

Fig. 2 Formation of lipid hydroperoxides (PV) (a,c) and Anisidine values (AnV) (b,d) of bulk tuna oil and tuna oils-encapsulated powder in the absence and presence of α-tocopherol during storage at 25 °C (a and b) and at 37 °C (c and d). Data shown are the average of duplicates

Outer Microstructure-Scanning Electron Microscope (SEM)

The structure of the microencapsulated particles after freeze drying as determined by SEM is presented in Fig. 3 (a-c). All powder samples had spherical or oval shapes with some surface wrinkles which was similar to the results of other previous carbohydrate-based encapsulation reports. Sheu and Rosenberg indicated that microcapsules made from WPI/MD (DE=5) had surface dents and large capsules exhibited "caps" within dents. [29] Similarly, Kagami *et al.* have found the formation of dents on the outer surfaces of fish oil microcapsules formed by MD (DE=18) / sodium caseinate as wall materials. [6] In addition, Klinkeson *et al.* showed the outer morphology of tuna oils microcapsules made from CS mixed with corn syrup solid (DE=36) by spray drying with some wrinkles and pores on the surface. [12] Fig. 3(a) shows the high oil content covered on the surface of the encapsulated particle as compared to those made from CS mixed with WPI or MD (Fig. 3(b) and Fig. 3(c), respectively). This finding supports the results of the emulsion stability study described by Klaypradit and Hung that CS alone is unable to produce a stable emulsion and it is presumably that the oil droplets were not completely encapsulated within the CS and some oil droplets were still on the surface of the particles. [8] 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.[11] Heinzelmann *et al.* 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. [10] 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, Fig. 3 (b) and (c) show very few oils on the droplet surface which we hardly found any pores. It is possible to suggest 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 also supports oxidative stability test of encapsulated powder previously discussed that showed the ability of the wall materials to reduce the oxidation rate compared to bulk oil.

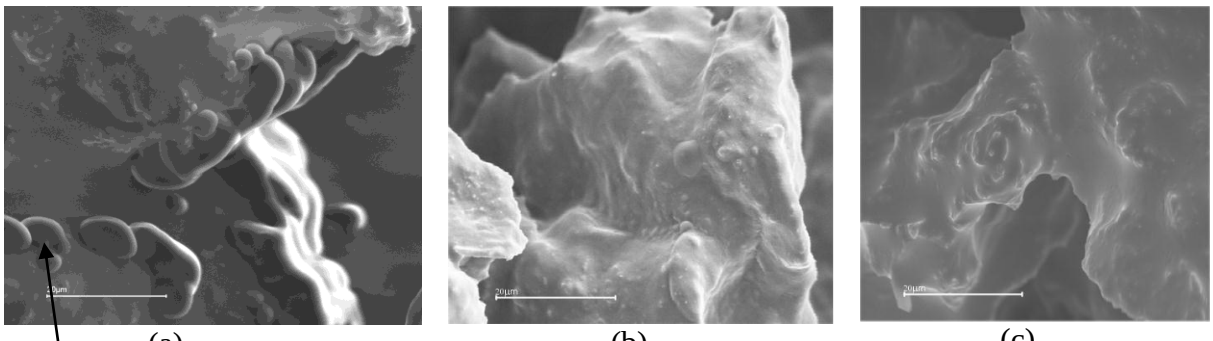
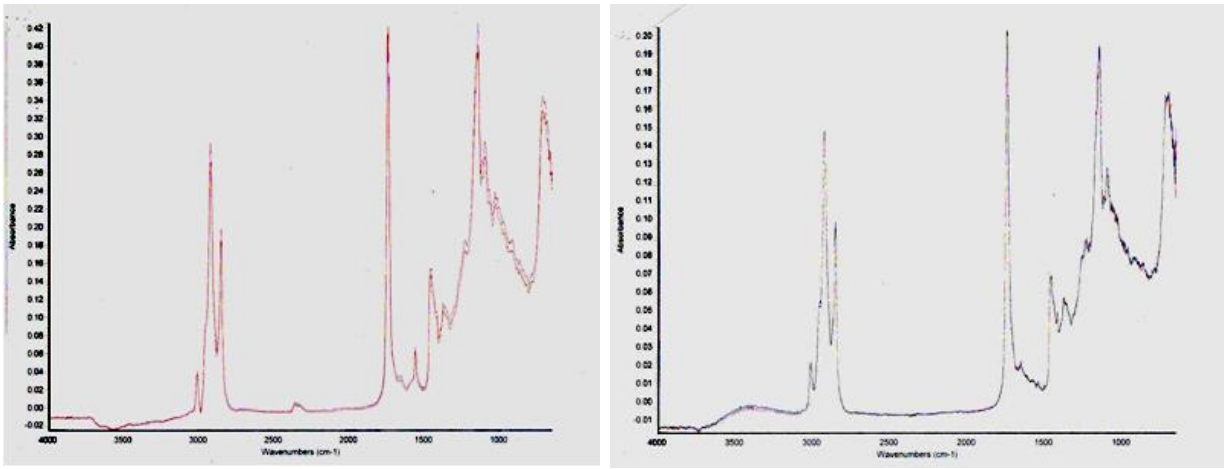


Fig. 3 Scanning electron micrographs of tuna oils encapsulated powder made from CS 1.0 g/100g (a), CS 1.0g/100g mixed with WPI 1.0g/100g (b) and CS 1.0 g/100g mixed with MD 10.0g/100g (c) at 5000X

Fourier Transform Infrared Spectroscopy (FT-IR)

In this study, the FT-IR technique was applied to investigate if tuna oils in the microspheres are physically encapsulated in the wall matrix made from CS and MD. The FT-IR spectrum of microparticle obtained from the combination of CS 1.0 g/100g and MD 10.0 g/100g without tuna oils (standard) and of tuna oils encapsulated powder made from the combination of CS 1.0 g/100g and MD 10.0 g/100g is presented in Fig. 4 (a) and (b), respectively. FT-IR is a fast and dynamic technique for collecting infrared spectra of an enormous variety of compounds for a wide range of industries. It 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. 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 our study, absorption peaks derived from the tuna oils/CS-MD microspheres (Fig. 4(b)) were almost in a similar fashion to those of the CS-MD molecule (Fig. 4(a)). Similar findings were reported by Zaleska and his colleagues. [18] According to these authors the spectra of complexes presented almost precise superposition of the IR spectra of WPI and WPI-starch. Therefore, our results could demonstrate that most oil contents in the microspheres are physically encapsulated in the CS-MD matrix. Some related work have been documented the application of FT-IR. [5-6, 15-16]

In conclusion, this study has shown that CS mixed with WPI or MD was successfully used as the wall materials for tuna oils microencapsulation. Oxidative stability of the encapsulated powder was improved, especially when adding more α-tocopherol compared to bulk tuna oils. CLSM, SEM, and FT-IR are useful tools to explain the characteristics of tuna oils encapsulated powder. Clearly, the fluorescence of lipid-Nile red mixtures is very sensitive to the oil phase and can be localized by CLSM. 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 application of FT-IR to discover if the encapsulated phase is physically encapsulated in the wall matrix has been reported.



(a) (b)

Fig. 4 FT-IR spectrum of microparticles obtained from combination of 1.0 g/100g CS and 10.0 g/100g MD without tuna oils (a) and obtained from combination of 1.0 g/100g CS and 10.0 g/100g MD (b)

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