

# Characterization of Xanthone in OSA-Black Glutinous Rice Flour Microcapsules by FTIR and XRD Methods

Paponpat Pattarathitawat<sup>1</sup> and Pilairuk Intipunya<sup>1,2\*</sup>

<sup>1</sup>*Division of Food Science and Technology, Faculty of Agro-Industry, Chiang Mai University 50100, Thailand*

<sup>2</sup>*Lanna Rice Research Center, Chiang Mai University, Chiang Mai 50200, Thailand*

*\*Corresponding author. E-mail: pilairuk.intipunya@cmu.ac.th*

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## ABSTRACT

*Octenyl succinic anhydride is often used to modify starch for encapsulation purposes. Xanthone and its derivatives exhibit a wide range of biological and pharmacological activities, including antimicrobial, antioxidant, and antitumor. This study investigated the effect of starch modification by octenyl succinic anhydride on xanthone encapsulation efficiency. The starch sample was added with 0.4% xanthone as core material. We analyzed the encapsulation efficiency and important characteristics of microcapsules, such as xanthone content, crystallinity of amylose, and chemical structure. Black glutinous rice flour modified by octenyl succinic anhydride showed higher encapsulation efficiency than the native black glutinous rice flour. Starch-xanthone complexation resulted in an A-type starch as identified by XRD. The FTIR pattern showed shifts in the absorbance peaks at 3,000-3,700 cm<sup>-1</sup>, which indicated that xanthone was adequately complexed in the flour structure.*

**Keywords:** OSA-modified rice flour, Black glutinous rice, Xanthone encapsulation, FTIR, XRD

## INTRODUCTION

Rice starch has increasingly been used to encapsulate edible and important substances, such as flavors, lipids, and carotenoids (Fang and Bhesh, 2010). It can act as a wall or shell material around substances to protect active ones, prolong or control their release, mask undesirable tastes, and/or improve handling. Even though native rice flours or starches are abundant and low cost, they are not efficient for some encapsulation purposes. Instead, they are usually modified by chemicals, physical treatments, enzymatic treatment, or hydrophobic modification methods to improve their encapsulating-related properties, such as viscosity, process tolerance, stability, digestion profile, and crystalline structure properties (Li, 2014). This research selected chemical modification using octenyl succinic anhydride (OSA), because this hydrophobic treatment in combination with hydrolysis creates a very active starch surface (Li, 2014). The esterified starch attracts hydrophobic molecules, such as food flavoring compounds, antimicrobial agents, and other fat-soluble compounds. As such, OSA-modified starch offers a high loading capacity of core material (Sriroth and Kuakoon, 2009).

Encapsulation of hydrophobic compounds, such as xanthone, in OSA-modified starch occurs through a process of starch-core inclusion complexation. Amylose chains form a helix around a hydrophobic core or ligand, resulting in crystalline-like structures (Song et al., 2006). The evidence of complexation into crystalline structure and chemical interactions between the starch and core compound can be analyzed by X-ray diffractometer (XRD) and Fourier transform infrared (FTIR) spectroscopy.

This study used native and OSA-modified black glutinous rice flours as wall material to encapsulate xanthone. Xanthone was selected because it has been shown to have a wide range of biological and pharmacological activities, including antimicrobial, antioxidant, and antitumor (Negi et al., 2008). Xanthone has a strong inhibition effect against *Collectotrichum musae* (Ungkanethiwat et al., 2010), *Staphylococcus aureus* (Srisuwan et al., 2009), and gram-positive bacteria (Negi et al., 2008). FTIR and XRD were employed to understand the chemical and structural changes that occurred, which could help design more effective micro encapsulation techniques and microcapsules.

## MATERIALS AND METHODS

### Materials

Black glutinous rice flour (*Oryza sativa L. indica*) was purchased from a market in Doisaket District, Chiang Mai Province, Thailand. Xanthone (Sigma-Aldrich, USA), octenyl succinic anhydride (Sigma-Aldrich, USA), and other chemicals were reagent grade and used without further purification.

### Flour modification by octenyl succinic anhydride

Black glutinous rice flour (30 g) was suspended in distilled water at 35% w/w with agitation. The pH of the suspension was adjusted to 7.0 by adding 3% NaOH solution. A weighed quantity of OSA (15% v/v in absolute alcohol) was slowly added within 2 hours. After reaction, pH was adjusted to 6.5 with 3% HCl solution. The mixture was centrifuged,

and washed two times with distilled water (250 ml) and two times with 70% aqueous alcohol (100 ml). The solid sample was dried at 40°C for 24 hours, followed by grinding and passing through a 180-mesh sieve (90 µm opening) (Song et al., 2006).

### **Encapsulation by freeze drying**

The OSA-modified and native black glutinous rice flours were used as wall materials for encapsulation by inclusion complexation method. The flour sample (100 g) was dissolved in distilled water at a 1:1 ratio. Xanthone at 0.4% w/w of the dry flour sample was dissolved in 10 ml of absolute ethanol (95%) and added to the flour solution. The mixture was mixed well, followed by heating at 80°C until gel formation was completed. The sample was cooled, frozen, and freeze dried using a drying chamber (at -20°C) and a cooling unit (at -50°C) under vacuum pressure of  $10^{-2}$  mbar (Marefati et al., 2015). Drying was carried out for eight hours. The freeze dried samples were ground and kept in a sealed laminated aluminum bag and stored at -18°C until further use.

### **Xanthone content analysis by HPLC method**

A high-performance liquid chromatograph (Shimadzu, Japan) equipped with an UV-detector set at 319 nm wavelength was used for the analysis of xanthone content (Thongthammachat and Jarudeelokoon, 2011). The chromatographic separation was performed at room temperature on an HPLC column ZORBAX, Rx-C18, 4.6 x 250 mm, analytical column (Agilent Technologies, USA). The sample injection volume of 20 µl was analyzed using a mobile phase flow rate of 1 ml/min.

The standard stock solution of xanthone (internal standard) was prepared by dissolving accurately weighed xanthone, with methanol added to make a final volume of 1.0 mg/ml. The xanthone stock solution was sequentially diluted with methanol (95% purity) to prepare a series of working standard solutions in the concentration range of 0.1-0.8%. All solutions were stored at 4°C and brought to room temperature before use. From the recorded peak areas in chromatograms of each standard solution, the ratios of xanthone to internal standard were calculated and plotted against xanthone concentrations. The calibration curve was constructed from triplicate analysis of the standard solutions. The linearity was evaluated by least square regression.

### **Determination of xanthone in microcapsules by HPLC method**

**Surface xanthone content analysis.** The microcapsule samples were accurately weighed into 10.0 ml volumetric flask and adjusted to the mark with methanol (95% purity), then rigorously mixed by vortex 10 times. The solution was filtered through a 0.45 µm nylon syringe filter. The chromatographic analysis was performed at room temperature by injecting 20 µl and mobile phase flow rate of 1 ml/min. The eluent was monitored at 319 nm. The mobile phase consisted of methanol:water (95:5% v/v). The surface xanthone content was calculated from peak area as compared to the standard calibration curve. All samples were analyzed in duplicate.

**Encapsulated xanthone content analysis.** The microcapsule sample obtained after surface extraction was put in a 10.0 ml volumetric flask and methanol (95% purity) was added to the marked volume. The flask was sealed with paraffin film and stored for one night at room temperature. The solution was filtered through a 0.45  $\mu\text{m}$  nylon syringe filter. Chromatographic analysis was performed as carried out for surface xanthone content analysis. All samples were analyzed in duplicate.

**Encapsulation efficiency (EF).** The encapsulation efficiency was calculated using xanthone content following the equation of Thongthammachat and Jarudeelokoon (2011) as follows:

$$EF (\%) = \frac{\text{Encapsulated xanthone content}}{\text{Total xanthone content}} * 100$$

### Determination of crystallinity of microcapsules

Microcapsule samples were subjected to crystallinity analysis using X-ray diffractometer (XRD). The samples were scanned with Cu-K $\alpha$  radiation at 35 kV and 20 mA. The intensity was recorded at the rate 3 $^\circ$ /min between 5 $^\circ$  to 60 $^\circ$  (2 $\theta$ ) (Haque and Roos, 2005).

### Analysis of chemical structure

The change in chemical structure of the starch was quantitatively analyzed using a Fourier transform infrared (FTIR) spectroscope. Infrared light absorbance of each sample was measured over the wavelengths between 400 to 4,000  $\text{cm}^{-1}$ .

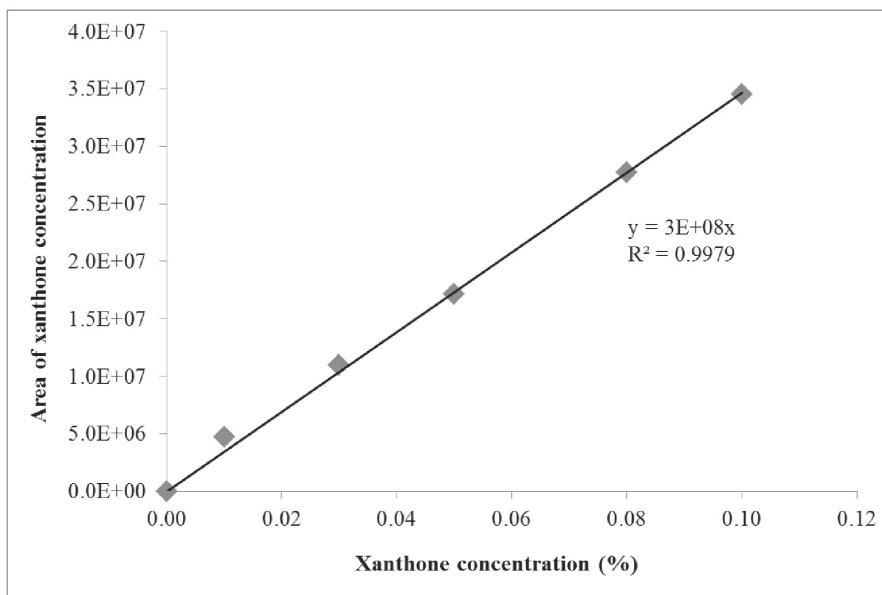
### Analysis of physical structure

The morphology of flour was examined using a scanning electron microscope (SEM, JEOL JSM-5910LV, Japan) operated at 15 kV accelerating voltage. Then, samples were fixed on the SEM stub, which were subsequently coated with gold to provide a reflective surface for the electron beam. The gold-coated samples were then viewed under the microscope.

## RESULTS

### Xanthone content in microcapsules

The calibration curve for the xanthone content was linear over the concentration range from 0.01% to 0.10%. The linear regression equation of the calibration curve was  $Y = 3E+08X$ , with  $R^2$  of 0.9979. The linear calibration curve for xanthone is shown in Figure 1.



**Figure 1.** Calibration curve of xanthone standard with concentration ranging from 0.01% to 0.10%.

The microcapsule with OSA-modified black rice flour as wall material showed higher xanthone content than that with native black rice flour (Table 1). With the same amount of xanthone added to the flours (0.4%), the encapsulation performance of the native and OSA-modified black glutinous rice flours differed. The native rice flour sample contained significantly higher surface xanthone content ( $P \leq 0.05$ ), but less encapsulated xanthone content ( $P \leq 0.05$ ) than the OSA-modified rice flour; as a result, the native rice flour had significantly lower encapsulation efficiency ( $P \leq 0.05$ ). The encapsulation efficiency of xanthone in OSA-modified black glutinous rice flour and native rice flour was 25.00 and 23.75%, respectively. The encapsulation efficiency was calculated from the encapsulated xanthone content. The surface xanthone content was considered non-encapsulated; it is susceptible to undesirable deterioration due to direct exposure to the surrounding environment.

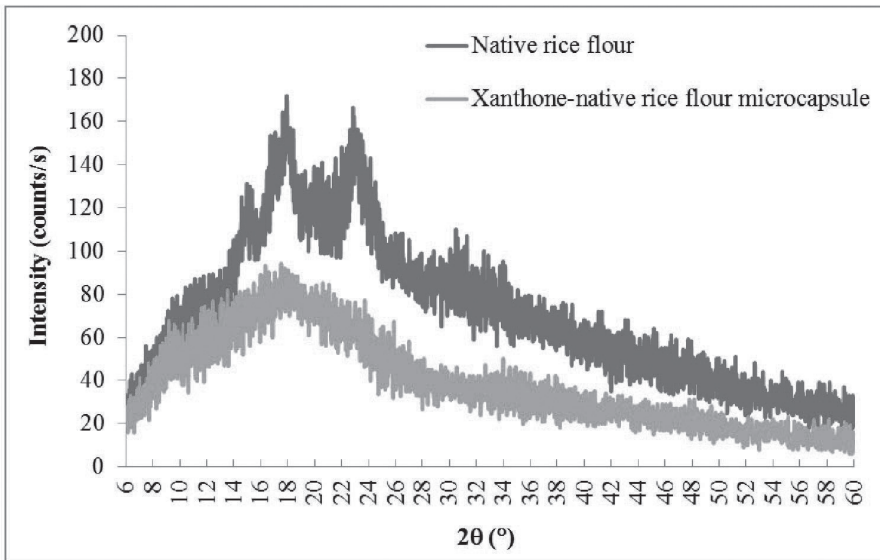
**Table 1.** Xanthone content and encapsulation efficiency.

Treatment	Total xanthone added (%)	Surface xanthone content (%)	Encapsulated xanthone content (%)	Encapsulation efficiency (%)
Black glutinous rice flour	0.4	0.125 <sup>a</sup> ± 0.000	0.095 <sup>b</sup> ± 0.000	23.75 <sup>b</sup> ± 0.00
OSA-modified black glutinous rice flour	0.4	0.022 <sup>b</sup> ± 0.000	0.100 <sup>a</sup> ± 0.000	25.00 <sup>a</sup> ± 0.00

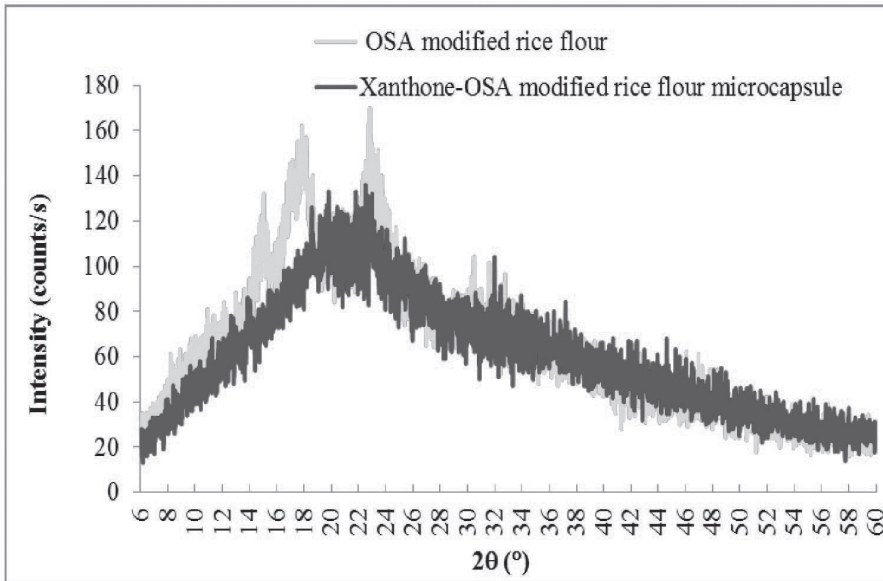
Note: Data presented in the table are mean ± SD from duplicate measurements. Different superscripts in the same column indicate statistical difference among mean values at the 95% confidence level ( $P \leq 0.05$ ).

### Crystallinity of amylose in microcapsules by XRD

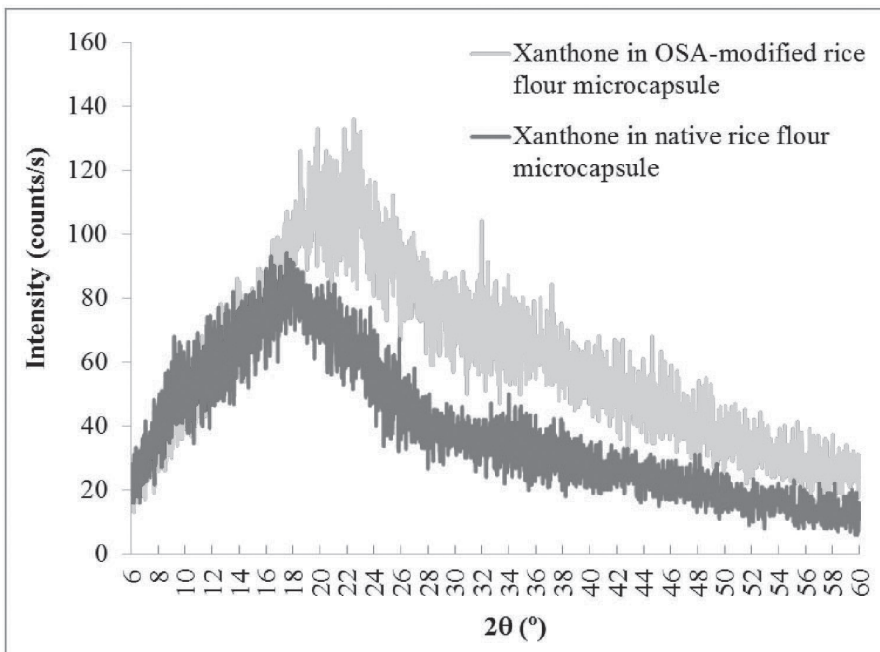
The XRD patterns were used to examine the crystalline structure of the microcapsules of xanthone in rice flours. The results were related to the encapsulation efficiency. Native black glutinous rice flour and OSA-modified black glutinous rice flour showed peak intensity at  $2\theta$  around  $15^\circ$ ,  $17^\circ$ , and  $23^\circ$  (Figures 2 and 3). Esterification using OSA did not change the crystalline pattern of the black glutinous rice flour. In both Figures 2 and 3, the intensity decreased at  $2\theta$  around  $15^\circ$ ,  $17^\circ$ ,  $18^\circ$ ,  $20^\circ$ , and  $23^\circ$  for the microcapsule samples as compared to XRD patterns of the wall materials. The peak intensity of the xanthone microcapsules in native flour was lower than that of the microcapsules in OSA-modified flour (Figure 4). This could mean that microcapsule in the native flour existed mostly in an amorphous state, with less helical complexation. A better xanthone inclusion complexation with the amylose in OSA-modified rice flour occurred due to substitution of the hydroxyl groups in the starch by the ester group in the OSA. The crystallinity is shown in Table 2.



**Figure 2.** XRD patterns of native black glutinous rice flour and xanthone microcapsules in native black glutinous rice flour.



**Figure 3.** XRD patterns of OSA-modified black glutinous rice flour and xanthone microcapsules in OSA-modified black glutinous rice flour.



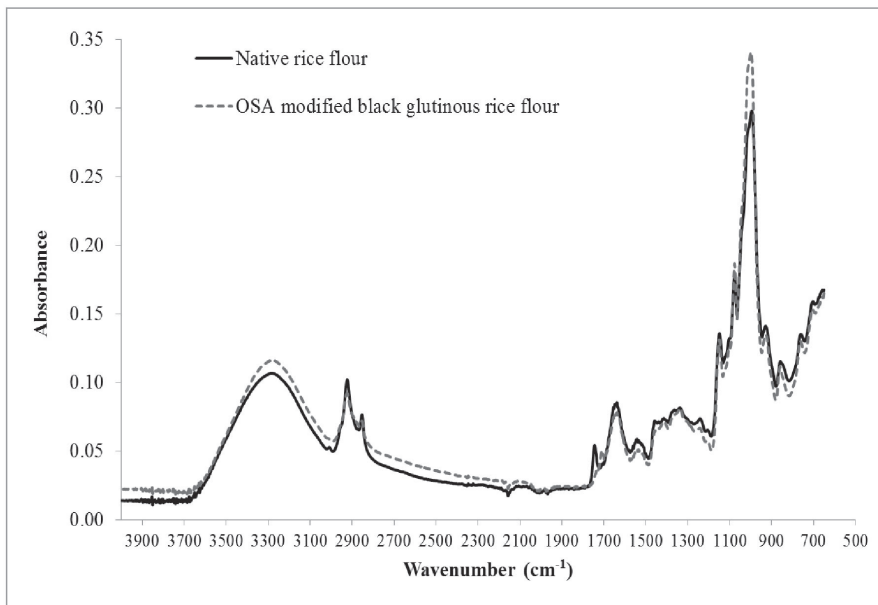
**Figure 4.** Comparison of XRD patterns of microcapsules of xanthone in OSA-modified black glutinous rice flour and in native rice flour.

**Table 2.** Crystallinity.

Treatment	Crystallinity (%)
Native black glutinous rice flour	15.94
OSA black glutinous rice flour	18.62
Native black glutinous rice flour microcapsule	9.89
OSA black glutinous rice flour microcapsule	11.47

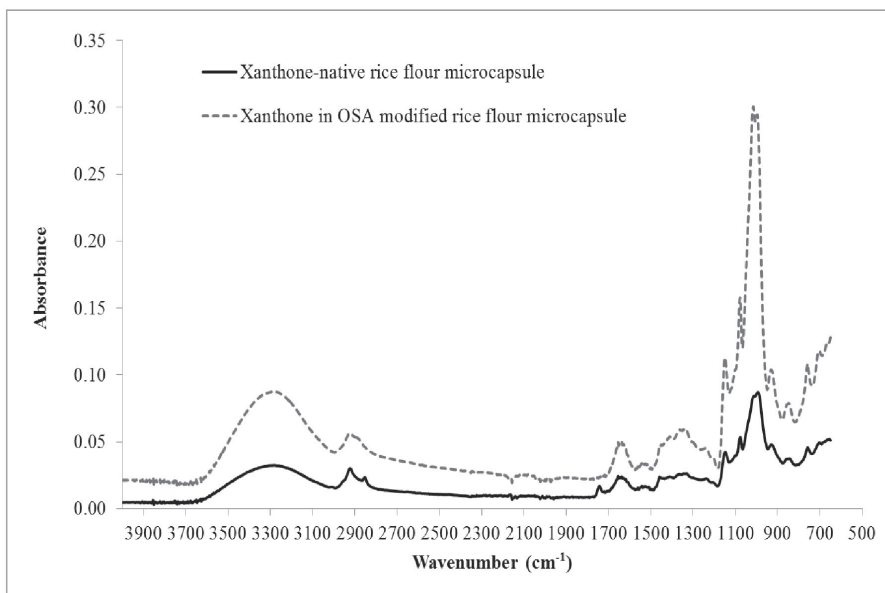
### Chemical structure obtained by FTIR

FTIR data showed differences in chemical conformation of rice flours and microcapsule samples (Figures 5 and 6). The FTIR pattern showed peaks at wavelengths around 860, 930, 1,015, 1,078, 1,150, 1,250, 1,540, 1,650, 2,850, 2,930, and 3,300  $\text{cm}^{-1}$ . Peak absorbance occurred at a wavelength of 1,750  $\text{cm}^{-1}$  for the native rice flour, but shifted to a wavelength of 1,700  $\text{cm}^{-1}$  for the OSA-modified rice flour (Figure 5). The FTIR pattern of OSA-modified black glutinous rice flour showed a higher peak absorbance at a wavelength of 1,000  $\text{cm}^{-1}$  compared to the native rice flour. These results showed that the ester groups of OSA replaced the hydroxyl groups in the starch molecules.



**Figure 5.** FTIR pattern of native and OSA-modified black glutinous rice flours.



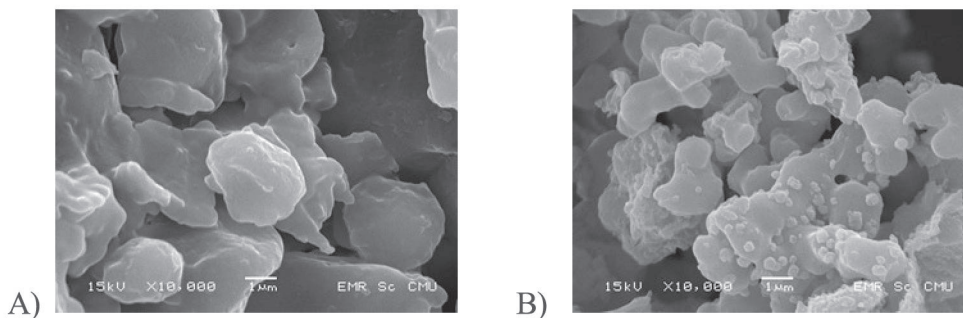


**Figure 6.** FTIR patterns of xanthone microcapsules in native and OSA-modified rice flours.

Figure 6 shows FTIR patterns of xanthone microcapsules encapsulated by native and OSA-modified rice flours. When the OSA-modified rice flour was used as wall material for encapsulation, higher absorbance at wavelengths around 930, 1,000, 1,150, 1,250, 1,650, and 3,300  $\text{cm}^{-1}$  was detected, as compared to the pattern for microcapsules in native flour. Microcapsules of xanthone in OSA-modified rice flour did not show peak absorbance at a wavelength of 1,700  $\text{cm}^{-1}$ , while microcapsule in native flour showed peak absorbance at 1,750  $\text{cm}^{-1}$ .

### Physical structure obtained by SEM

The SEM micrographs highlighted the effects of freeze drying on surface morphology of microcapsule particles (Figure 7). Freeze-dried powders exhibited slight variations in their surface morphology due to the properties of the encapsulating material. Many studies have reported a similar morphology for freeze dried microcapsules (Anandharamakrishnan et al., 2010). All freeze-dried powders produced with different modified rice flour showed a similar particle shape. Many cracks occurred on the particle surface of most freeze-dried powders, which may affect xanthone stability during storage because of oxygen permeability. SEM did not show residual xanthone on the surface of the encapsulated sample; this may due to the xanthone being included in the rice flour.



**Figure 7.** SEM of xanthone microcapsules in A) native and B) OSA-modified rice flours.

## DISCUSSION

Encapsulation efficiency of xanthone was calculated as the percentage of encapsulated xanthone content per total xanthone added. The surface xanthone content is considered non-encapsulated; this is susceptible to deterioration and undesirable reactions (Tonon et al., 2012). Higher encapsulation efficiency was obtained when OSA-modified glutinous rice flour was used as the wall material for encapsulation, because the flour contained an added ester group at the  $-OH$  site that assisted entrapment of core material by hydrophobic force (Song et al., 2006). Such hydrophobic force may enable effective inclusion complexation of xanthone in the amylose component of the rice flour (Song et al., 2006; Li, 2014). Retention and release of encapsulated compounds in food carbohydrates depend on the physicochemical properties of compounds and type of carbohydrates. High hydrophobicity and the presence of an alcohol group will increase the retention of the compounds (Okano et al., 2001; Wulff et al., 2005; Blaszcak et al., 2007). Because of this, native black glutinous rice flour, which is less hydrophobic, showed higher surface content of xanthone than OSA-modified rice flour.

Starches can be divided into two types: crystalline (type A) and non-crystalline (type B). Crystalline starches bind compounds less than non-crystalline types. Crystalline starches have low encapsulation efficiency because their starch granules are packed in a monoclinic unit cell. In contrast, non-crystalline starches are polymorphic with a more open structure (Naknean and Meenune, 2010). Black glutinous rice flour is classified as A-type polymorphic; which was confirmed by the XRD data (Figure 3). The A-type starch has a peak intensity at  $2\theta$  of  $15^\circ$ ,  $17^\circ$ , and  $23^\circ$  (Song et al., 2006). We also found these intensity peaks for both native and modified glutinous rice flours, which agreed with Song et al. (2006) and Wang and Wang (2002), who found that OSA did not change the crystalline pattern of starch. However, we found that peak intensity decreased after chemical modification. This could be because the crystalline structure transformed into an amorphous structure. The heat treatment required to modify rice flour melted the crystalline region and transformed it into rubbery state. Upon cooling, the rubbery state transformed into an amorphous, dry state. This structural transformation significantly changed the XRD patterns (Dufresne, 2014). Therefore, the OSA-modified black glutinous rice flour had a more amorphous structure than the native black glutinous rice flour, although it retained its A-type (crystalline) structure. The encapsulated samples also showed less intensity at  $2\theta$  of  $15^\circ$ ,  $17^\circ$ ,  $18^\circ$ ,  $20^\circ$ , and  $23^\circ$ , as compared to the non-encapsulated rice

flour. From Figure 4, microcapsules of native rice flour were more amorphous than the sample with modified flour; this indicated less inclusion complex formation. Because OSA-modified flour is more hydrophobic, it can form more inclusion complex with xanthone, hence showing high intensity at specific  $2\theta$ . However, the native rice and OSA-modified flour structures displayed small amounts of a V-pattern, as shown by the peaks at  $2\theta$  of  $12.9^\circ$  and  $19.8^\circ$ . The encapsulated sample did not have this V-pattern. Therefore, it can be suggested that xanthone inclusion complexation either did not form a V-pattern structure or it formed in insufficient quantity to be detected by the analytic method (Luo et al., 2016).

From the FTIR patterns, a peak at wavelengths between  $1,015$ - $1,160$   $\text{cm}^{-1}$  indicated the presence of C-O and C-O-C bonds in polysaccharides (Simsek et al., 2015). A peak around  $1,510$   $\text{cm}^{-1}$  represented C=C aromatic rings of polyphenols (Lazano et al., 2015). A peak at wavelengths between  $1,078$   $\text{cm}^{-1}$  and  $1,149$   $\text{cm}^{-1}$  indicated the presence of  $\alpha$ -1,4 glycosidic bonds (Falade and Christopher, 2015); a peak at  $1,270$   $\text{cm}^{-1}$  indicated C-O bonds of polyphenol aromatic rings. Absorption at wavelengths of  $1,640$   $\text{cm}^{-1}$  and  $2,930$   $\text{cm}^{-1}$  represented the existence of bound water and C-H<sub>2</sub> bonds in the sample (Zhang et al., 2011; Joshi et al., 2013). The peak found at a wavelength between  $3,000$ - $3,700$   $\text{cm}^{-1}$  indicated an interaction that could reflect the presence of either a complex from intermolecular hydrogen bonding (Yu and Huang, 2010) or O-H bond stretching vibration (Li et al., 2016). As shown in Figures 5 and 6, the peak absorbance of the native flour samples were lower than that of the OSA-modified samples due to less complexation. In our experiment, modifying flour with OSA resulted in ester groups replacing the hydroxyl groups. Therefore, we detected carbonyl groups at wavelengths of  $1,150$ ,  $1,080$ ,  $1,000$ , and  $930$   $\text{cm}^{-1}$ . Li et al. (2013) also reported that OSA-modified starch showed carbonyl (C=O) stretching vibration of ester group at wavelengths around  $1,725$   $\text{cm}^{-1}$ . From Figure 4, new absorption at about  $1,700$   $\text{cm}^{-1}$  was shown; this indicated that the OSA had been successfully incorporated into the starch structure. In the microcapsules of xanthone in the OSA-modified flour, the absorption at about  $1,750$   $\text{cm}^{-1}$  disappeared, but absorption at  $1,700$   $\text{cm}^{-1}$  increased, perhaps due to complexation of xanthone in an amylose helix at the carbonyl of the ester group.

## CONCLUSION

Modification of black glutinous rice flour with octenyl succinic anhydride improved the encapsulation efficiency of xanthone. Hydrophobicity arising from replacement of the hydroxyl group in the flour by the ester group in OSA enabled better xanthone-amylose inclusion complexation. FTIR patterns confirmed the inclusion complexation of xanthone with amylose. XRD patterns showed that the black glutinous rice flour was an A-type (crystalline) starch with its crystallinity shown at specific  $2\theta$ . OSA modification of black rice flour caused the starch's crystalline structure to partially transform into an amorphous structure, which facilitated the xanthone-starch complexation. Because rice flour is readily available and inexpensive, its modification for encapsulation purposes offers promise in food and other industries.

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