Article

Crystallization of All *trans–β*–carotene by Supercritical Carbon Dioxide Antisolvent via Co–axial Nozzle

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Abstract. The crystallization of β–carotene through supercritical antisolvent process with carbon dioxide (CO_2) as an antisolvent has been demonstrated. The experiments were conducted at temperatures of $40 - 60$ ^{o}C and pressures of $10 - 14$ MPa at a constant CO_2 flow rate. As a starting material, β–carotene powder was dissolved in dichloromethane (DCM). Results of UV–vis spectrophotometry and GC–MS analysis showed that there was no remaining DCM solvent in the β–carotene particles products. It showed that CO_2 has successfully removed DCM from β–carotene particles products. The product characterization by using fourier transform infrared spectroscopy (FT–IR) and X-ray diffraction (XRD) showed that the CO_2 solvent did not impregnate to the β–carotene particles products. Results from scanning electron microscope (SEM) images showed that the β–carotene particles products were successfully prepared in plate–like shape morphologies with size around 1 μm.

Keywords: $SCCO_2$, Crystallization, β–carotene, DCM, supercritical antisolvent.

1. Introduction

Carotenoids have been known as one of phytochemicals that contribute to their status as functional foods due to the major contributor to their purported health benefits. These compounds are a diverse and ubiquitous yellow, orange, and red pigments found in nature. Due to their chemical structure, the carotenoid molecules allowed to absorb light and to quench singlet oxygen and free radicals. Therefore, in nature, these compounds may provide two essential functions as accessory pigments in photosynthesis, and in photoprotection [1, 2]. β–carotene, as one of natural compounds of carotenoids, is an organic, strongly colored red–orange pigment that found abundantly in plants and fruits. Similar to other carotenoids, this compound is also a fat–soluble vitamin. β–carotene is the most prominent carotenoid and was used as a source of vitamin A for most of the people in the world [2].

It has been known that the fine particles production process is very important due to its biocompatibility to related end applications, especially in food and pharmaceutical industry [3, 4]. Here, supercritical carbon dioxide (SCCO$_2$) would be used as a media to produce particles from β–carotene dissolved in dichloromethane (DCM) in nano– and microscales. Supercritical fluid including SCCO$_2$ can be described as any fluid which is at conditions above its critical point. It has liquid–like densities with gas–like transport properties and moderate solvent power, which can be adjusted with shifts in pressure and temperature. Therefore, supercritical fluid can be applied in different fields for different applications, such as extractions, chromatography, or particle generation [4–7]. When the supercritical fluid was applied to particles generation, the organic solvent used as a solvent media can be removed completely from the particles products due to the high solubility of this solvent in supercritical fluid. Moreover, the amount of organic solvent used also can be reduced.

Table 1. β–carotene solubility in different toxicological classes of organic solvents.

Solvents	Solubility [g l^{-1}]	Solvent class*
Acetone	0.2	3
Acetonitrile	0.01	2
Benzene	4	1
Chloroform	2	2
Cyclohexane	2	2
Cyclohexanone	2	–
Dichloromethane	6	2
N,N–Dimethylformamide	0.2	2
Dimethyl sulfoxide	0.03	3
Ethanol	0.03	3
Ethyl acetate	0.5	3
Ethyl ether	1	3
Hexane	0.6	2
2–Propanol	0.04	3
Methanol	0.01	2
tert–Butylmethyl ether	1	3
Tetrahydrofuran	10	2
Toluene	4	2

*Class 1: solvents to be avoided; Class 2: solvents to be limited; Class 3: solvents with low toxic potential.

There are two main techniques to form particles by using supercritical fluid: rapid expansion of supercritical solutions (RESS) and supercritical antisolvent (SAS) techniques. In RESS technique, supercritical fluid was applied as a solvent while it would be as an antisolvent in SAS technique [8]. In this work, CO$_2$ was employed as an antisolvent to form particles from β–carotene in DCM via a coaxial tube under supercritical conditions. This coaxial device consisted of two capillary tubes that allows to deliver the feed solution and

the CO_2 as an antisolvent continuously. By using this device, the agglomeration of particle products also can be minimized due to the two coaxial flows velocities can be controlled independently [9,10]. In this process, the chemical or the physical degradation of β–carotene which affected by the presence of light, moisture, oxygen, and high temperature can be avoided. Thus, it is required to perform the additional in vitro and in vivo testing before the β–carotene particles products were marketed for human consumption. However, Miguel *et al.* [11] reported that there was no degradation on the β–carotene via isomerization and oxidation reaction during SCCO$_2$ antisolvent precipitation at temperatures of $35 - 45$ ºC and pressures of $7 - 15$ MPa. They explained that CO_2 as an environment precipitation process is inert and the contact between CO_2 and β–carotene may occur in the short period of time prior to micronization takes place. Cardoso *et al.* [12] conducted experiments for SCCO$_2$ antisolvent micronization of β–carotene at temperatures of $35 - 60$ ºC and pressures of $6.5 - 13$ MPa with tetrahydrofuran as a solvent. They confirmed that there was no degradation occurred on the β–carotene during the process micronization at these operating conditions. There was no effect on the β–carotene properties, hence, in this work the in vitro or in vivo of β–carotene was not performed.

DCM was chosen as the most suitable solvents for easy dissolution of the carotenoids including β–carotene. Table 1 lists the solubility of β–carotene in some organic solvents. β–carotene seemed to have high solubility in DCM [12]. As shown in this Table, DCM is a class 2 solvent which means that DCM can be used on the products for human consumption. Tsivintzelis *et al.* [13] studied the phase compositions for the system CO_2–DCM at various temperatures and pressures. They informed that CO_2 apparently had enough affinity to bring portion of the DCM. Franceschi et al. [14] reported that the adding β–carotene solute to the binary system consisting of DCM as an organic solvent and CO_2 had negligible effect on the transition pressures. Therefore, this solvent has been applied as a solvent to dissolve β–carotene for fine particles production by SCCO$_2$ anti solvent [14–16].

2. Experimental Section

2.1. Materials and Chemicals

β–carotene (purity > 80 %) was purchased from Wako Pure Chemical Industries, Ltd., Japan. It was directly dissolved in dichloromethane (DCM; CH_2Cl_2, purity > 99 %) which purchased from Kanto Chemical Co., Inc., Japan and used as a starting material. The concentrations of β–carotene in DCM were 0.5 and 1.5 mg ml^{-1}. Carbon dioxide (CO_2; 99%) was supplied by Sogo Kariya Sanso, Inc. Japan. This concentration was selected based on the previous report [17]. Fig. 1 illustrates the SEM image of β–carotene without any treatment.
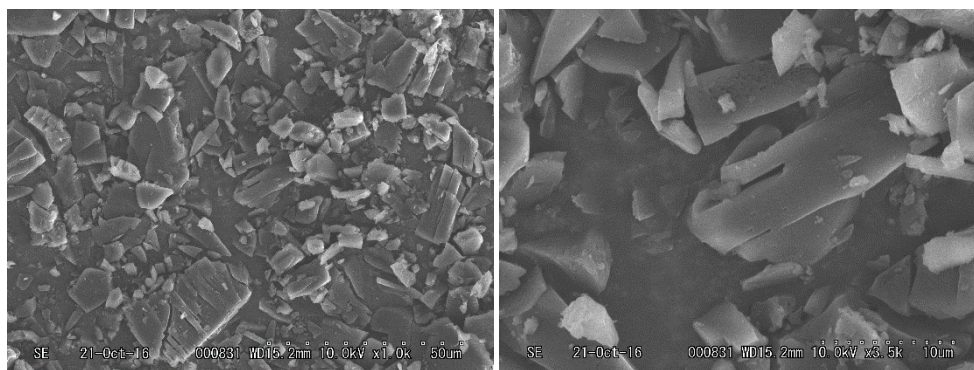

Fig. 1.   SEM image of raw material β–carotene.

2.2. Experimental Setup and Procedure

The experiments for particles formation from β–carotene via SCCO$_2$ antisolvent were performed in a homemade supercritical antisolvent equipment that illustrated schematically in Fig. 2. This apparatus system

comprises the following main components: two high–pressure pumps, one for CO_2 (PU–2086, Jasco, Japan) and the other for the solution (LC–20AT, Shimadzu, Japan), a heating chamber (EI–700B; ETTAS, AS–ONE, China), a nozzle device (SUS–316, 0.5 mm internal diameter) and back pressure regulator (BPR; AKICO, Tokyo, Japan). The nozzle consists of a 1/16–in tube (SUS–316, 0.5 mm internal diameter) for the feed solution, placed inside a 1/8–in tube (SUS–316, 2.17 mm internal diameter) for the CO_2 antisolvent. By using union Tee (SS–200–3, Swagelok), this nozzle was connected to a 1/8–in tube (SUS–316, 2.17 mm internal diameter) with 5 m of length that deployed as a precipitator unit. In the heating chamber, the coil preheater made of 1/8 inch stainless–steel tubing (SUS316) with 300 cm length was placed to introduce the CO_2 before entering to the nozzle device. K–type thermocouples were inserted in the preheater and attached in the nozzle device to monitor the temperature during experiment. To monitor the particles generation pressures, the pressure gauge (GLT–21–25 MPa, Migishita Seiki MFG co., ltd, Japan) was assembled on the 1/16 inch stainless–steel tubing (SUS316) and placed between particles products collector and BPR.

The experiments were performed at temperatures of $40 – 60$ ºC and pressures of $10 – 14$ MPa. The CO_2 and the β–carotene feed solution flow rates were 15 ml min^{-1} and 0.25 ml min^{-1}, respectively. The experiments can be described briefly as follow. Initially, the power of heating chamber was turned on to heat the nozzle device including preheater to a desired temperature. Once the desired temperature was reached, CO_2 was pumped into the micronization apparatus system via the 1/16 inch stainless–steel capillary tube at a desired pressure. A BPR was used to keep a constant pressure during micronization process. Next, the β–carotene feed solution was injected into the nozzle device upon the attainment of the desired conditions. The delivery of β–carotene feed solution was finished after 60 min; the fresh CO_2 was still pumped at around 60 min to remove the residual DCM solvent in the particles products. This step was needed to avoid the re-dissolved of particles products in DCM solvent during depressurization process. After the process was completed, the CO_2 flow was stopped and the micronization apparatus system was slowly depressurized to atmospheric pressure. Then the particles products were collected in the bottles and stored in vacuum desiccator at room temperature. These processes were conducted until analysis.
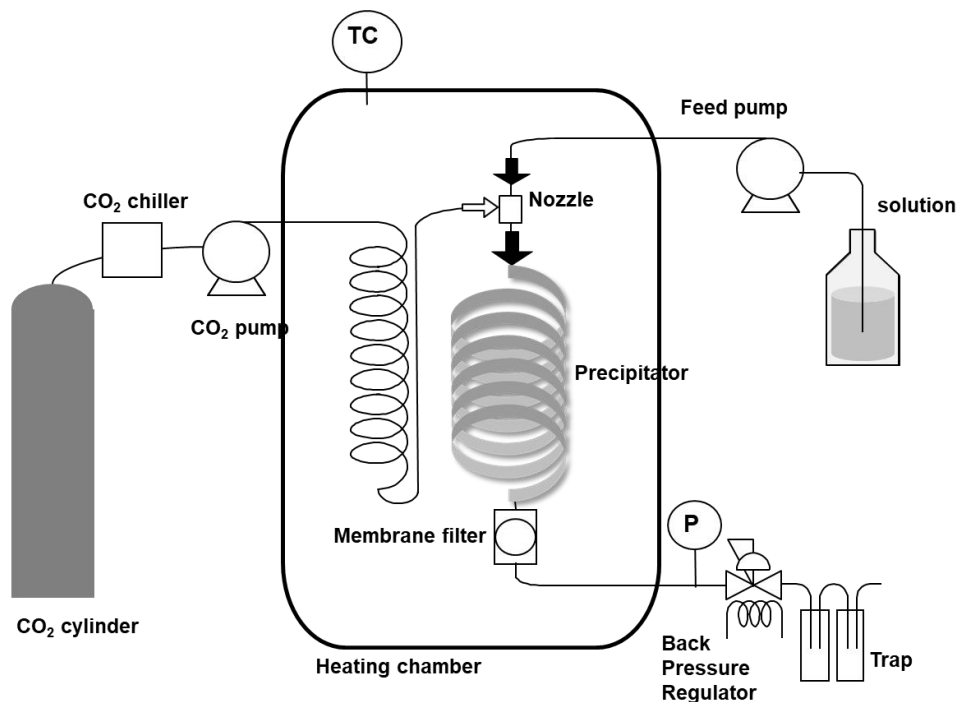

Fig. 2.   Experimental apparatus.

2.3. Analytical Methods

In order to understand the remaining DCM solvent in product, the β–carotene particles products were dissolved in hexane and analyzed by UV–vis spectrophotometry (V–550, Jasco Corporation, Japan). This analysis is simple to be used and most analytes can be detected. The solution of β–carotene particles products

was placed and analyzed in a quartz cuvette with a 1 cm path length. By using a PC–driven scanning spectrophotometer operating in the fast scan mode, the allowing spectra of between 190 and 800 nm with 10 nm min⁻¹ of bands were monitored and recorded. The β–carotene particles products was also analyzed by using GC–MS (gas chromatography mass spectrometry) Hewlett Packard model 6890 series GC system and 5973 mass selective detector with a DB–5 MS capillary column (J&W Scientific, length 30 m, i.d. 0.25 mm, film 0.25 μm). Helium gas was used as carrier gas; and 1 μl a solution in hexane was injected [18]. The injector temperature was 250 ºC and the chromatographic exit to mass spectrometer interface temperature was 300 ºC. The GC oven temperature was held at 40 ºC for 1 min then programmed to raise at 5 ºC/min to 300 ºC. The NIST (National Institute of Standards and Technology) library of mass spectroscopy was applied to identify the compounds. The analysis using a Spectrum Two FT–IR spectrophotometer (PerkinElmer Ltd., England) was applied on the β–carotene particles products collected at each operating condition to observe the structure of β–carotene after treatment by SCCO₂ antisolvent. The spectra were measured in attenuated total reflectance (ATR) mode (golden single reflection ATR system, P/N 10500 series, Specac) at 4 cm⁻¹ resolution. The scanning wave number ranged from 4000 to 400 cm⁻¹. X–ray diffraction (XRD) analysis was also applied to observe the β–carotene after SCCO₂ treatment. A Rigaku FR–E X–ray diffractometer with Cu Kα radiation (λ = 1.542 Å) was employed with a beam size of approximately 300 μm × 300 μm, with a camera length of 70 mm. The powder samples (β–carotene before or after SCCO₂ treatment) were submitted in a capillary tube (φ1.0 mm) and were then irradiated with the X–ray beam without further adjustments. The morphology of the β–carotene particles products was observed using a scanning electron microscope (SEM; S–4300, Hitachi, Japan) after gold coating. The diameters of them were measured from the SEM images using image analyzer software (Image J 1.42). Due to the limitation of the analytical apparatus, the dynamic light scattering (DLS) analysis was not conducted to characterize the β–carotene particles products.
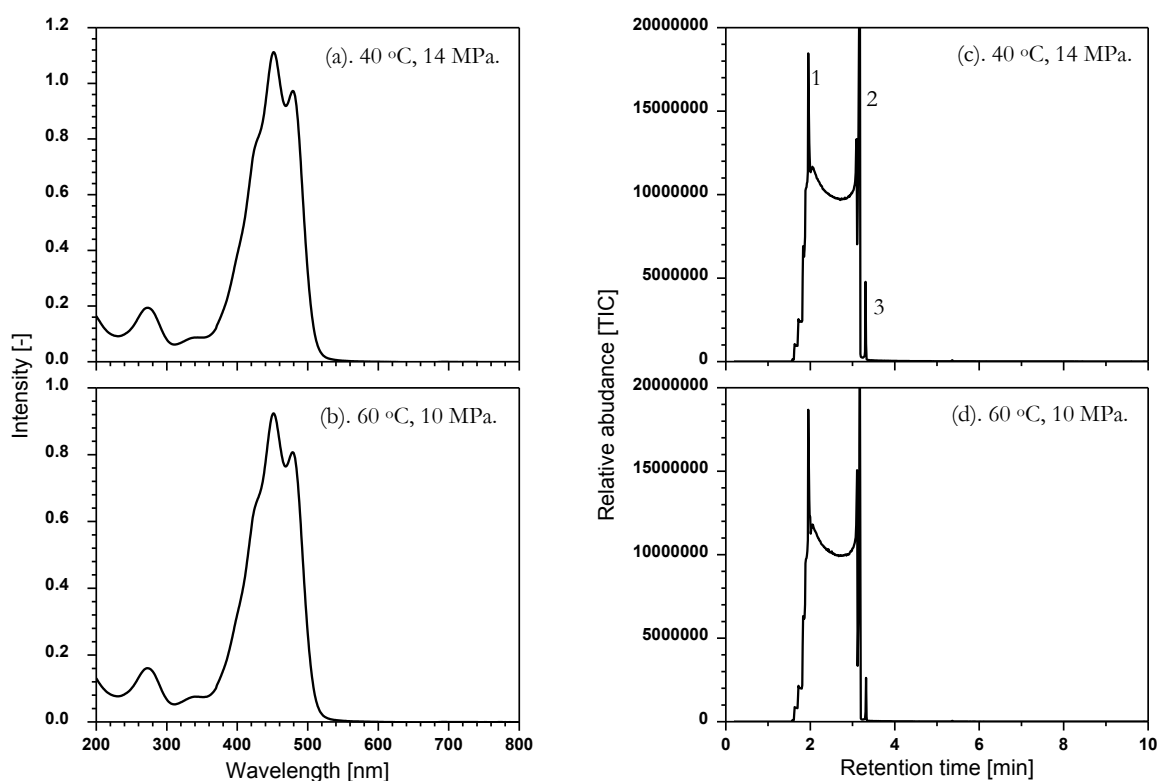
3. Results and Discussion

Fig. 3. UV–vis spectras (a and b) and GC–MS chromatograms (c and d) of β–carotene.

Fig. 3 (a and b) shows the UV–vis spectra of β–carotene particles before and after SCCO₂ antisolvent treatment at temperatures of 40 and 60 ºC and pressures of 14 and 10 MPa, respectively. Prior to analysis,

about 0.1 mg of the β–carotene particles products was dissolved with 5 ml hexane at room temperature. This analysis is a non–destructive analytical technique and also an easy in sample preparation. Generally, the UV–vis spectrophotometry for pharmaceutical applications concern light in the wavelength range 190 – 800 nm, qualitatively, β–carotene in hexane will be identified at 454 and 480 nm [19]. As described in Fig. 3 (a and b), the peak intensity at around 454 and 480 nm correspond to the β–carotene compound in the hexane solvent was clearly detected. Other peaks were not detected. It indicated that the DCM solvent in the β–carotene particles products which retained in the particles products collector by placing stainless filter was successfully removed with the flowing CO_2 under supercritical fluid conditions. Mattea et al. [18] conducted $SCCO_2$ antisolvent to produce β–carotene nanoparticle from an emulsion as a feed solution at pressures of 8 – 13 MPa and temperatures of 35 – 50 °C. They informed that there was evolution on the DCM concentration during precipitation process of β–carotene from feed solution when the DCM solvent was deployed as a solvent media. This phenomenon could be found on the β–carotene particle products which obtained in all operating conditions. Wahyudiono et al. 2014 [20] also informed that CO_2 may remove DCM solvent completely from the fiber products when they conducted electrospinning to generate fibers from polymer solution under pressurized CO_2. The same result was also obtained when the similar aliquots were introduced in the GC–MS device (see Fig. 3 (c and d)). The DCM solvent peak was not found at each chromatogram, however, (1) hexane and its derived compounds peaks such as (2) 2,2,3–trimethylhexane and (3) cyclohexane were detected prominently. It should be noted that the DCM solvent can be identified clearly by GC–MS when the DCM–β–carotene–hexane mixed solution was injected in the GC–MS apparatus. These analysis suggested that the DCM solvent in the collected β–carotene particles products has been removed with the flowing CO_2 under these conditions.

Table 2.   FT–IR spectroscopy of organic functional groups.

Wave number [cm^{-1}]	Functional groups
3500 – 3100	O–H group
3032.9 – 2818.5	C–H stretch
1736.0 – 1714.4	C=O acetate
1624.9 – 1614.0	Conjugated C=O
1559.6 – 1440.1	CH$_2$ stretch
1391.3 – 1390.4 and 1366.8 – 1366.5	Geminal methyl
1315.9	C–O acetate
1171.5 – 949.3	Trans–distributed –C=C–
900 – 700	Bending of aromatic C–H groups

To observe the possibility change structure of β–carotene molecules after $SCCO_2$ antisolvent treatment, the particles products were characterized by FT–IR spectroscopy, in the wave numbers region of 4000 – 400 cm^{-1}. This analytical method allows to identify the unknown substances and the types of chemical bonds of the compounds in those substances content. β–carotene which used as a starting material was deployed directly as a control. Fig. 4 showed the FT–IR spectra of β–carotene particles before and after $SCCO_2$ antisolvent treatment. Similar to other molecule, β–carotene is also comprised of many different chemical bonds, and these bonds are slightly elastic and can stretch, bend, or vibrate. In principle, the same organic group in different compounds may absorb approximately in the same spectral region. Table 2 listed the main regions of an infrared spectrum for single, double, or triple or bonds to hydrogen and others [21]. As shown in this figure, it seemed that the β–carotene before and after treatment by $SCCO_2$ antisolvent exhibited the strongest absorption peaks at 964.29 and 964.11 cm^{-1}. This band related to the trans conjugated alkene CH out of plane deformation mode which found in the β–carotene structure [22, 23]. Baranska et al. [22] informed that the bands at 949.29 – 949.05 cm^{-1} indicated that the β–carotene particles were not pure and contained small amounts of another carotenoid. These functional groups may absorb radiation in certain frequencies, therefore some differences exist at each FT–IR spectra due to their structure properties. However, as shown in Fig. 4, there was no diversity on the FT–IR spectra of β–carotene particles before and after $SCCO_2$ antisolvent treatment. It indicated that the β–carotene particles products generated by $SCCO_2$ antisolvent

treatment are within a similar functional group as β–carotene particles before CO_2 treatment. It also indicated that CO_2 did not impregnate to the β–carotene particles products or there was no remaining DCM solvent in the β–carotene particles products. Similar result was also found when the β–carotene particles products were characterized by X–ray diffraction (see Fig. 5) and compared to the raw material β–carotene. There are no different on the two X–ray diffraction patterns. It was clearly that the sharp diffraction peaks were found at each pattern. The β–carotene particles products showed the X–ray diffraction pattern similar to the raw material β–carotene. It confirmed that the β–carotene that dissolved in DCM solvent was not change after recrystallization process by $SCCO_2$.
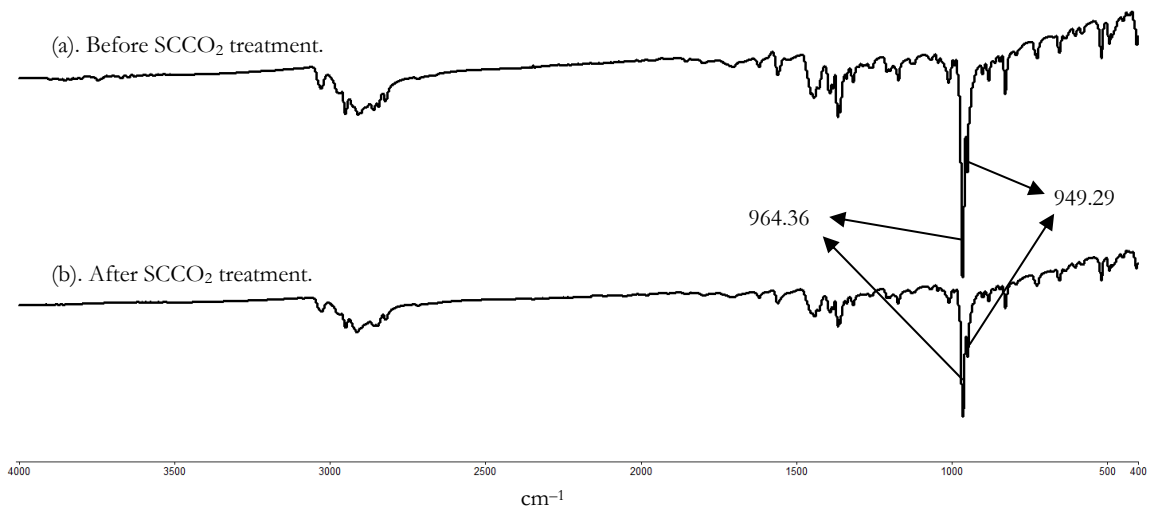

Fig. 4.   FT–IR spectrum of β–carotene particles (a) before and (b) after treatment by $SCCO_2$ antisolvent at pressure of 14 MPa and temperature of 40 °C.

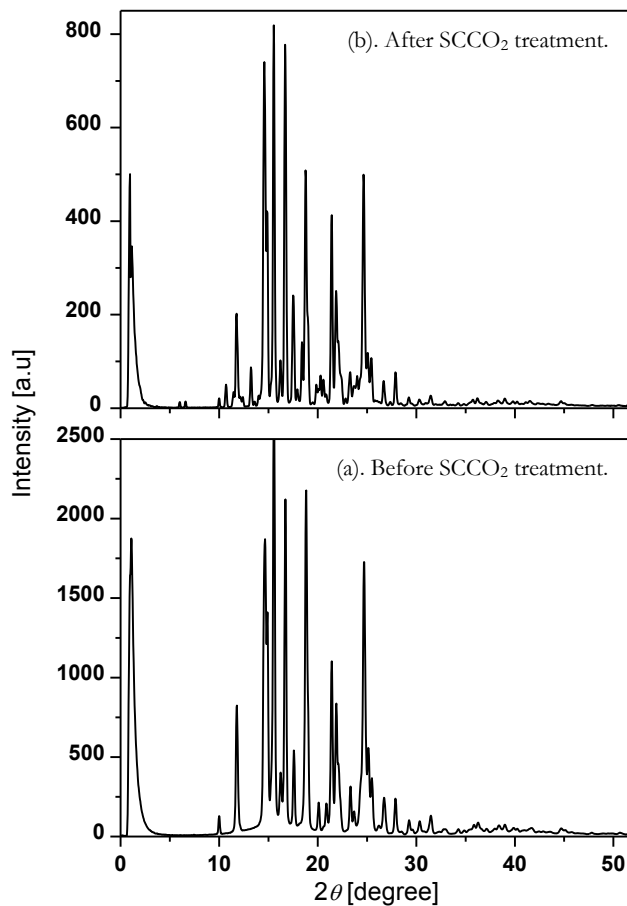Fig. 5. XRD patterns of β–carotene (a) before and (b) after SCCO$_2$ treatment at temperature of 60 ^{o}C and pressure of 10 MPa.
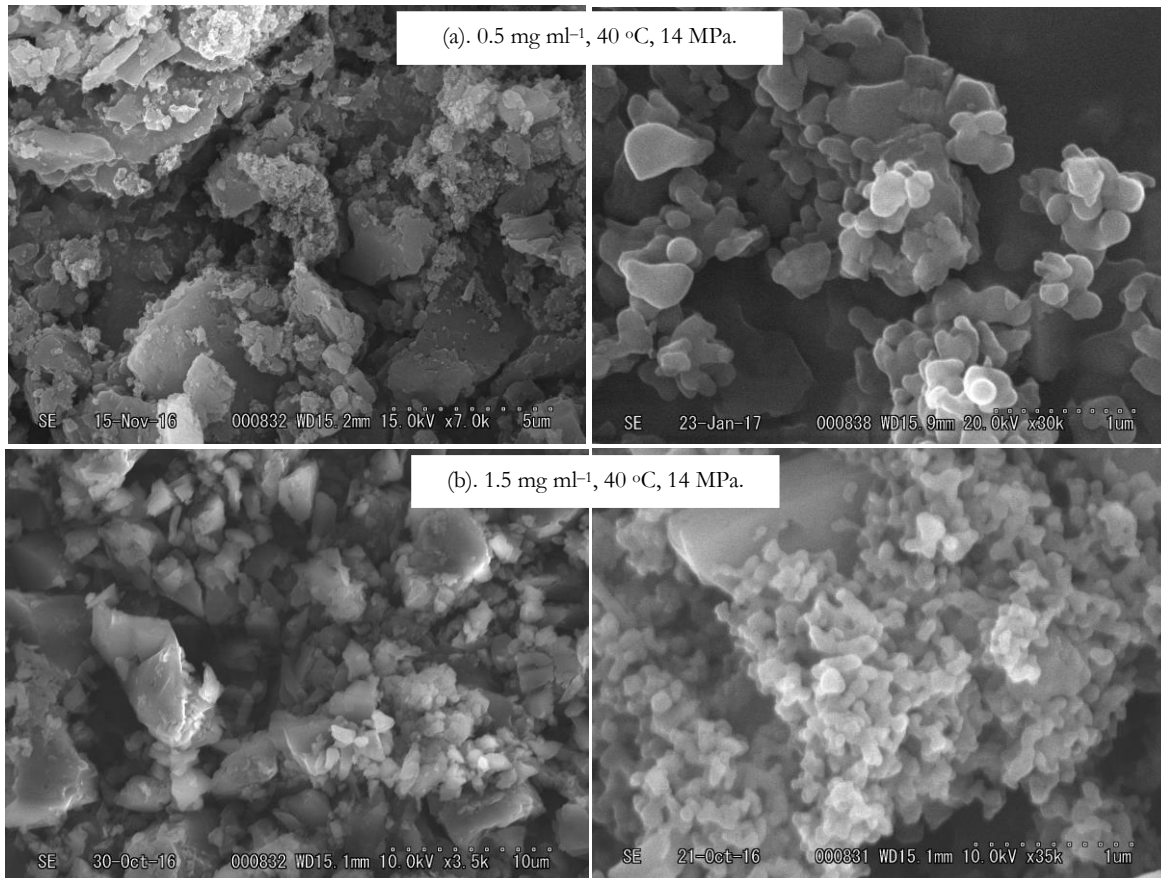
Fig. 6. SEM images of β–carotene particles products obtained by SCCO$_2$ antisolvent at different feed solution concentrations.

Like the particle formation from other chemical compounds by SCCO$_2$ antisolvent [24–26], the particles formation from β–carotene solution by SCCO$_2$ antisolvent may be affected by the feed solution concentration of β–carotene injected in the SCCO$_2$ antisolvent apparatus system. Fig. 6 shows the SEM images of β–carotene particles products found in the sample collector when the experiment was conducted at pressure of 14 MPa and temperature of 40 ᵒC with concentrations of (a) 0.5 and (b) 1.5 mg ml^{-1}, respectively. As seen from the SEM images, the different morphologies of β–carotene particles products were not found. Their morphologies are almost irregular flake–like or plate–like shapes, but the β–carotene particles products seemed to have spherical–like shape morphologies in higher magnification. However, due to the limitation of the SEM analytical equipment, the high magnification of β–carotene particles products were not presented in the next section. The SEM images also showed that the unexpected morphology of β–carotene particles products were still occurred on random occasion due to the agglomeration process during formation of β– carotene particles at each concentration. It indicated that the β–carotene particles formation from β–carotene solution by using SCCO$_2$ antisolvent on the different concentrations is essentially the same process. This process comprised of mixing of feed solution and antisolvent, generation of supersaturation, nucleation, and growth by coagulation and condensation, followed by agglomeration [27]. Li *et al.* [25] conducted SCCO$_2$ antisolvent to produce microparticles from puerarin solution. They reported that the similar morphology of puerarin particles products were obtained when the different feed solution concentration of puerarin was fed in the SCCO$_2$ antisolvent. Similar results were also found when Chhouk *et al.* 2017 [26 conducted experiment to generate curcumin particles via SCCO$_2$ antisolvent. They reported that the morphologies of curcumin particles products had almost spherical shape at various feed solution concentrations. Therefore, it could be said that the feed solution concentration does not affect significantly on the morphology of particles products.
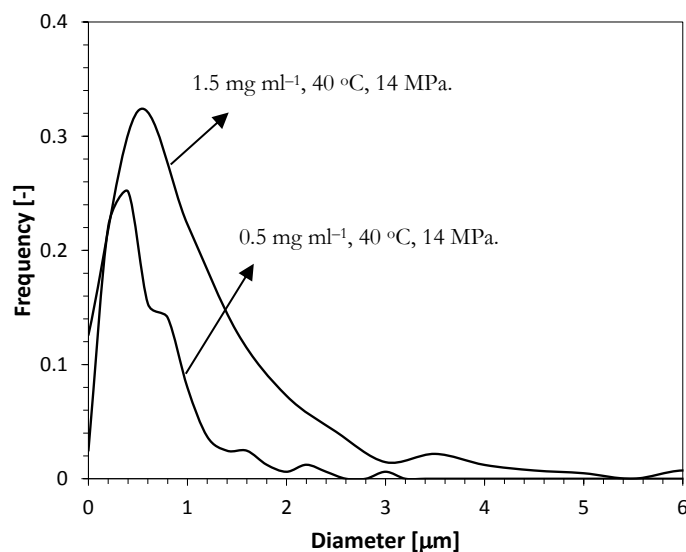
Fig. 7. Particle size distribution of β–carotene particles products obtained by SCCO$_2$ antisolvent at different feed solution concentrations.

In most cases, the increasing feed solution concentration in SCCO$_2$ antisolvent process may give several effects. An increase in feed solution concentration at a constant CO$_2$ flow rate will enhance the viscosity and surface tension of the solution, next, the injection velocity at a given pressure drop across a nozzle will be declined to result the change of particles size products [28]. Chen *et al.* [28] explained that besides the increasing feed solution concentration affects the viscosity and surface tension of the feed solution, the increasing feed solution concentration will promote the supersaturation. The nucleation will occur soon to form the crystals, as a result, the particles with larger diameter will be generated. In this work, using the Image J 1.42 tool, from each image illustrated in Fig. 6, at least 250 different β–carotene particles products were randomly chosen, and their size were measured to obtain the particle size distribution. As shown in Fig. 7, the β–carotene particles products produced from 1.5 mg ml^{-1} feed solution concentration had bigger diameter than the β–carotene particles products formed from 0.5 mg ml^{-1} feed solution concentration. It indicated that the increasing concentration of β–carotene in feed solution may promote the supersaturation conditions to yield faster nucleation, therefore the bigger size of β–carotene particle products with wide particle size distribution were formed. Based on the result, 0.5 mg ml^{-1} β–carotene feed solution concentration was fed as a feed concentration for further experiments.

Figures 8 and 9 also show the effect of operating temperature on the β–carotene particles products. It seemed that the increasing operating temperature of SCCO$_2$ antisolvent treatment at the same operating pressure tended to result larger β–carotene particles products and plate–like morphologies. The change of β–carotene particles products morphology might be caused by the change of volume expansion during SCCO$_2$ antisolvent process [14]. Cocero and Ferrero [15] reported that the increasing operating temperature in SCCO$_2$ antisolvent had high responsibility for the ultimate extent of agglomeration achieved. As a result, at higher operating temperature, the plate–like morphologies of β–carotene particles products were found prominently. In the case of β–carotene particles products size, they increased with the rising operating temperature as shown in Fig. 9. At 40 °C, the β–carotene particles products with size ranges of 0.4 − 1.2 μm with an average particles size of 0.84 μm were obtained. While their size ranges of 0.4 − 1.8 and 0.4 − 1.8 μm with average particles sizes were 0.88 and 1.11 μm were found when the experiments were performed at operating temperatures of 40 and 50 °C, respectively. It shows that the increasing operating temperature leads to increase the β–carotene particles products size. At these conditions, the increasing operating temperature leads to the decreasing CO$_2$ density, thus the dissolving ability of CO$_2$ also decreases. Then supersaturation will decrease and the bigger size of β–carotene particles products will be formed [17, 25, 29, 30]. Imsanguan *et al.* [29] investigated the effect of operating temperature on the SCCO$_2$ antisolvent to precipitate andrographolide. They conducted experiments at operating temperatures of 35 − 50 °C and operating

pressures of 8 – 20 MPa. They explained that there are two competition phenomena in the particle formation as the temperature changes under $SCCO_2$ conditions. First, a decline in $SCCO_2$ density promotes to the $SCCO_2$ diffusivity to result in high mass transfer rates and decreasing particle size products. Second, an increase in the solute solubility will slow the supersaturation attainment and an increase in the particle size products. However, they informed that the increasing particle product size seems to be dominantly with increasing operating temperature. Li *et al.* [25] also confirmed that the bigger puerarin particles products were produced because the supersaturation decreased when the higher operating temperature was applied on the recrystallization puerarin under $SCCO_2$ conditions. Judging from the results, it could be said that the β–carotene particle product size seemed increase with an increasing operating temperature.
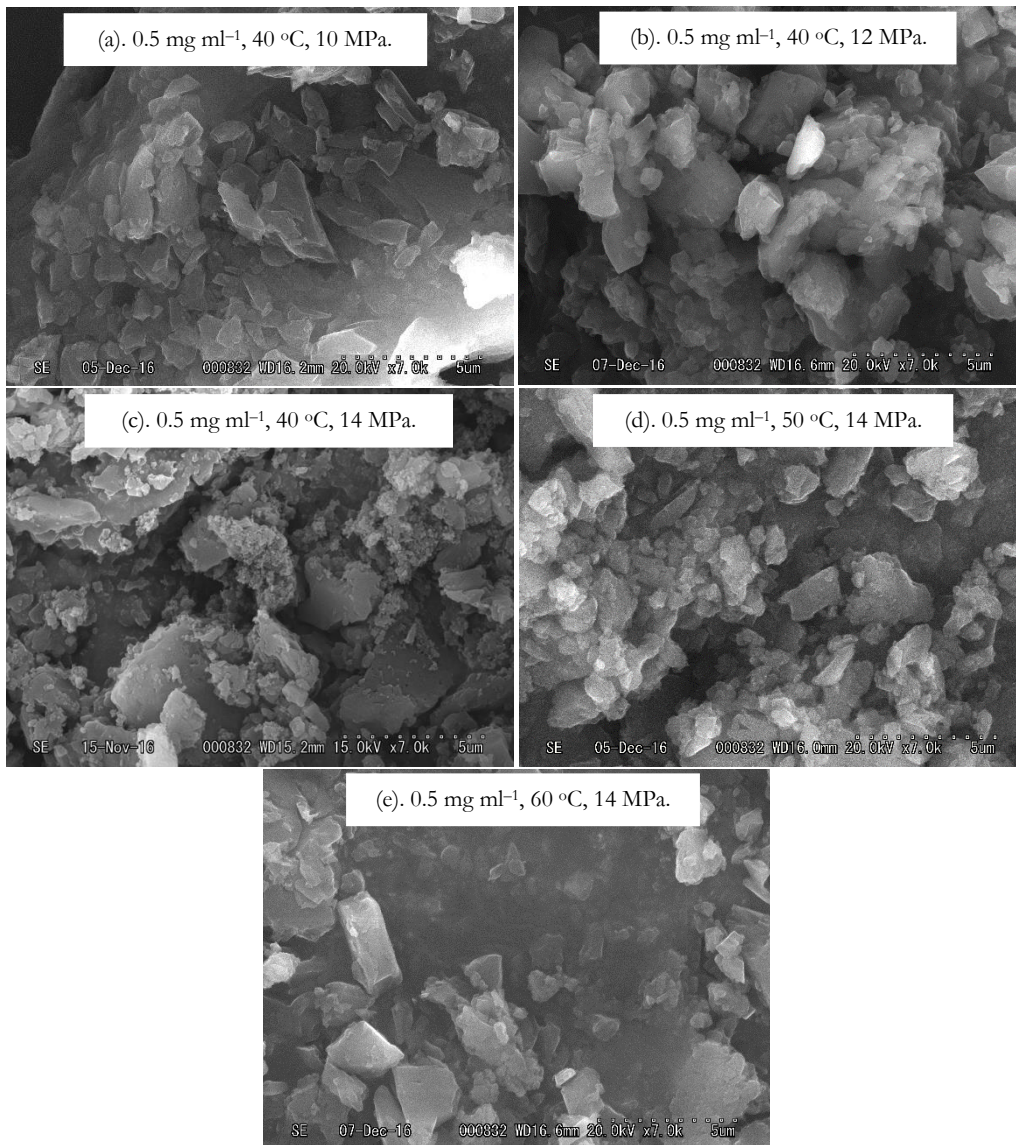

Fig. 8. SEM image of β–carotene particles products obtained by $SCCO_2$ antisolvent at different operating conditions.
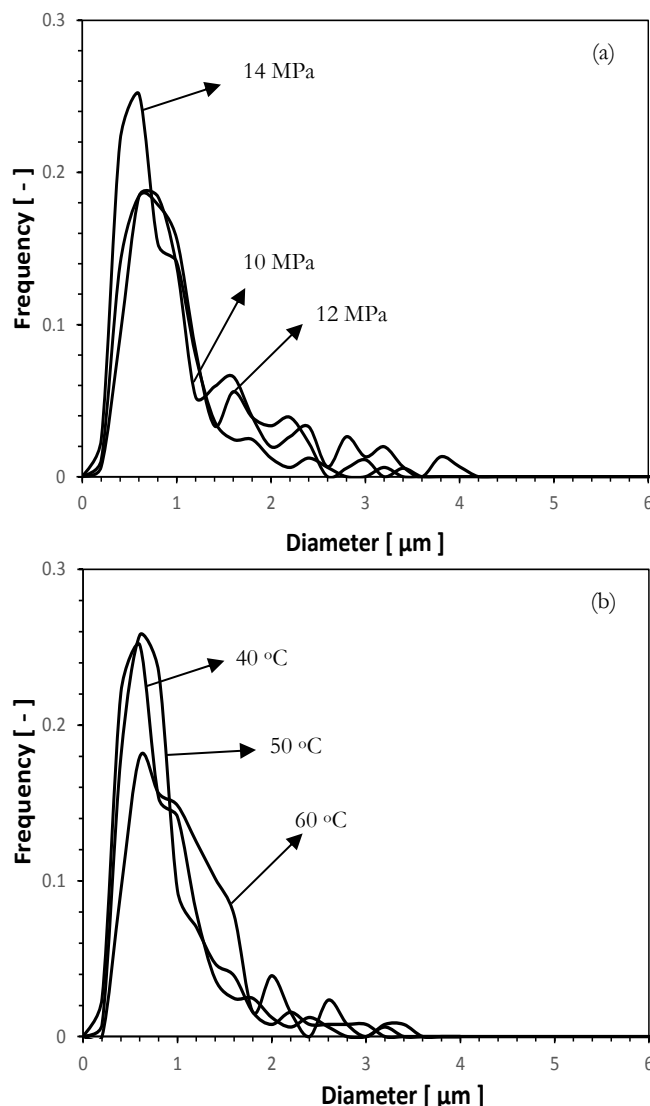
Fig. 9.   Particle size distribution of β–carotene particles products obtained by SCCO$_2$ antisolvent at various (a) pressures and (b) temperatures.

4.   Conclusions

The generation of β–carotene particles via supercritical antisolvent process with CO$_2$ as an antisolvent has been performed. The experiments were conducted at temperatures of 40 – 60 ^{o}C and pressures of 10 – 14 MPa. The β–carotene powder dissolved in DCM was deployed as a starting material. The spectra of UV–vis and GC–MS analysis showed that there was no remaining DCM solvent in the β–carotene particles products. It indicated that CO$_2$ has successfully removed DCM from β–carotene particles products. The product characterization by using FT–IR and XRD showed that the CO$_2$ solvent did not impregnate to the β–carotene particles products. The SEM images showed that the β–carotene particles products were successfully produced in plate–like shape morphologies with size around 1 μm. Finally, it could be said that this process seems a powerful method and to be an apt for size reduction of β–carotene powder.

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