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Supercritical CO_2 fluid extraction of crystal water from trehalose dihydrate. Efficient production of form II (T_α) phase

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Abstract

Form II is a kind of metastable crystalline form of trehalose anhydrate, and it is easily converted to the dihydrate crystal by absorbing water in moist atmosphere at room temperature (Akao et al., *Carbohydr. Res.* **2001**, 334, 233–241). It can be utilized as an edible and nontoxic desiccant, and thus its efficient production from the dihydrate is significant from a viewpoint of industrial applications. In this study, we attempt to extract crystal water from the dihydrate using supercritical CO_2. We examine the dependence of extraction efficiency on the extraction time, the temperature and pressure of the fluid. Then, FTIR measurements are carried out to detect the extracted water and to identify the polymorphic phase of the sugar sample after the extraction treatment. In particular, the so-called first derivative euclidean distance analysis for IR spectra is shown to be quite useful for the structural identification. Consequently, we demonstrate that form II is produced from the dihydrate through supercritical CO_2 fluid extraction if appropriate temperature and pressure conditions (around 80 °C and 20 MPa) are maintained.

Keywords: Trehalose; Supercritical fluid CO_2 extraction; Dehydration; Vitrification; FTIR

1. Introduction

Many seeds, some plants, yeast cells and fungal spores are capable of surviving almost complete dehydration.[1] These organisms synthesize large quantities of trehalose (α-D-glucopyranosyl α-D-glucopyranoside) in intracellular media in response to desiccation stress. Trehalose appears to act as a substitute for water and thus maintains the life of such organisms. This sugar has no toxicity and is currently being used in several industries, such as food, medical, cosmetic and other bio-industries, to preserve biomaterials under dry conditions. Recently, it has been reported that trehalose can greatly improve the survival of manmalian cells during cryopreservation and desiccation.[2,3]

Over the past few decades, much attention has been given to the elucidation of the molecular mechanism of desiccation tolerance induced by trehalose. At least two hypotheses have been proposed. One is the water-replacement hypothesis[4,5] and the other is the vitrification hypothesis.[6] However, these hypotheses alone may not explain the better properties of trehalose as a biostabilizer because other saccharides interact with membranes and proteins,[7–9] and they are vitrified under desiccation conditions.[10,11] It has been reported that some forms of anhydrous trehalose can readily take up water to form the dihydrate, resulting in stabilization of the vitrification state of trehalose.[12,13] Most recently, we have demonstrated that anhydrous form II, first identified by a Raman and IR study of trehalose,[14] is converted to the original dihydrate form under environmental-like conditions (humidity of 40% and room temperature).[15] Form II is produced by vacuum heating of the dihydrate at moderate temperatures (lower than 85 °C)[14] or more easily by gentle heating of its powder in the air.[15]

In the previous report,[15] we described the possibility that form II is also produced in the drying (or lyophilizing) process of biomaterials including trehalose as a stabilizer (vitrification agent). If so, it acts as a water

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absorber to produce the dihydrate. As a consequence, the water absorption into the glassy part of trehalose, leading to devitrification of the dried material, would be avoided, which retards the lowering of storage lifetime.[15] X-ray and thermodynamic studies[16–18] have also found the anhydrous crystalline form, called T_α, which is produced by gentle dehydration and possesses the hygroscopic property similar to form II, namely reversibly absorbs water to produce the dihydrate. It is supposed at present that T_α is identical with form II.[18] The special ability of trehalose as a biostabilizer may be attributed to the occurrence of such a unique polymorphic form. Form II is an edible and nontoxic desiccant. It is thus of great importance to develop a method available to efficiently produce form II in the light of industrial applications of trehalose. The dihydrate is appropriate as a starting material because it is already mass-produced.

The phase-transition behavior of trehalose dihydrate depends on several parameters such as heating rate, particle size, dehydration methods (vacuum heating or hot air heating) and so on.[16–20] Thus, a highly regulated dehydration method is required to efficiently produce form II. Currently, supercritical fluid CO_2 is utilized to extract objectionable compounds from biomaterials in the food industry, for example, caffeine from coffee,[21] tocopherol from wheat[22] and so on. In the supercritical fluid CO_2 extraction (SFE) process, the extraction conditions such as temperature, pressure, and entrainment are easily controllable. The SFE method is expected to be available for extraction of crystal water from trehalose dihydrate.

Herein we report that the SFE method is a powerful one to extract crytalline water from the dihydrate. The primary purpose of this study is to obtain basic information on how the production of form II is affected by the extraction conditions, such as temperature and pressure, and not to search the optimal conditions for mass-production of form II. In addition, we show that FTIR spectroscopy combined with a simple data analysis method is very useful for rapid identification of products obtained from the SFE process.

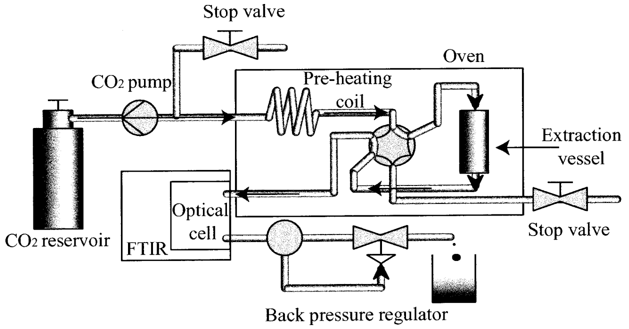

Fig. 1. A block diagram of the SFE–FTIR instrument

2. Experimental

Trehalose dihydrate and maltose monohydrate were purchased from Wako Chemical Co., Tokyo. Each sugar sample was ground using a mortar and a pestle until the particle size became less than 50 μm, and then 50 mg of the resulting powder was placed into an extraction vessel made of stainless steel with an inside diameter of 4.6 mm, an outward diameter of 6.3 mm and a length of 50 mm. Fig. 1 shows the block diagram of a supercritical extraction system attached to a FTIR spectrometer (SFE–FTIR). Supercritical CO_2 fluid was allowed to flow through the extraction vessel containing the sugar sample by using a pump (JASCO SCF-Get). The temperature inside the extraction vessel was controlled by using a column oven (JASCO CO-2065). The pressure inside the extraction vessel was controlled by a backpressure regulator (JASCO SCF-Bpg). Extracted materials are transferred to the cell of an FTIR spectrometer (JASCO FT/IR-680 spectrometer). The window of the cell was made of zinc sulfate, and the temperature inside the cell was controlled by using a water circulator. Time-resolved IR spectra were obtained using an interval measurement program (released from JASCO Co., Tokyo).

In this study, we carried out the following four kinds of experiments. First, to examine the properties of water dissolved in supercritical CO_2 fluid, we measured the FTIR spectra of a supercritical CO_2–water mixture, which was prepared in advance by mixing CO_2 and a trace amount of water (2–3 μL) under the temperature and pressure conditions appropriate for keeping CO_2 in the supercritical state. Second, we measured the FTIR spectra of water extracted from the sugar sample contained in the extraction vessel. For this purpose, supercritical CO_2 was allowed to flow at a rate of 2 mL $\min^{-1}$ through the extraction vessel. In this experiment, the sample temperature was changed from 60 to 90 °C in a stepwise manner (see dashed line in Fig. 3). In the third experiment, during the extraction process (120 min) the temperature of the sample was kept at 60, 70, 80 or 90 °C and the pressure was kept at 20 MPa. After the end of the extraction, the samples present in the vessel were picked up, and their IR spectra were measured by the attenuated total refraction (ATR) method using a JASCO FT/IR-680 spectrometer with an ATR attachment (PIKE: MIRacle). Then 32 scans were performed for each measurement at a resolution of 4 cm^{-1}. In the fourth experiment, the effect of pressure was examined under constant-temperature conditions (70 °C). The pressure was controlled to be 10, 12, 14, 16, 18 or 20 MPa. FTIR measurements were continued for 120 min under such conditions. After the finish of the FTIR measurements, the samples present in the vessel were picked up and measured by the ATR method. After measurement of the IR spectra, the

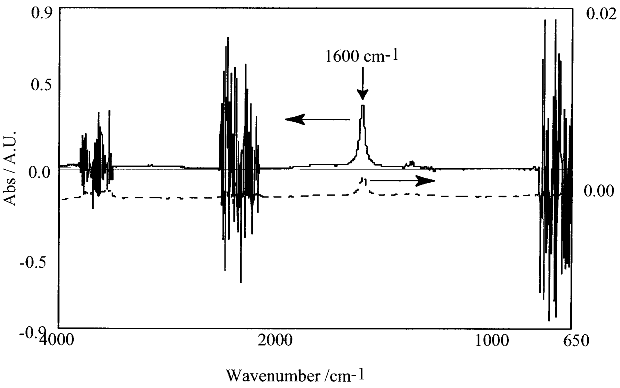

Fig. 2. IR spectrum (solid curve) of a trace amount of H_2O added in the supercritical CO_2 fluid and IR spectrum (broken curve) of materials extracted from trehalose dihydrate.

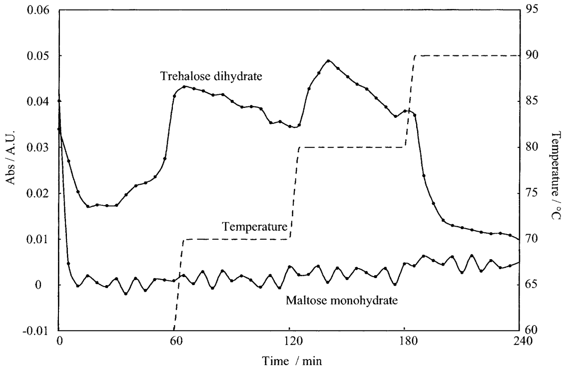

Fig. 3. Time dependence of the IR absorption at 1600 cm^{-1} of water extracted from trehalose dihydrate and maltose monohydrate. Temperature of the extraction vessel was increased form 60 to 90 °C in a stepwise manner (broken line).

samples were stored for 1 week in a shielded box in which temperature and relative humidity were kept constant at 25 °C and 40%, respectively. One week after, the IR spectra of the samples were again measured by the ATR method. Thirty-two scans were performed for all the ATR measurement at a resolution of 4 cm^{-1}.

To identify the polymorphic forms of the sugar samples from their ATR-IR spectra, we carried out the so-called euclidiean distance analysis, where the root-mean-square deviation is calculated between the spectrum of an unknown sample and that of a selected reference sample over all the sampling data points. The root-mean-square deviation can be also obtained for all the first-derivative values defined between neighboring data points (see Eq. (1)), a method called the first-derivative euclidiean (FDE) distance analysis. It is superior to direct analysis for the original spectra because one can escape from the effect of baseline drifts, and therefore it was adopted here. The FDE distance I is defined as follows:

$$I = \sqrt{\sum_{i=1}^{N-1} \left\{ \frac{U_{i+1} - U_i}{|U|} - \frac{R_{i+1} - R_i}{|R|} \right\}^2} \tag{1}$$

$$|U| = \sqrt{\sum_{i=1}^{N-1} (U_{i+1} - U_i)^2} \tag{2}$$

$$|R| = \sqrt{\sum_{i=1}^{N-1} (R_{i+1} - R_i)^2} \tag{3}$$

where N, U_i and R_i are the number of data points, the i-th data value of unknown sample and the i-th data value of reference sample, respectively. As will be described in the next section, the reference data corresponds to the IR spectrum of each polymorphic form obtained by usual transmittance IR measurements. The FDE distance was evaluated using the Spectral Search program (released from JASCO Co., Tokyo).

To examine the confidence limit of the FDE distance analysis, we evaluated the I value for two repeated ATR experiments on the dihydrate. Such a measurement was performed five times for different samples, and the resultant values were within ± 0.5.

3. Results

Fig. 2 shows an IR spectrum of water dissolved in supercritical CO_2 at 20 MPa. There is only one peak at 1600 cm^{-1}. Noisy peaks around 3800, 2200 and 600 cm^{-1} are due to the absorption of CO_2. As is well known, water has three normal vibration modes: two of them are assigned to symmetric and antisymmetric stretching vibrations and the other to a bending vibration of the HOH angle. In the gas phase, the former two bands appear at 3652 and 3756 cm^{-1}, respectively.[23] In Fig. 2, these bands cannot be observed due to the presence of the strong absorption of CO_2 around 3800 cm^{-1}. The bending band appears at 1595 cm^{-1} in the gas phase,[23] in good agreement with the value (1600 cm^{-1}) observed in Fig. 2. Thus, the physicochemical state of water in the supercritical CO_2 is similar to that in the gas phase. The dashed line of Fig. 2 shows an example of the IR spectra of materials extracted from the trehalose dihydrate sample by supercritical CO_2 fluid. Obviously, a peak assigned to the bending motion of water is observed at 1600 cm^{-1}, which indicates that crystal water was extracted from the dihydrate.

Time dependence of the IR absorption of water extracted from the trehalose dihydrate and maltose monohydrate samples is shown in Fig. 3, where the absorbance values at 1600 cm^{-1} are plotted against extraction time. The temperature of the extracted vessel was increased from 60 to 90 °C in a stepwise manner (see dashed line). From 0 to 15 min, the absorbance decreased quickly in both cases of trehalose dihydrate and maltose monohydrate, which implies that the water adsorbed on these samples was extracted first. From 15 to 185 min, the absorbance for the trehalose sample increased in a stepwise manner with changing temperature. In contrast, the absorbance for the maltose sample exhibited no significant change from 15 to 185 min. Fig. 4 shows the effect of temperature on the supercritical extraction of crystal water from trehalose dihydrate. At 60 °C the absorbance at 1600 cm^{-1} decreased quickly from 0 to 10 min. After then, it exhibited no significant change. At 70 °C, the absorbance at 1600 cm^{-1} also decreased quickly from 0 to 10 min and did slowly from 10 to 40 min. In contrast, at 80 and 90 °C the time dependences of the absorbance exhibit convex curves. The maximum appears at shorter time in the case of the 90 °C experiment than in the case of the 80 °C experiment.

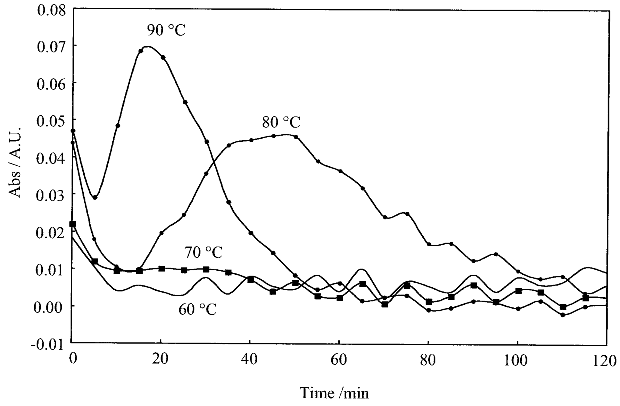

Fig. 4. Effect of temperature on the time course of supercritical fluid extraction of water from the trehalose dihydrate sample.

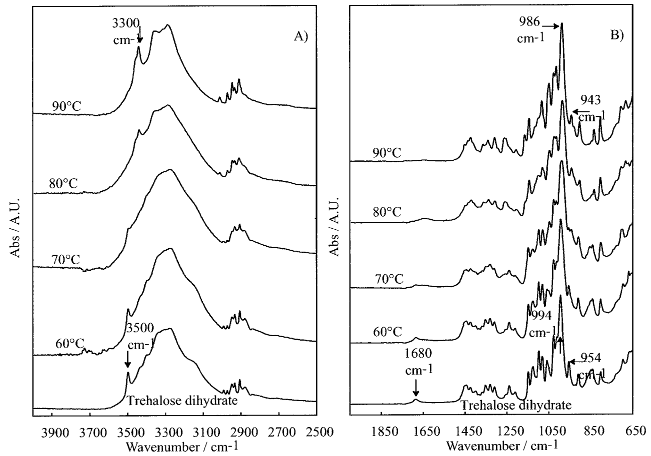

Fig. 5. The IR spectra of the trehalose samples obtained after 2 h extraction at different temperatures at 20 MPa.

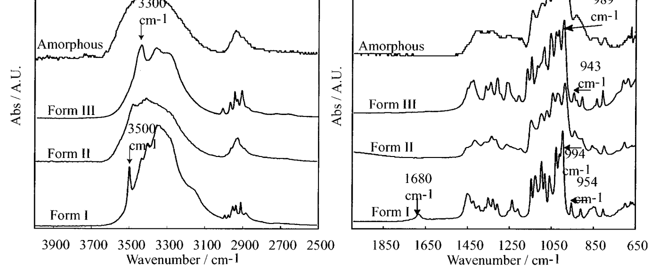

Fig. 6. The IR spectra of different polymorphic forms of trehalose. All the spectra were obtained by usual transmittance method.

Table 1

The results of the FDE distance analysis for the temperature-dependence experiments at a constant pressure (20 MPa)

Temperature (°C)	Reference polymorphic phase			
	Form I	Form II	Form III	Amorphous
60	81	101	102	126
70	98	79	102	114
80	137	81	86	105
90	129	99	77	121
Before extraction	67	115	103	138

Figs. 5A and 5B show the IR spectra of the trehalose samples obtained after supercritical extraction for 2 h at different temperatures at 20 MPa. For comparison, the spectrum of trehalose dihydrate before supercritical extraction is shown at the bottom. As described in the experimental section, all the IR spectra of the sugar samples were obtained by the ATR method, because the sample can be subjected to IR measurements as it is, different from the case of usual transmittance method. The spectra of the sample obtained after the extraction are clearly different from that of the original dihydrate sample. For comparison, the IR spectra of different polymorphic forms of trehalose, including amorphous forms, are depicted in Figs. 6A and 6B, where all the spectra were obtained by the usual transmittance method, and the notations of form I and form III correspond to dihydrate and anhydrate T_β crystals, respectively.[15] However, a visual comparison between Figs. 5 and 6 does not enable us to correctly identify the polymorphic form of each sample, because the relative peak heights in an ATR-IR spectrum often undergo some distortions due to surface effects. Instead, we attempted the FDE distance analysis by using Fig. 6 as reference data.

Table 1 summarizes the results for the FDE distance (parameter I in Eq. (1)) between the spectra in Fig. 5 and those in Fig. 6. As can be seen from Eq. (1), the smaller the FDE distance is, the more similar the two spectra are. For the original dihydrate sample (the bottom row of Table 1), the FDE distance takes the smallest value (67) when form I (dihydrate) is chosen as reference. This is quite reasonable. For the sample obtained after the 60 °C extraction, the smallest I value also occurs for form I, but the values for form II and the amorphous form slightly decrease compared to the corresponding values in the case of the original sample. These results indicate that a major product is identified as form I, but small amounts of form II and the amorphous form may be present. In contrast, a major product after the 70 °C extraction is identified as form II. Form III (the anhydrous T_β crystal) is formed at 90 °C. It is reasonable to judge that the sample after the 80 °C experiment is a mixture of forms II and III.

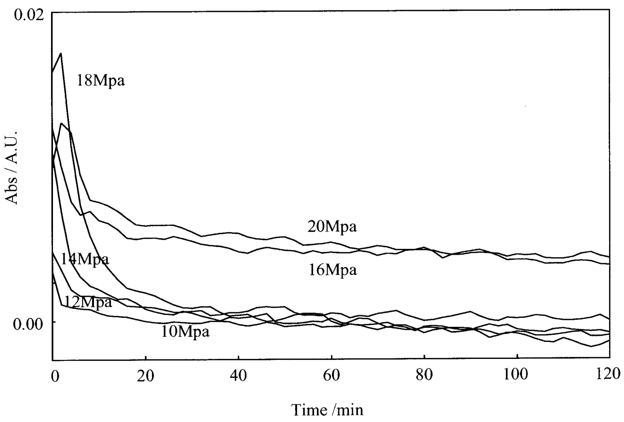

Fig. 7. Effect of pressure on the time course of supercritical fluid extraction of water from the trehalose dihydrate sample.

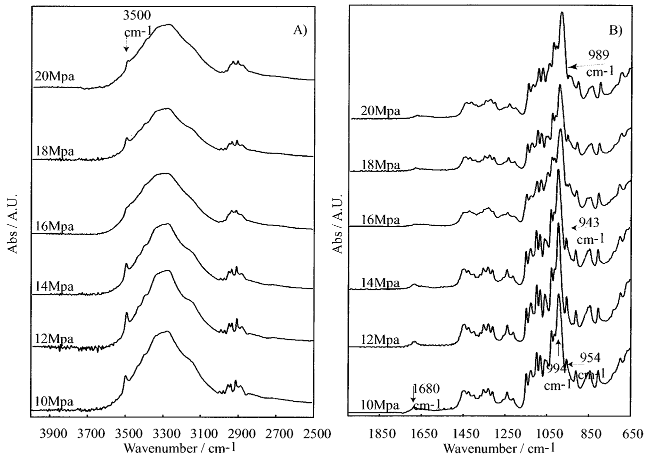

Fig. 8. IR spectra of the trehalose samples obtained after 2 h extraction at different pressures at 70 °C.

Table 2
The results of the FDE distance analysis for the pressure-dependence experiments at a constant temperature (70 °C)

Pressure (MPa)	Reference polymorphic phase			
	Form I	Form II	Form III	Amorphous
10	80	101	103	126
12	69	114	103	138
14	75	104	102	130
16	110	70	106	107
18	78	100	102	125
20	98	79	102	114

Table 3
The results of the FDE distance analysis for the samples obtained after 1 week of a hygroscopic treatment[a]

Pressure (MPa)	Reference polymorphic phase			
	Form I	Form II	Form III	Amorphous
10	73	113	103	137
12	75	113	103	138
14	76	112	103	136
16	73	113	103	138
18	72	114	103	138
20	77	109	102	134

[a] At 25 °C and 40% relative humidity.

Fig. 7 shows the effect of pressure on the supercritical extraction from trehalose dihydrate. The absorbance at 1600 cm^{-1} decreased quickly from 0 to 10 min under all the pressure conditions. After then, the absorbance was nearly zero when the given pressure was 10, 12, 14 or 18 MPa. In contrast, the absorbance of 1600 cm^{-1} at 16 and 20 MPa was kept to be a nonzero value. Figs. 8A and 8B show the IR spectra of the samples obtained after extraction at different pressures for 2 h, where all the extraction experiments were carried out at 70 °C. Table 2 shows the results for the FDE distance analysis between the spectra in Fig. 8 and those in Fig. 6. Obviously, form II was formed as a major product at 16 and 20 MPa. In contrast, the initial form (form I) was kept to be unchanged during the extraction experiments at 10, 12, 14 and 18 MPa.

The FDE distance analysis does not necessarily allow absolute assignment of various forms. Thus, the above results should be supported by other data. In the previous study,[15] we demonstrated that form II absorbs water to easily return to form I. To confirm that the samples judged above to be form II have such a property, we carried out the hygroscopic assay. The post-extraction samples in the experiments of Fig. 7 were stored for 1 week in a shielded box in which temperature and relative humidity were kept constant at 25 °C and 40%, respectively. Table 3 shows the results for the FDE distance analysis for the resultant samples. Obviously, the samples obtained after extraction at 16 and

20 MPa were changed into form I. Therefore, the FDE distance analysis is sufficiently reliable to discriminate the different polymorphic phases of trehalose.

4. Discussion

The above results clearly indicate that the supercritical CO_2 fluid extraction method is available for efficiently extracting the crystal water from trehalose dihydrate. In particular, the fact that form II was produced is of great significance in light of industrial applications as described in the introduction. In addition, it is very interesting from a physicochemical viewpoint that the crystal water of trehalose dihydrate is more easily dehydrated than that of maltose monohydrate (Fig. 3). Due to the ease of dehydration, trehalose crystals may exhibit complicated phase behavior as previously reported by other workers.[14–20]

An advantage of the utilization of the SFE method is that one can easily regulate the extraction conditions such as temperature, pressure, flow rate, heating rate, and so on. In general, the extraction efficiency highly depends on the temperature, diffusion coefficient and density of the supercritical fluid used, and the latter two quantities depend on pressure. Thus, in this study we focused our attention only on the effects of temperature and pressure. In the experiments of Fig. 3, the temperature was increased in a stepwise manner, while in that of Fig. 4 the sample temperature was rapidly increased to the objective values. Obviously, the history of temperature-treatment affects the temperature at which the dehydration begins: in the former and latter cases the dehydration begins 70 and 80 °C, respectively. In any case, a temperature higher than 70 °C would be required for rapid and full extraction of the crystal water. The value of 70 °C is a critical one from a physicochemical viewpoint. In our previous FTIR study,[24] the crystal water in trehalose dihydrate was shown to undergo a phase transition from ice-like state to liquid-like state at 70 °C. The above results indicate that the crystal water cannot be extracted until the mobility of the water becomes high enough to break the ice-like hydrogen bonds. Next, we discuss the effect of pressure. It was expected that the higher the pressure is, the higher the conversion efficiency from form I to form II is. However, as can be seen from Fig. 7 and Table 2, some irregular results were obtained: the conversion from form I to form II was observed at 16 MPa, but not at 18 MPa. We repeated the experiment two times, and such irregularity was reproduced. Thus, the phenomenon is not due to any procedural errors, but due to the intrinsic property of the supercritical CO_2 fluid. The density of the supercritical CO_2 fluid increases in a nonlinear fashion with increasing pressure.[25,26] Similarly, its diffusion coefficient decreases in a nonlinear fashion with increasing pressure.[25,26] In both cases, the non-linearity becomes remarkable in a pressure range of 15–20 MPa. On the other hand, the extraction efficiency is determined by the synergistic effect of the density and diffusion coefficient. Therefore, there is a possibility that the extraction efficiency does not monotonously change with pressure in the above range.

According to Taylor and co-workers,[19,27] the dehydration of the dihydrate using a simple hot stage produces the amorphous phase when small-size crystals (< 45 μm) are used. In contrast, as evident from Tables 1 and 2, the SFE method used here produced form II from small-size crystals that were fully ground (< 50 μm). In addition, the anhydrate crystal (T_β) was also formed from similar, small-size crystals (Table 1), although in the case of hot stage heating, it is formed only from large-size crystals (> 425 μm). These results indicate the advantage of using the SFE method: namely the crystalline structure is kept more stable, and the properties of the products are easily controllable by tuning the thermodynamic parameters (temperature and pressure).

For practical application of the SFE method, the nondestructive and rapid method for checking products would be required. Our results shown in Tables 1–3 indicate that the ATR-IR measurements, combined with the FDE distance analysis, are suitable for this purpose. The supercritical CO_2 fluid extraction is a nontoxic process. Therefore, the resultant form II is expected to be utilized as a nontoxic desiccant in the food industry.

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