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One-Step Preparation of *Z*-Isomer-Rich β-Carotene Nanosuspensions Utilizing a Natural Catalyst, Allyl Isothiocyanate, via Supercritical CO_2

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Abstract: This study aims to improve the production efficiency of β-carotene suspensions using a naturally occurring *Z*-isomerization-accelerating catalyst, allyl isothiocyanate (AITC), via supercritical CO_2 (SC-CO_2). Namely, utilizing solubility improvement of β-carotene with the *Z*-isomerization by adding AITC in the SC-CO_2-used dispersion process, the encapsulation efficiency of β-carotene was enhanced. The dispersion of β-carotene was conducted by ultrasonic treatment, and there was no involvement of organic solvents in the whole process. When 100 mg of AITC was added in the dispersion process, the encapsulation efficiency (β-carotene content in resulting suspension) was approximately 3.5 times higher than that without addition of the catalyst. Moreover, the *Z*-isomer ratio of β-carotene in the suspensions significantly improved, that is, it was approximately 12 times higher than the raw β-carotene material. Since *Z*-isomers of β-carotene are known to have higher antiatherosclerotic and antiatherogenic activities compared to the all-*E*-isomer, this one-step method not only efficiently produces β-carotene suspensions without organic solvents but also enhances the bioactivities of them.

Keywords: *E*/*Z*-isomerization; β-carotene; supercritical CO_2; ultrasonic treatment; encapsulation efficiency; nanosuspensions

1. Introduction

In recent years, synthetic food colorants have not been extensively accepted by consumers. Therefore, the demand for natural pigments such as carotenoids is increasing annually. β-Carotene is a natural fat-soluble carotenoid containing 11 conjugated double bonds (Figure 1) abundantly contained in fruits and vegetables with a deep orange color, such as mangos, carrots, and pumpkins [1,2]. Since β-carotene has multiple health benefits, such as antioxidant and antiatherosclerotic activities as well as provitamin A activity, the carotenoid is used as a safe and high value-added food colorant all over the world [3,4]. Due to high hydrophobicity, high crystallinity, and the poor water solubility of β-carotene, it is problematic to utilize it for food formulations [5,6]. Moreover, the low solubility in water of fat-soluble components such as carotenoids could be prone to reducing the bioavailability [7]. Thus, in many food industrial cases, β-carotene is used after the water-soluble preparation (distributed processing) using emulsifiers. Several studies successfully obtained carotenoid nanosuspensions by emulsification-evaporation technique as follows: (1) dissolution of the target carotenoid in an organic

solvent; (2) distribution processing in water with a dispersant; (3) solvent evaporation under reduced pressure (Figure 2a) [8,9]. However, generally, these distribution methods use toxic organic solvents, e.g., ethyl acetate, chloroform ($CHCl_3$), and hexane, to dissolve carotenoids, and consequently the residual solvent often becomes a major problem in food industry.

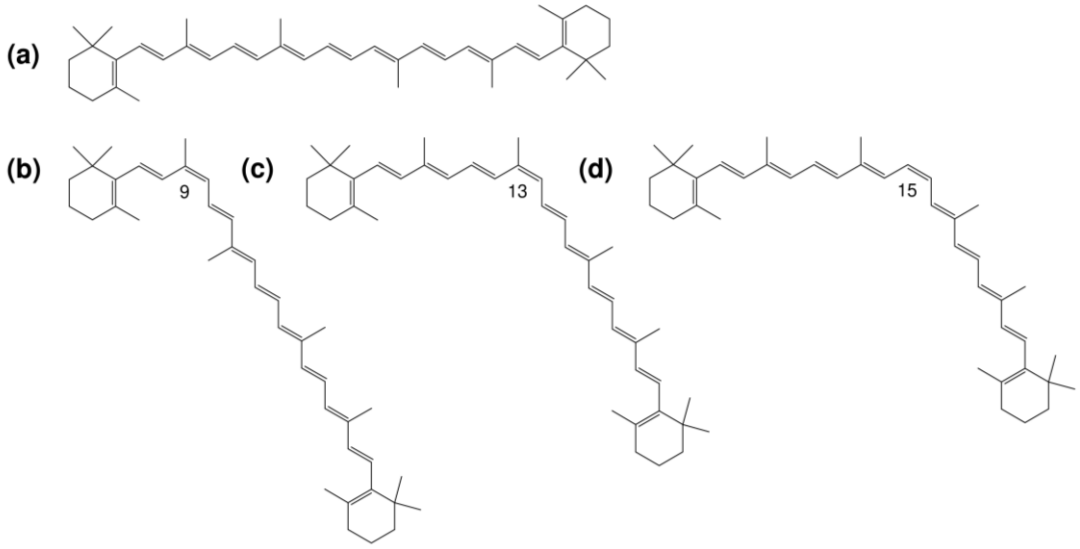

Figure 1. Chemical structures of typical β-carotene isomers: (**a**) (all-*E*)-β-carotene; (**b**) (9*Z*)-β-carotene; (**c**) (13*Z*)-β-carotene; (**d**) (15*Z*)-β-carotene.

Recently, we successfully produced *β*-carotene suspensions using supercritical CO_2 (SC-CO_2), which is non-toxic and easily separated from the products, as an alternative to organic solvents [10]. However, when we produced the β-carotene suspensions using SC-CO_2 as the organic phase, since (all-*E*)-β-carotene (Figure 1a), which is the most predominant geometric isomer in nature, is extremely low solubility in SC-CO_2, the *Z*-isomerization treatment was performed before the distributed processing (Figure 2b). *Z*-Isomers of β-carotene (Figure 1b–d) are more soluble in organic solvents and SC-CO_2 than the all-*E*-isomer [5,6,10–13]. For example, Honda et al. reported that the solubility of *Z*-isomers of β-carotene in ethanol is approximately 250 times higher than that of the all-*E*-isomer [7]. In addition, Gamlieli-Bonshtein et al. showed that the solubility of (9*Z*)-β-carotene in SC-CO_2 was approximately four times higher than that of the all-*E*-isomer [11]. Thus, it is very effective to use *Z*-isomers of β-carotene to increase the efficiency of β-carotene dispersion. Moreover, since *Z*-isomers of β-carotene exhibit greater antiatherosclerotic and antiatherogenic activities than the all-*E*-isomer, enrichment of β-carotene *Z*-isomers in the suspensions can be expected to enhance the health benefits of them [14,15]. Normally, *Z*-isomers of β-carotene are obtained by thermal treatment in toxic organic solvent such as dichloromethane (CH_2Cl_2) and $CHCl_3$ [5,10,16]. Although (all-*E*)-carotenoids can be thermally isomerized to the *Z*-isomers in SC-CO_2, 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 could enhance *Z*-isomerization of (all-*E*)-carotenoids [16,18,19]. Hence, we considered that, when preparing β-carotene suspensions via emulsification-evaporation technique using SC-CO_2, by adding the *Z*-isomerization-accelerating catalyst to the process, the solubility of β-carotene in SC-CO_2 could be enhanced with the *Z*-isomerization reaction, and the production efficiency of β-carotene suspensions would be improved (Figure 2c). This study aims to improve the performance of the production of β-carotene nanosuspensions using plant-derived *Z*-isomerization-accelerating catalyst via SC-CO_2 as an organic phase. In this work, allyl isothiocyanate (AITC) was utilized as the catalyst, which is found in abundance in mustard seed and is commercially available in a highly pure and relatively inexpensive form. Additionally, AITC is less toxic (note that AITC has irritation smell and skin

irritation) and has several health benefits for humans, such as anticancer and anti-inflammatory activities [20,21]. Furthermore, since AITC has a relatively low boiling point [22], it could be easily removed by, for example, using a vacuum oven.

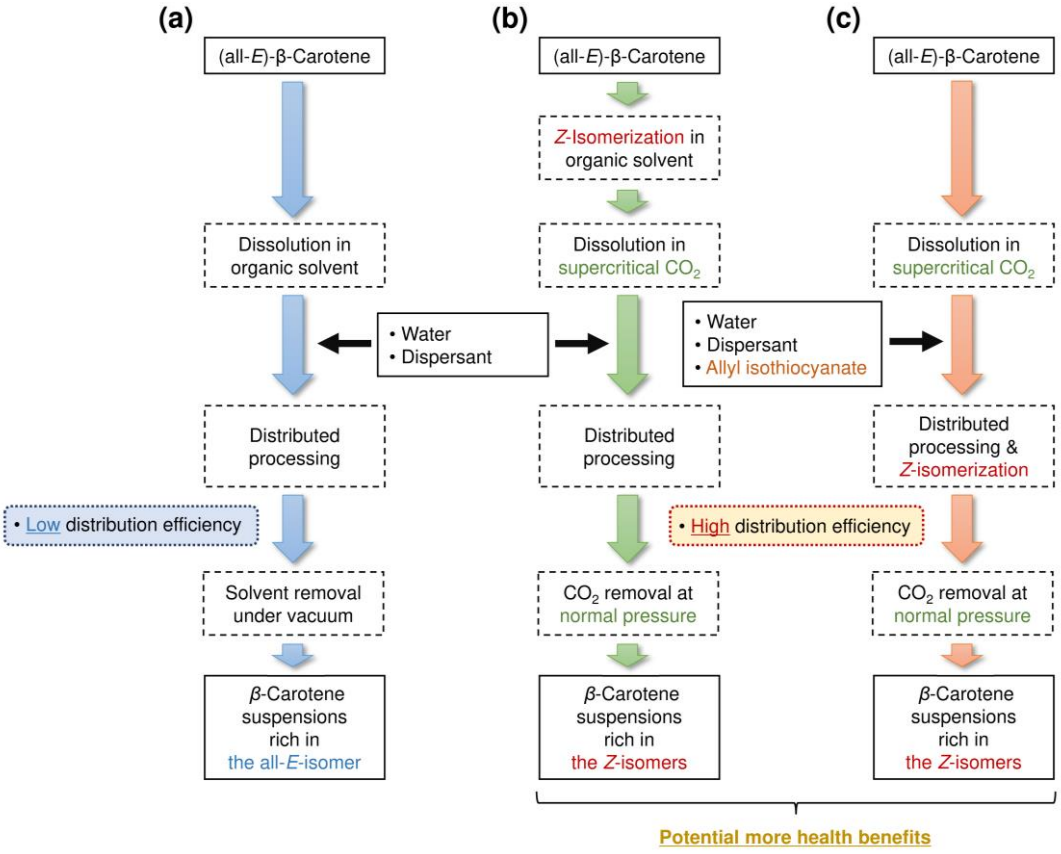

Figure 2. Increase in the efficiency of distributed processing utilizing *Z*-isomerization-induced alteration in solubility: (**a**) general distribution method for carotenoids (emulsification-evaporation technique using organic solvents) [8,9]; (**b**) emulsification-evaporation technique using SC-CO_2 as the organic phase containing a process of *Z*-isomerization treatment before dispersion process [10]; (**c**) emulsification-evaporation technique using SC-CO_2 as the organic phase and conducted *Z*-isomerization in dispersion process by adding allyl isothiocyanate (this study's new method).

2. Materials and Methods

2.1. Reagents

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). Polyoxyethylene sorbitan monolaurate (Tween 20) and AITC were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Carbon dioxide was supplied by Tomoe Shokai Co., Ltd. (Tokyo). Distilled water was utilized in all experiments.

2.2. Isomerization and Distributed Processing of β-Carotene

The distributed processing of β-carotene was carried out according to the method described by Tan and Nakajima [9] and Ono et al. [10]. A schematic diagram of the distribution process 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 high-pressure vessel (SUS-316 stainless steel; GL Sciences Inc., Tokyo, Japan) equipped with two 2 μm filters, an ultrasonic generator

(W-118, Honda Electronics Co., Ltd., Toyohashi, Aichi, Japan), and a back-pressure regulator (BPR; Akico Co., Ltd., Tokyo, Japan). Fifteen mg of crystalline β-carotene and 13.5 mL of distilled water containing 0.5 wt% of Tween 20 (emulsifier) were added into the vessel. CO_2 was bubbled through water solution to get rid of dissolved oxygen in the water solution, and then AITC (50 or 100 mg) was added into the vessel. Then, the vessel containing the solution was connected into the system. Liquid CO_2 in the cylinder was cooled by the chiller, and it was pumped into the vessel via a high-pressure pump. Successively, the upper space in the vessel was filled with liquified CO_2. The pressure of the system was kept at 20 MPa by BPR, and the vessel was preheated for 30 min at 50 °C in a water bath to transform liquid CO_2 into the supercritical state. The volume proportion of SC-CO_2/aqueous phase (distilled water) was adjusted to 1:9 [9,23]. The β-carotene suspensions were produced by ultrasonic treatment at 45 kHz for 180 min. After the dispersion treatment, the pressure of the system was slowly reduced to atmospheric pressure via BPR, and the suspensions were brought out together with CO_2 through a 2 μm filter and were collected in recovery vial. The non-suspended crystalline β-carotene was removed by the 2 μm filter.

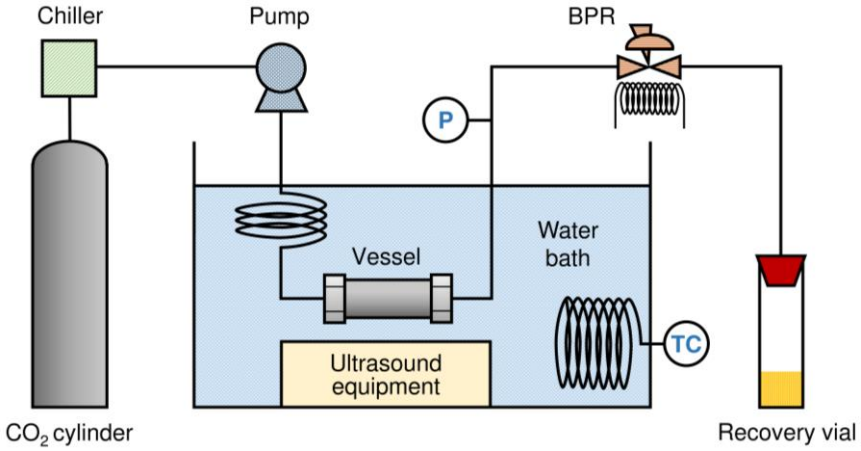

Figure 3. Schematic diagram of the β-carotene dispersion process.

2.3. Evaluation and Characterization of β-Carotene Suspensions

2.3.1. *Z*-Isomer Ratio of β-Carotene in Suspensions

The total *Z*-isomer ratio of β-carotene in resulting suspensions was determined by reversed-phase HPLC with a triacontyl-bonded silica (C_{30}) column (250 × 4.6 mm i.d., 5 mm, YMC Co., Ltd., Kyoto, Japan) [10,24,25]. The extraction of β-carotene isomers from the suspensions was performed using the mixture of methanol and hexane (1:1, v/v) as described previously [10]. The mixture of methanol and MTBE (89:11, v/v) were used as the mobile. The flow rate and the column temperature were set at 1 mL/min and 40 °C, respectively. The detection and the quantification of β-carotene isomers were carried out by peak area integration at 453 nm by a UV-vis detector (UV-2075 Plus, Jasco Co., Tokyo). The peaks of β-carotene isomers such as (all-*E*)-, (9*Z*)-, and (13*Z*)-isomers were identified by the HPLC retention times, the visible spectral data (absorption maxima and shape of spectrum), and the relative intensities of the *Z*-peak at around 340 nm to the main absorption peak of the isomer (% D_B/D_{II}) as described previously [10,16,24–26]. The total *Z*-isomers ratio (%) of β-carotene was the ratio of peak areas of *Z*-isomers of β-carotene to peak areas of all β-carotene isomer.

2.3.2. 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) as previously described [8,10,27]. The amount of dispersed β-carotene in the solution is proportional to the absorbance. Meanwhile, particles of crystalline β-carotene contribute little to the absorbance. In this work, pure

(all-*E*)-β-carotene dissolved in ethanol with five different concentrations was used for the calibration curve [10].

2.3.3. Absorption Spectra of β-Carotene Suspensions

To evaluate the color of resulting β-carotene suspensions, the absorption spectra of the suspensions as well as β-carotene crystalline, AITC, and Tween 20 water solutions were measured using a UV-vis spectrophotometer (V-550, Jasco Co., Tokyo) ranging from 300 nm to 800 nm. All suspensions and reagents were dissolved in distilled water. The absorption spectra of the suspensions were obtained from the colors of resulting suspensions and crystalline β-carotene, but the latter contributes little to the absorption [10].

2.3.4. Particle Size Analysis

The average diameters of resulting β-carotene suspensions were measured by dynamic light scattering ranging from 0.3 nm to 10 μm (Zetasizer Nano ZS, Malvern Instruments, Ltd., Worcestershire, UK). The refractive index of β-carotene in water was set to 1.47 [10], and every sample was measured 3 times.

2.4. Statistical Analysis

All experiments were carried out in triplicates, and the results were expressed in the mean ± standard deviation. The differences in mean values were analyzed by Tukey's multiple comparison test. Significant differences were calculated at $p < 0.05$.

3. Results and Discussion

3.1. Effects of AITC on Z-Isomer Ratio and β-Carotene Content in Nanosuspensions

Z-Isomers of β-carotene are more soluble in solvents, including SC-CO_2, than the all-*E*-isomer [5,11]. Recently, by utilizing the solubility improvement, we successfully improved the production efficiency of β-carotene suspensions with emulsification-evaporation technique [10]. However, our previous study used a toxic solvent (CH_2Cl_2) to isomerize (all-*E*)-β-carotene to the *Z*-isomers, and the isomerization reaction was carried out in a separate step with the dispersion process. In this study, we attempted to carry out isomerization and dispersion in the same process using a naturally occurring *Z*-isomerization-accelerating catalyst, AITC, that is, there was no involvement of organic solvents in the whole process.

Typical chromatograms of β-carotene isomers in resulting suspensions as well as raw β-carotene material (crystalline β-carotene) are shown in Figure 4. In the intact crystalline β-carotene, more than 90% of β-carotene was detected as the all-*E*-isomer, whereas in the suspensions, the peak number and area from β-carotene *Z*-isomers markedly increased. Ample studies showed that (all-*E*)-carotenoids thermally isomerized to the *Z*-isomers in solvents, including SC-CO_2 [16,17]. Thus, thermal *Z*-isomerization reaction of (all-*E*)-β-carotene would proceed in the dispersion process. When we added AITC in the dispersion process, the thermal *Z*-isomerization of β-carotene was markedly promoted. Interestingly, (9*Z*)-β-carotene was not detected in the suspensions without AITC, whereas when we added AITC, a large amount of the 9*Z*-isomer was produced (Figure 4 and Table 1). It is considered that AITC promotes thermal isomerization by formation of radical-ion intermediates, and this reaction pattern would enhance 9*Z*-isomerization of β-carotene [16,28,29]. Several studies showed that (9*Z*)-β-carotene exhibited greater antiatherosclerotic and antiatherogenic activities than the all-*E*-isomer [14,15]. Hence, the use of AITC may improve the pharmacological activity of resulting β-carotene suspensions.

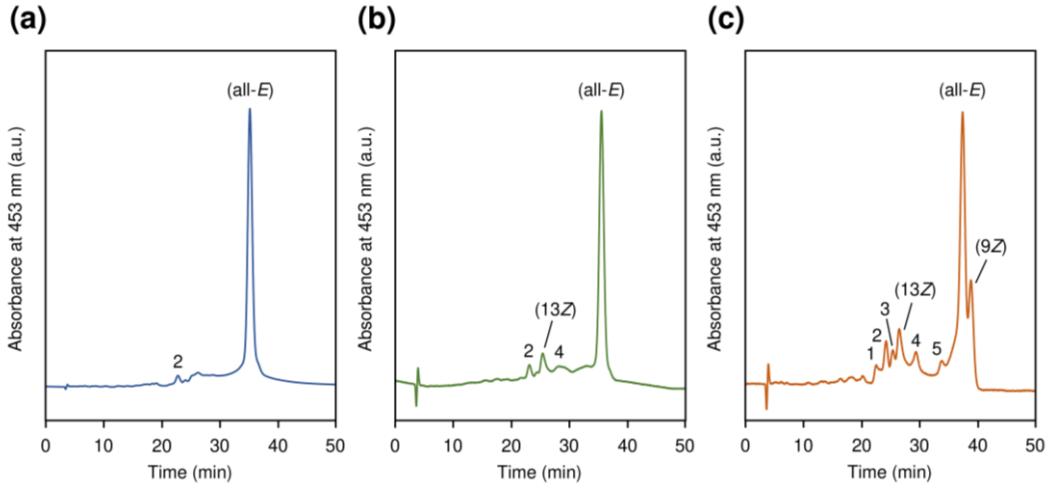

Figure 4. Reversed-phase HPLC chromatograms of (**a**) intact crystalline β-carotene and β-carotene isomers in the resulting suspensions (**b**) without allyl isothiocyanate (AITC) and (**c**) with 100 mg of AITC. The suspensions were obtained by the 180 min ultrasonic treatment at 50 °C and 20 MPa. (all-*E*)-, (9*Z*)-, and (13*Z*) designated in the chromatograms were identified according to previous studies [10,16,24–26]. Some of the peaks (1–5) were tentatively identified as shown in Table 1.

Table 1. Absorption maxima (λ_{max}) and relative intensities of *Z*-peak (%D_B/D_{II}) of geometrical β-carotene isomers separated and observed using reversed-phase high-performance liquid chromatography.

Peak No.	β-Carotene Isomer [a]	λ_{max} (nm)		% D_B/D_{II}	
		In-Line	Reported [a]	In-Line	Reported [a]
1	UZ	336,458,483,582	–	56.7	–
2	UZ	342,434,452,484	–	55.6	–
3	UZ	329,393,442,486	–	64.1	–
	(13*Z*)	336,425,442,472	339,420,445,470	44.2	37.1
4	UZ	332,412,442,484	–	70.1	–
5	UZ	338,442,462,473	–	61.7	–
	(all-*E*)	423,450,473	426,452,478	ND	ND
	(9*Z*)	338,420,446,470	340,422,447,473	6.6	9.4

Values and peak designations (1–5) were obtained from the chromatograms in Figure 4. –, not assigned. UZ, unidentified *Z*-isomer of β-carotene. ND, not detected substantially. [a] Tentatively assigned by the literatures [10,16,24–26].

The total *Z*-isomer ratio and the β-carotene content in resulting suspensions are summarized in Table 2. The addition of 50 mg or more of AITC to 15 mL of SC-CO_2/distilled water (9:1, v/v) successfully improved the *Z*-isomer ratio of β-carotene. In addition, the β-carotene content, i.e., encapsulated β-carotene, was markedly improved by adding AITC in the dispersion process. For example, when we added 100 mg of AITC in the process, the encapsulated β-carotene was approximately 3.5 times higher than that where no AITC was added. This improvement should be due to the increase of β-carotene solubility in SC-CO_2 with its *Z*-isomerization [10,16,17]. This study is the first example of performing isomerization and dispersion in one step without using any organic solvents. The resulting suspensions had the smell of AITC. However, since AITC is highly volatile, it could be easily removed by decompression treatment [22]. On the other hand, since AITC has several useful bioactivities, such as anticancer and anti-inflammatory activities [20,21], a synergistic health effect would be expected when it is taken at the same time as β-carotene.

Table 2. Total *Z*-isomers ratio (%) and β-carotene content (mg/L) in suspensions.

Amount of AITC Added (mg)	*Z*-Isomers Ratio (%)	β-Carotene Content (mg/L)
0	15.1 ± 2.0 [a]	29.6 ± 19.1 [a]
50	31.8 ± 1.5 [b]	66.4 ± 8.1 [b]
100	37.4 ± 1.7 [c]	102.5 ± 21.7 [b]

[abc] Mean values within a column with no common superscript differ significantly ($p < 0.05$, $n = 3$).

3.2. Characterization of β-Carotene Suspensions

3.2.1. Color

The color of resulting β-carotene suspensions with or without AITC was evaluated by the appearances and the absorption spectra (Figure 5). The suspensions were obtained by the 180 min ultrasonic treatment at 50 °C and 20 MPa. In the case of adding no AITC, the appearance of β-carotene suspensions was a light reddish yellow. On the other hand, when we added 100 mg of AITC in the dispersion process, that of the resulting suspensions was a deep yellow. Silva et al. reported that β-carotene suspensions obtained by organic solvent-used emulsification-evaporation technique showed a similar color appearance [23]. Thus, by utilizing a *Z*-isomerization-accelerating catalyst, AITC, SC-CO_2 can be used as an alternative to organic solvents in this technique. The resulting absorption spectrum patterns also showed that the encapsulation of β-carotene was successfully carried out by using AITC. Namely, water dispersions of β-carotene, AITC, and Tween 20 did not have absorption spectrum in the range of 350–700 nm, and β-carotene suspensions without adding AITC showed a slight absorption in the range of 400–550 nm. However, β-carotene suspensions with adding 100 mg of AITC showed strong absorption in the range of 400–550 nm. The absorption spectrum around 400–550 nm is a characteristic one of β-carotene, and its observation indicated that β-carotene is well-dispersed in water and exhibits coloration [10,27,30].

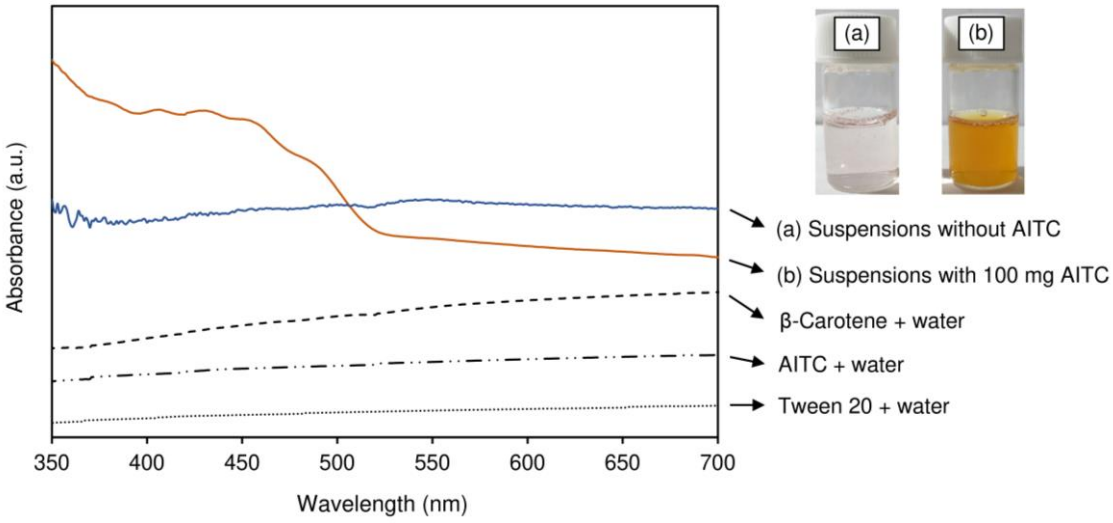

Figure 5. Appearances and absorption spectra of β-carotene suspensions with and without 100 mg of AITC as well as water dispersions of β-carotene, AITC, and Tween 20.

3.2.2. Size Distribution

The particle size distributions of β-carotene suspensions obtained by ultrasonic treatment for 180 min at 50 °C and 20 MPa with or without AITC are shown in Figure 6. Two peaks at around 100 and 700 nm were observed in both suspensions. This dispersion pattern was also observed in our previous study that prepared β-carotene suspensions by SC-CO_2-used emulsification-evaporation technique [10]. A peak at around 700 nm may have been of crystalline β-carotene that did not dissolve in SC-CO_2,

whereas the suspensions that were successfully dispersed by this method may have had a peak around 100 nm. In fact, Tan and Nakajima reported that, when they prepared β-carotene suspensions by organic solvent-used emulsification-evaporation technique, the particle size of the resulting suspensions was at around 100 nm [8]. The mean particle diameters of β-carotene suspensions prepared without AITC and with 50 and 100 mg of AITC were 212.1 ± 32.8, 186.1 ± 84.4, and 101.0 ± 44.4 nm, respectively. By adding AITC in the process, the particle diameters of β-carotene suspensions significantly reduced. This would have been because the solubility of β-carotene in SC-CO_2 increased by the *Z*-isomerization; thus, the dispersion process was successfully carried out. In addition, since *Z*-isomers of β-carotene have lower crystallinity than that of the all-*E*-isomer, the increase in particle size due to crystal growth could be suppressed [5,6]. Several studies indicated that nano-sized carotenoid suspensions at around 100 nm enhanced their bioavailability [31,32]. Therefore, the use of AITC in this method may contribute to improve the β-carotene bioavailability.

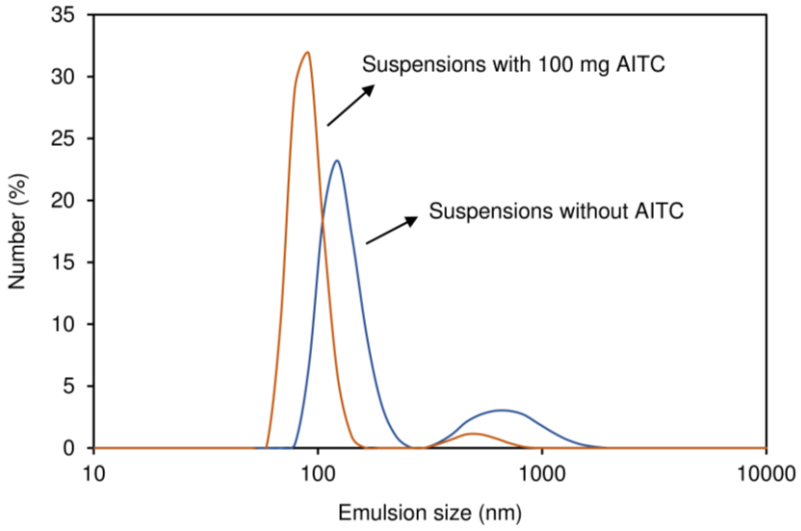

Figure 6. Particle size distributions of β-carotene suspensions with and without adding AITC in the dispersion process.

4. Conclusions

β-Carotene nanosuspensions were successfully prepared by SC-CO_2-used emulsification-evaporation technique with a natural *Z*-isomerization-accelerating catalyst, AITC. For example, when 100 mg of AITC was added in the dispersion process, the β-carotene content in the water phase was approximately 3.5 times higher than that without addition of the catalyst, and approximately 100 nm of β-carotene nanosuspensions was obtained. This would have been because the solubility of β-carotene in SC-CO_2 increased by the *Z*-isomerization; thus, the dispersion process was efficiently carried out. In fact, the total *Z*-isomer ratio in the resulting suspensions markedly increased. Interestingly, by using AITC, (9*Z*)-β-carotene content, which exhibits greater antiatherosclerotic and antiatherogenic activities than the all-*E*-isomer, was mainly increased. Thus, this one-step dispersion method is very useful not only for productionβ-carotene suspensions without organic solvents but also for enhancement of β-carotene bioactivity.

Author Contributions: Conceptualization, Y.Z., M.H., and M.G.; formal analysis, Y.Z.; investigation, Y.Z., M.H., and W.; writing—original draft preparation, Y.Z., M.H., and W.; writing—review and editing, H.K., T.F., and M.G.; project administration, M.G.; funding acquisition, M.H. and M.G. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AITC, allyl isothiocyanate; BPR, back-pressure regulator; $CHCl_3$, chloroform; CH_2Cl_2, dichloromethane; HPLC, high-performance liquid chromatography; λ_{max}, absorption maximum; MTBE, methyl *tert*-butyl ether; Tween 20, poly oxyethylene sorbitan monolaurate; $\%D_B/D_{II}$, relative intensities of *Z*-peak; SC-CO_2, supercritical CO_2.

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