Enhanced production of β-carotene suspensions using supercritical CO₂ via naturally occurring Z-isomerization-accelerating catalyst

Yelin Zhang¹, Masaki Honda²,*, Wahyudiono¹, Hideki Kanda¹ and Motonobu Goto¹,*

¹Department of Materials Process Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan
²Faculty of Science & Technology, Meijo University, Shiogamaguchi, Tempaku-ku, Nagoya 468-8502, Japan
* Correspondence: honda@meijo-u.ac.jp; Tel.: +81-52-838-2284 (M.H.); goto.motonobu@material.nagoya-u.ac.jp; Tel.: +81-52-789-3392 (M.G.)

Abstract. β-carotene has high antioxidant activity and its adequate intake can reduce the risk of various diseases. Thus, β-carotene can be utilized as a dietary supplement and valuable natural food colorant. Z-isomerization of β-carotene can reduce the crystallinity of (all-E)-β-carotene and improve the solubility of it in solvents. Preparing nanosuspensions of Z-isomer-rich β-carotene can improve the dispersibility of β-carotene in water and bioavailability of it. In previous work, Z-isomerization and encapsulation process of β-carotene was implemented in two separated steps. In this work, organic catalyst derived from plants was utilized for Z-isomerization of β-carotene. The Z-isomerization and emulsification-evaporation process were conducted simultaneously under supercritical CO₂ assisted by ultrasound. β-carotene suspension produced in this work was characterized by UV-vis, HPLC and DLS. The nanosuspensions rich in Z-isomers of β-carotene has been successfully prepared using allyl isothiocyanate (AITC) as the catalyst for Z-isomerization. When the distributed processing was performed with 50mg, 100mg AITC, the encapsulation efficiency and Z-isomerization ratio in the suspensions were approximately 6 times and 13 times higher than those of no catalyst. Adding AITC in the emulsification-evaporation process using SC-CO₂ as a Z-isomerization-accelerating catalyst improved not only the Z-isomerization ratio of β-carotene but also the encapsulated β-carotene content in suspensions.

KEYWORDS: Carotenoid, E/Z-isomerization, allyl isothiocyanate, supercritical carbon dioxide; suspension

1. Introduction

In recent years, synthetic food colorants haven’t been extensively accepted by consumers so that the demand for natural pigments such as carotenoids is increasing year by year. β-Carotene is a natural fat-soluble carotenoid containing 11 conjugated double bonds (Figure 1) found abundantly in vegetables and fruits with a deep orange-yellow color such as carrots and pumpkins
Since β-carotene has multiple health benefits such as antioxidant capacity and antiatherosclerosis activity as well as provitamin A activity, the carotenoid is used as safe and high value-added food colorant all over the world. Due to high hydrophobicity and high crystallinity and the poor water solubility of β-carotene, it is problematic to utilize it for food formulations. In addition, the low solubility in water of lipid bioactive compounds would be prone to reduce the bioavailability. Thus, in many cases, β-carotene is used by formulation into a water-soluble preparation using emulsifier in food industry. Several studies successfully obtained carotenoid suspensions by emulsification-evaporation technique as following: 1) Dissolution of the target carotenoid in an organic solvent; 2) Distributed processing of the solution with water containing a dispersant; 3) Solvent evaporation under reduced pressure. However, generally, these distribution methods use toxic organic solvents such as hexane and ethyl acetate to dissolve carotenoids, and thus the residual solvent often becomes a major issue.

Figure 1. Chemical structures of typical β-carotene isomers: (a) (all-E)-β-carotene; (b) (9Z)-β-carotene; (c) (13Z)-β-carotene; (d) (15Z)-β-carotene.

Figure 2. Increase in the efficiency of distributed processing utilizing Z-isomerization-induced alteration in solubility: (a) general distribution method for carotenoids (emulsification-evaporation technique); (b) emulsification-evaporation technique using supercritical CO₂ (SC-CO₂) as organic phase and conducted Z-isomerization pre-treatment; (c) emulsification-evaporation technique using SC-CO₂ as organic phase and conducted Z-isomerization in distribution process by adding allyl isothiocyanate (new method in this study).

Very recently, we successfully produced β-carotene suspensions using supercritical CO₂ (SC-CO₂), which is non-toxic and can be easily separated from the products, as an alternative to
organic solvents [10]. When we produced the β-carotene suspensions by the method, since (all-
E)-β-carotene (Figure 1a), which is the most predominant geometric isomer in nature, is poorly
water-soluble in SC-CO₂, the Z-isomerization treatment was performed before the distributed
processing (Figure 2b). Z-Isomers of carotenoids including β-carotene (Figure 1b–d) are more
soluble in organic solvents and SC-CO₂ than the all-E-isomers [5,6,11–13]. For example, Honda
et al. [7] reported that the solubility in ethanol of β-carotene is approximately 250 times higher
than that of the all-E-isomer. In addition, Gamlieli-Bonshtein et al. [11] showed that the solubility
of (9Z)-β-carotene in SC-CO₂ was nearly four times higher than that of the all-E-isomer. Thus, to
increase the efficiency of β-carotene distribution using organic solvents and SC-CO₂ as the
organic phase, it is very effective to use Z-isomers of β-carotene. Moreover, since Z-isomers of β-
carotene would have higher antiatherogenesis and antiatherosclerosis activities [14,15], the
addition of Z-isomerization process can also be expected to enhance the health benefits of the
obtained suspensions. However, to obtain Z-isomers of β-carotene, thermal treatment in toxic
organic solvent such as dichloromethane (CH₂Cl₂) and chloroform (CHCl₃) is required [5,10,16].
Although (all-E)-carotenoids are thermally isomerized to the Z-isomers in SC-CO₂, the efficiency
is very low and high temperature heating is required [17]. Recently, we revealed that some plant-
derived compounds such as isothiocyanates and polysulfides enhanced Z-isomerization of (all-
E)-carotenoid [18,19]. We considered that in the case of making β-carotene suspensions via
eumulsification-evaporation technique, by adding the Z-isomerization-accelerating catalyst to the
organic phase, the solubility of β-carotene is increased associated with the Z-isomerization and
dispersing efficiency would be improved (Figure 2c). Therefore, this study aims to improve
production of β-carotene suspensions using SC-CO₂ as an organic phase via naturally occurring
Z-isomerization-accelerating catalyst. As the catalyst, we used allyl isothiocyanate (AITC)
because food-grade AITC derived from mustard seed is already available commercially and that
is relatively inexpensive and has high purity. In addition, since AITC is non-toxic and have several
health benefits for human such as anti-cancer and anti-inflammatory [20,21]. Furthermore, since
AITC have relatively low boiling point [22], that could remove by heating under vacuum.

2. Materials and Methods

2.1 Materials

High-performance liquid chromatography (HPLC)-grade organic solvents (acetone, hexane,
methanol, methyl tert-butyl ether [MTBE]) and (all-E)-β-carotene (crystalline β-carotene) were
obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Poly oxyethylene
sorbitan monolaurate (Twee 20) and AITC were purchased from Tokyo Chemical Industry Co.,
Ltd. (Tokyo, Japan). Carbon dioxide was supplied by Tomoe Shokai Co., Ltd. (Tokyo, Japan).

2.2 Isomerization and distributed processing of β-carotene

The distributed processing of preparing β-carotene was carried out according to the method
described by Tan and Nakajima [9] and Ono et al. [10]. In order to isomerize (all-E)-β-carotene
to the Z-isomers to improve the solubility in SC-CO₂ [5,6,10], a Z-isomerization-accelerating
catalyst, AITC [18], was added into the reactor directly. A schematic diagram of the dispersion
process of β-carotene is shown in Figure 3. The apparatus consists of a chiller (TBG020AA,
Advantec Toyo Kaisha, Ltd., Tokyo, Japan), a high-pressure pump (PU-980, Jasco Co., Tokyo,
Japan), a 15-mL SUS-316 stainless steel high-pressure vessel equipped with a 2-μm filter (GL
Sciences Inc., Tokyo, Japan), an ultrasound equipment (W-118, Honda Electronics Co., Ltd.,
Toyohashi, Aichi, Japan), and a back-pressure regulator (BPR; Akico Co., Ltd., Tokyo, Japan).
In brief, 15mg of (all-E)-β-carotene and 13.5mL of distilled water containing 0.5wt% Tween 20
were put in the vessel. CO₂ was bubbled through water solution to get rid of oxygen dissolved in
water and then AITC (50 mg or 100 mg) was added into the vessel. The volume proportion of
SC-CO₂/aqueous phase was adjusted to 1:9 [9,23] and the head space in the vessel was filled with
CO₂ gas. Liquid CO₂ in cylinder was cooled with chiller and it was pumped into the system via the high-pressure pump. The pressure of the system was kept at 20 MPa by BPR and the vessel was preheated for 30 minutes in 50 °C water bath. Liquid CO₂ was then transformed into the supercritical state in the vessel under 20 MPa and 50 °C. The suspension of β-carotene was performed using the ultrasound equipment at 45 kHz for 3 h. After the ultrasound treatment, the pressure of the system was reduced to atmospheric pressure via BPR slowly, and the suspension was brought out together with CO₂ through the 2-µm filter and collected in recovery vial. The non-suspended β-carotene crystals were removed. All experiments were carried out in triplicate and expressed as the mean ± standard deviation.

**Figure 3.** Schematic diagram of the β-carotene dispersion process. A 2-µm filter was inserted at the outlet of the vessel.

### 2.3 Evaluation and characterization of β-carotene suspensions

#### 2.3.1 Absorption spectra analysis of β-carotene suspensions

The absorption spectra of β-carotene suspensions as well as β-carotene crystal and Tween 20 were measured by a UV-vis spectrophotometer (V-550, Jasco Co., Tokyo, Japan) ranging from 300 nm to 800 nm. All suspensions and reagents were dissolved in distilled water.

#### 2.3.2 Z-isomers content of β-carotene

Methanol and hexane (1:1, v/v) was utilized to separate β-carotene isomers from tween 20 as tween 20 dissolves in methanol and β-carotene dissolves in hexane. The upper layer solution of hexane and β-carotene was collected. Hexane was evaporated under reduced pressure and the remained β-carotene was dissolved in acetone for HPLC analysis. The Z-isomer content of β-carotene was determined by reversed-phase HPLC with a C₃0 carotenoid column (250×4.6mm i.d., 5 mm, YMC Co., Ltd., Kyoto, Japan). The HPLC analysis was conducted as method reported previously [10,24,25]. The quantification of Z-isomers of β-carotene was determined by a UV-vis detector (UV-2075 Plus, Jasco Co., Tokyo, Japan) with detection wavelength at 453 nm. The isocratic elution consisted of 11:89 (v/v) MTBE/methanol and the flow rate of isocratic elution was set at 1 mL/min. The column temperatur was set at 40 °C. β-Carotene isomers were dissolved in acetone and filtered via a 0.2-µm polytetrafluoroethylene (PTFE) membrane filter (Advantec Co., Ltd.) before the sample was injected into HPLC. The peaks of β-carotene isomers such as (all-<i>E</i>)-9Z-, and 13Z-isomers were identified by the HPLC retention times and absorption maximum of the isomer and relative intensities of the Z-peak as %DB/DII (Q-ratios) as described previously [24,26]. The Z-isomer content (%) of β-carotene was estimated as the peak area of β-carotene Z-isomers to the peak area of total β-carotene isomers including the all-<i>E</i>-isomer.

#### 2.3.3 Encapsulated β-carotene content

The encapsulated β-carotene contents in resulting suspensions were determined by the absorbance of the solutions at 453 nm using UV-vis spectrophotometer (V-550, Jasco Co., Tokyo, Japan) as previously described [8,10,27]. The amount of dispersed β-carotene in the solution is proportional to the absorbance via UV-vis but particles of crystalline β-carotene did not contribute
to the absorbance. Certain amounts of pure (all-E)-β-carotene were dissolved in ethanol and used for preparing the calibration curve due to the convenience using UV-vis for the determination of β-carotene in suspensions [10].

2.3.4 Emulsion size analysis
The average diameters of emulsion and size distributions of resulting suspensions were measured by dynamic light scattering (DLS) ranging from 0.3 nm–10 μm (Zetasizer Nano ZS, Malvern Instruments, Ltd., Worcestershire, United Kingdom). The refractive index of β-carotene in water was set to 1.47 [10] and every sample was measured for 3 times.

3. Results and Discussion
3.1 Profile of β-carotene isomers in the suspensions with or without AITC
The reverse-phase HPLC charts of crystalline β-carotene (mainly the all-E-isomer) and β-carotene suspensions with and without AITC are shown in Figure 4. Although raw-material crystalline β-carotene contained only 3.2 % of the Z-isomers, proportion of Z-isomers in the suspension of β-carotene increased to 15.0 ± 2.0%. The increase in Z-isomers content may attribute to the thermal treatment that was conducted during the process, as it has been reported that thermal treatment of carotenoids in SC-CO₂ enhanced the Z-isomerization [28]. When 100 mg AITC was loaded into the vessel, the Z-isomerization proportion of β-carotene was improved to 38.3 ± 4.1%. Recently, we found that AITC can enhance thermal Z-isomerization of a carotenoid, lycopene [18]. Thus, AITC also acted as a Z-isomerization-accelerating catalyst for β-carotene. Specifically, when AITC and β-carotene dissolved in SC-CO₂, the Z-isomerization reaction of (all-E)-β-carotene was enhanced in SC-CO₂. In addition, it is possible that the existence of AITC in SC-CO₂ would improve the solubility of β-carotene in SC-CO₂, and thus Z-isomerization is promoted. Ample studies reported that (all-E)-carotenoids were easily Z-isomerized in their dissolved state [6,16,29].

Table 1. Absorption maxima (λ_max) and relative intensities of Z-peak (%D₀/DII) of geometrical β-carotene isomers separated and observed using reversed-phase HPLC.

| Peak | β-Carotene isomer | Observed | Reported* | Observed | Reported* |
|------|-------------------|----------|-----------|----------|-----------|
| a    | UZ                | 336,458,483,582 | -         | 56.7     | -         |
| b    | (15Z)             | 342,434,452,484 | 338,424,449,474 | 55.6     | 49.7      |
| c    | (13Z)             | 329,393,442,486 | -         | 64.1     | -         |
| d    | UZ                | 336,425,442,472 | 339,420,445,470 | 44.2     | 37.1      |
| e    | UZ                | 332,412,442,484 | -         | 70.1     | -         |
|     | (all-E)           | 338,442,462,473 | -         | 61.7     | -         |
|     | (9Z)              | 423,450,473     | 426,452,478 | -        | -         |

*Values and peak designations were obtained from the chromatograms in Figure 4. –, not assigned. ND, not detected substantially.
Tentatively assigned in the literatures [10,24–26].

Figure 4. Reversed-phase HPLC chromatograms of (a) (all-E)-β-carotene and the suspensions containing β-carotene (b) without allyl isothiocyanate and (c) with 100 mg allyl isothiocyanate. The suspensions were obtained by the 180-min ultrasound treatment at 50 °C and 20 MPa. (all-E)-, (9Z)-, and (13Z)-β-Carotene designated in the chromatograms were identified according to previous studies [10,24–26]. Some of the peaks (a–e) were tentatively identified as shown in Table 1.

3.2 Encapsulation efficiency of β-carotene and Z-isomer content in the suspensions

Table 2 shows β-carotene contents in suspensions when different amounts of AITC were added. In the absence of the catalyst, the content of distributed β-carotene in water phase was only 81.8 ± 8.5 mg/L, whereas the β-carotene content increased 485.5 ± 14.1 mg/L when 100 mg AITC was utilized in the vessel.

Table 2 Average emulsion size (nm) of suspensions and β-carotene content (mg/L) and Z-isomers content (%) in suspensions.

| Catalyst Content (mg) | Average emulsion size (nm) | β-carotene content (mg/L) | Z-isomers content (%) |
|-----------------------|-----------------------------|---------------------------|-----------------------|
| 0                     | 1087.7 ± 117.3              | 81.8 ± 8.5                | 15.0 ± 2.0            |
| 50                    | 775.4 ± 82.6                | 174.9 ± 4.4               | 35.4 ± 1.9            |
| 100                   | 654.6 ± 140.4               | 485.5 ± 14.1              | 38.3 ± 4.1            |

From 50 mg AITC to 100 mg AITC, even though the Z-isomerization ratio increased only 3%, the β-carotene contents of emulsion of 100 mg AITC were nearly 3 times higher than those of 50 mg AITC. The existence of a small amount of organic solvents and oily compounds in SC-CO₂ can improve the solubility of β-carotene in it [13,17,30]. In general, it has been reported that in the emulsification-evaporation process, if the lipid-soluble compound has higher solubility in solvent, it can be easily encapsulated into nanosuspension [8–10]. When the ultrasonication treatment started, β-carotene in SC-CO₂ close to the interphase boundary moved toward water phase, and it was encapsulated into micelle by Tween 20. Due to the hydrophilicity of micelles containing β-carotene, micelles moved towards and remained in water phase. As the Z-isomers in SC-CO₂ phase decreased, it induced to further Z-isomerization of β-carotene while AITC acting as the catalyst. Therefore, the isomerization process in SC-CO₂ and the process of emulsification in the interphase boundary repeated. The increased solubility of β-carotene in SC-CO₂ phase
enhanced the encapsulation efficiency using ultrasound and the encapsulation efficiency reached to 43.7% ± 1.3% of 100 mg AITC. As AITC also dissolved in SC-CO₂, when ultrasound was utilized to the vessel, AITC could also move towards water phase and be encapsulated by Tween 20 and the micelle containing AITC remained in water phase. When AITC amount decreased in SC-CO₂ phase, it reduced the Z-isomerization process and furthermore reduced the contents of Z-isomers in suspensions. This could lead to the lower isomerization ratio in this method than the previous work [10]. Moreover, compared with results of 100 mg AITC, suspensions of 50 mg AITC showed lower Z-isomerization ratio and β-carotene content, and this could also support that AITC was encapsulated by Tween 20 and suspended in water phase. Furthermore, sample being kept in the vessel by 2-μm filter didn’t give off the typical pungent smell of AITC and this could also reveal that AITC was brought out together with water phase.

3.3 Characterization of (all-E)-β-carotene and the Z-isomer suspensions

3.3.1 Color analysis of suspensions by UV-vis

The color of suspensions rich in (all-E)-isomers and Z-isomers with different AITC amount was characterized by their appearance and the absorbance using UV-vis spectrophotometry (Figure 5). The suspensions rich in (all-E)-isomers showed light reddish color but the suspensions rich in Z-isomers showed deep yellow color. The similar colors have been observed in Z-isomer-rich β-carotene suspensions in previous works [10,23] and it indicated that the Z-isomerization and encapsulation of β-carotene were successfully performed. In Figure 5, an obvious absorbance peak was observed in β-carotene suspensions with 100 mg AITC in the range of 400-500 nm, whereas the peak intensity was very small in the suspensions without AITC in the wavelength range. As has been reported, dispersed β-carotene exhibits a specific absorbance in this wavelength range [8,10]. Since there was a certain degree of Z-isomerization in SC-CO₂ without AITC, it accounted for the phenomenon of small absorbance peak of suspensions without catalyst-AITC. Thus, it implied that the water-soluble β-carotene suspension was successfully prepared, especially when AITC was used as a catalyst for Z-isomerization.

Figure 5. Appearances and absorption spectra of the β-carotene suspensions with and without allyl isothiocyanate (catalyst). The suspensions were obtained by the 180-min ultrasound treatment at 50 °C and 20 MPa.

3.3.2 Size distribution analysis of suspensions by DLS

The size distributions of β-carotene suspensions in the absence or presence of 100mg AITC are shown in Figure 6 and the average emulsion sizes of them are shown in Table 2. As the amount of adding AITC increased, the average emulsion size of the suspensions decreased. For example, in the absence AITC, the average emulsion size was 1087.7 ± 117.3 nm, whereas adding 50 mg and 100 mg of AITC, the sizes were 775.4 ± 82.6 and 654.6 ± 140.4 nm, respectively. The β-carotene suspensions with or without AITC showed two peaks around 100 nm and 700 nm and the number of emulsion size around 700 nm was very small; the majority of emulsion size was around 100 nm. According to previous researches, the emulsion of (all-E)-β-carotene showed one peak around
700 nm, and the emulsion size of Z-isomers was around 100 nm [9,10]. The most of the micelles in suspensions were rich in Z-isomers. In emulsification-evaporation technology, the purpose component was kept in dissolved state in organic solvent, and when ultrasound treatment started, the solution was suspended into aqueous phase and further stabilized in it [10]. Most Z-isomers was dissolved in SC-CO₂, and they were suspended into water phase by Tween 20 in ultrasound treatment. The majority of crystal (all-E)-isomers stayed in undissolved and crystal state, but it was also suspended slightly in the interphase boundary under ultrasound treatment. This may lead to larger emulsion size based on the crystalized all-E-isomers. As the existence of 100 mg AITC, when Z-isomers dissolved in SC-CO₂ phase as it was consumed and brought into water phase, AITC could help with Z-isomerization process in SC-CO₂ and supply larger amount of Z-isomers than those without AITC. To be concluded, the smallest average emulsion size was obtained when 100 mg AITC was utilized and the ratio of emulsion size around 100 nm showed nearly 32% in numbers in suspensions.

![Figure 6](image)

**Figure 6.** Emulsion size distributions of the β-carotene suspensions with and without AITC. The suspensions were obtained by the 180-min ultrasound treatment at 50 °C and 20 MPa.

3.3.3 Chaterization of β-carotene isomers by HPLC

The peaks of β-carotene isomers were determined as described previously [10,24–26]. The HPLC results of crystalline β-carotene (raw material) and suspensions with and without AITC are shown in Figure 4. A strong one peak was observed in β-carotene crystal and from the retention time and the absorbance maxima, the peak was determined in (all-E)-β-carotene (Figure 4a). As for β-carotene suspensions without AITC, in addition to a strong peak of (all-E)-β-carotene, some small peaks resulting from β-carotene Z-isomers, such as (13Z)-β-carotene, were detected. It indicated that under thermal treatment in SC-CO₂ in the vessel, (all-E)-β-carotene isomerized to the Z-isomers. Several studies using organic solvents also reported that thermal treatment induced Z-isomerization of (all-E)-β-carotene and mainly the 13Z-isomer was occurred [5,16]. When used AITC, many kinds of peaks resulting from β-carotene Z-isomers were observed as well as the peak of (all-E)-β-carotene. In particular, the 9Z-isomer was mainly obtained by using AITC. Several studies demonstrated that (9Z)-β-carotene showed higher antiatherogenesis activity and antiatherosclerosis activity than the all-E-isomers [14,15]. Thus, the use of AITC in the distributed processing of β-carotene would improve not only the encapsulation efficiency but also the functionalities of the resulting suspensions.

4. Conclusions

The nanosuspensions rich in Z-isomers of β-carotene has been successfully prepared in SC-CO₂ at 50 °C, 20 MPa, with 45 KHz ultrasound treatment for 3 hours by using AITC as a catalyst for Z-isomerization. The isomerization ratio of 100 mg AITC was 13 times of the raw (all-E)-β-carotene. Adding AITC in the emulsification-evaporation process using SC-CO₂ as organic solvent improved not only the Z-isomerization ratio of β-carotene but the encapsulated β-carotene content in suspensions. Moreover, the emulsion size in suspension was decreased and the average
micelle size was reduced by using AITC. It is the first experiment of managing $Z$-isomerization using AITC as a catalyst and encapsulation of $\beta$-carotene by Tween 20 simultaneously. This experiment can contribute to the utilization of $\beta$-carotene process without including toxic organic solvents in the whole process.

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