Fucoxanthin as a Major Carotenoid in Isochrysis aff. galbana: Characterization of Extraction for Commercial Application

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ORIGINAL ARTICLE

Fucoxanthin as a Major Carotenoid in *Isochrysis* aff. *galbana*: Characterization of Extraction for Commercial Application

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Abstract Fucoxanthin, a main marine carotenoid, in five species of fucoxanthin-containing microalgae, was quantified by high-performance liquid chromatography. Among the studied species, *Isochrysis* aff. *galbana* contained the highest amount of fucoxanthin (18.23 mg/g dried sample). This microalga showed good fucoxanthin extraction efficiency under the tested solvents (methanol, ethanol, acetone, and ethyl acetate), with the exception of *n*-hexane. In addition, most fucoxanthin (~95%) could be extracted by a single extraction in ethanol within 5 min, and only 15% degradation of fucoxanthin was detected during ethanol extraction for 24 h. The two-phase solvent system of *n*-hexane-ethanol-water with a volume ratio of 10:9:1 was determined to be the best system for the separation of fucoxanthin and lipids from extracts of *I.* aff. *galbana*. Under these conditions, fucoxanthin was fractionated in the hydroalcohol phase apart from the hexane phase containing lipids. These results imply that *I.* aff. *galbana* can be a commercial source for the spontaneous production of valuable fucoxanthins and lipids.

Keywords carotenoid · extraction · fucoxanthin · *Isochrysis* aff. *galbana* · lipid · microalgae · two-phase solvent system

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Introduction

Fucoxanthin, a major carotenoid present in the chloroplasts of brown seaweeds, contributes to more than 10% of the estimated total production of carotenoids in nature (Miyashita et al., 2011; Peng et al., 2011). This pigment is abundant not only in macroalgae such as *Eisenia bicyclis*, *Laminaria japonica*, and *Undaria pinnatifida*, but also in microalgae such as *Phaeodactylum tricornutum* and *Odontella aurita* (Peng et al., 2011). Fucoxanthin has a unique structure with an unusual allenic bond and a 5,6-monoepoxide in its molecular structure, and is metabolized into fucoxanthinol, amarouciaxanthin A, and halocynthiaxanthin after absorption into the human body (Sangeetha et al., 2010). A number of studies have examined the metabolism, safety, and bioactivities of fucoxanthin, including its anti-cancer, anti-obesity, antioxidant, anti-inflammatory, anti-diabetic, and anti-angiogenic activities (Miyashita et al., 2011; Peng et al., 2011).

However, although fucoxanthin is clearly a valuable pigment with various biological activities, its use has been limited due to the low extraction efficiency from marine materials and the difficulty of chemical synthesis (Yamano et al., 1995; Kanazawa et al., 2008; Kajikawa et al., 2012). Brown algae are a common source of industrial fucoxanthin production. In particular, various extraction characteristics of fucoxanthin from the waste parts of *L. japonica* have been investigated (Kanazawa et al., 2008). However, some microalgae are also known to possess fucoxanthin as a main carotenoid (Rijstenbil, 2003; Moreau et al., 2006; Iio et al., 2011). In addition, the industrial production of microalgae is ongoing for the commercial production of live food and lipids (Spolaore et al., 2006). In these respects, microalgae can be considered a potential source of fucoxanthin. Recently, our group found that fucoxanthin content in the microalga *P. tricornutum* is higher than that in macroalgae, suggesting that *P. tricornutum* can serve as a commercial source of fucoxanthin (Kim et al., 2012).

In the present study, fucoxanthin content was determined by

reversed-phase high-performance liquid chromatography (RP-HPLC) in five microalgae that possess fucoxanthin (Boczar and Prezelin, 1989; Ikeda et al., 2008; Peng et al., 2011; Kim et al., 2012). Based on the results, *Isochrysis* aff. *galbana* was selected as a potential source of fucoxanthin for commercial production. The effects of solvent type, extraction number, and extraction time on fucoxanthin extraction were assessed from freeze-dried *I.* aff. *galbana.* In addition, a two-phase solvent system for spontaneous production of fucoxanthin and lipids from ethanol extract was investigated to increase the utility of this microalga as a potential source of high-value constituents. The results are expected to be useful for commercial application of the microalga *I.* aff. *galbana*.

Materials and Methods

Algae samples and culture conditions. The marine microalga *I.* aff. *galbana* (CCMP1324) was provided by the National Center for Marine Algae and Microbiota. *P. tricornutum* (KMMCC-14), *Chaetoceros gracilis* (KMMCC-27), *I. galbana* (KMMCC-12), and *Nitzschia* sp. (KMMCC-308) were provided by the Korea Marine Microalgae Culture Center. The microalgae were cultivated in 30-L plastic cylinders at 20°C using f/2-Si medium (Guillard and Ryther, 1962) prepared from filter-sterilized seawater, and air was continuously supplied at 5 L/min by an air-lift pump. Light was provided by 60 W fluorescent lamps at an intensity of 2,500 lx. The culture was continuously active for 4–5 days after onset of the process. The cells were flocculated with 200 ppm $Al_2(SO_4)_3$ (v/v) and then recovered with centrifugation at 2,000 rpm using a basket centrifuge (Hanseong Co., Korea). The harvested biomass was freeze-dried at −70°C for 2 days.

Chemicals and reagents. For extraction and partition, analytical grade solvents purchased from Daejung (Korea) were used. Methanol and water for HPLC and liquid chromatography-mass spectrometry (LC-MS) were HPLC-grade solvents purchased from Fisher Scientific (USA). Standard fucoxanthin isolated by silica gel chromatography in a previous work was used for construction of the calibration curve (Kim et al., 2012).

Extraction of fucoxanthin from freeze-dried microalgae. For all extraction experiments, 100 mg dried powder of microalgae including *I.* aff. *galbana* was extracted with 4 mL tested-solvent in a 10-mL vial under various extraction conditions. Subsequently, the extract solution was filtered through a 0.42-μm filter prior to injection into the HPLC system for quantification and MS analysis. For determination of fucoxanthin contents from the five microalgae, the extraction was performed with ethanol at room temperature for 1 h. Effect of the solvent on fucoxanthin extraction from *I.* aff. *Galbana* was evaluated; extraction was performed with each conventional solvent (*n*-hexane, acetone, ethyl acetate, methanol, and ethanol) at room temperature for 1 h. For determination of fucoxanthin contents, depending on the number of extractions from *I.* aff. *galbana*, the dried powder of *I.* aff. *galbana* was extracted with ethanol at room temperature for 1 h and then filtered with suction over Whatman 3M paper on a Buchner funnel. The residue was collected, and extraction was repeated two more times under the same conditions. Each extraction solution was used for HPLC analysis after filtration through the 0.42-μm filter. Time-dependent fucoxanthin extraction with ethanol as a solvent was also assessed in *I.* aff. *galbana* by extracting for 5 min to 24 h at room temperature.

LC-MS analysis. The presence of fucoxanthin in each microalga was confirmed by LC-MS analysis. In brief, the ethanol extract solution was injected into a Varian HPLC-hyphenated MS system (USA) consisting of a ProStar 410 autosampler, two ProStar 210 pumps, and a 1200L triple quadrupole mass spectrometer. For HPLC, a flow rate of 1 mL/min of the mobile phase was controlled by binary pumps at 40°C. The mobile phase consisted of methanol and *tert*-butyl methyl ether (TBME). TBME was increased from 0 to 30% over a period of 45 min. YMC carotenoid column (250 mm length × 4.6 mm i.d. and 3 μm particle size; Waters, USA) was used for separation, and the chromatogram was detected at 445 nm. The atmosphere pressure chemical ionization (APCI) interface condition was as follows: drying gas of 12 psi at 150°C, nebulizing gas (N_2) of 60 psi, auxiliary gas of 17 psi, corona current of 5 μA, and housing temperature of 50°C. The positive ion mass spectra were acquired in the *m/z* range of 200–1000. The Varian MS workstation software (version 6.3, Varian Inc., USA) was used for data acquisition and processing.

HPLC quantification of fucoxanthin from microalgae. Fucoxanthin was quantified using an Agilent 1200 HPLC system (Agilent Technologies, USA). The HPLC system consisted of a G1312A binary pump, a G1367B auto sampler, a G1315D PDA detector, and a G1316A column oven. Separation was carried out with a YMC carotenoid column (250 mm length × 4.6 mm i.d. and 3 μm particle size; Waters). The mobile phase of methanol and water was eluted with a 1 mL/min flow rate by increasing the methanol from 90 to 100% over 30 min and holding for the following 10 min. A diode array detector (DAD) at a range of 200–800 nm was used, and the chromatogram was recorded at 445 nm. For construction of calibration curve, fucoxanthin was dissolved in ethanol at a range of 10–250 μg/mL, and each concentration was analyzed by HPLC as described above. All chromatograms were processed with Chemstation software (version B.03.02, Agilent Technologies).

Selection of the two-phase solvent system. To make an optimal two-phase solvent system for the separation of fucoxanthin and lipid compounds from the ethanol extract of *I.* aff. *galbana*, the partition coefficient (*K*) value of fucoxanthin was investigated by HPLC analysis as described in the literature (Kim et al., 2011). An *n*-hexane-ethanol-water system was selected, and the volume ratio of ethanol and water was serially changed from 9:1 to 1:9 (v/v), with the *n*-hexane volume fixed. One milligram of fucoxanthin was dissolved in each trial and equilibrated for 10 min after vigorous mixing. The amount of fucoxanthin in the upper and lower phases was analyzed separately by HPLC as described above. The *K* value was calculated as the amount of fucoxanthin

in the upper phase divided by that in the lower phase.

Preparation of crude fucoxanthin and lipid fractions. To evaluate the commercial feasibility of the spontaneous production of fucoxanthin and lipids from *I.* aff. *galbana*, the weights of the crude extract and its partition phases were measured, and fucoxanthin content in each sample was determined by HPLC. Dried *I.* aff. *galbana* powder (1g) was extracted three times with 100 mL ethanol for 3 h. The combined extract solution was evaporated and weighed. The crude extract (CE) was then dissolved in 5 mL of 90% aqueous ethanol and partitioned with 5 mL *n*-hexane to yield the *n*-hexane phase fraction (HX) and hydroalcohol phase fraction (HA). The partition step was repeated four more times, and then the combined HX and the remaining HA were concentrated and weighed. Fucoxanthin contents in CE, HX, and HA were determined using 10 mg/mL sample solution in ethanol by HPLC as described above.

Statistical analysis. All data were determined from three independent experiments. Mean values and standard deviations were calculated with Microsoft Excel software, and the data are expressed as the means ± standard deviation (SD).

Results and Discussion

Determination of fucoxanthin contents of microalgae. Fucoxanthin has mainly been isolated from marine brown seaweeds such as *Eisenia bicyclis*, *Laminaria japonica*, and *Sargassum horneri*, because the harvesting of biomass is more feasible in brown algae than microalgae (Peng et al., 2011). However, as reported in our previous study on fucoxanthin contents, more fucoxanthin is present in microalgae than in brown algae (Kim et al., 2012). Several species of microalgae are currently being cultivated at a pilot-plant scale for commercial purposes (Spolaore et al., 2006). However, none of these microalgae have been reported as a commercial source of fucoxanthin, except for our previous report (Kim et al., 2012). Thus, more microalgae were screened as potential commercial sources of fucoxanthin. In addition, to reduce cost and labor in the cultivation of microalgae, five microalgae were selected, including *P. tricornutum*, *C. gracilis*, *I. galbana*, *I.* aff. *galbana*, and *N.* sp., as potential sources of fucoxanthin on the basis of a literature search (Boczar and Prezelin, 1989; Ikeda et al., 2008; Peng et al., 2011; Kim et al., 2012). After cultivation and harvest, the biomass was freeze-dried and used for the extraction. Although there are several conventional solvents (e.g., acetone, methanol, and ethanol) for carotenoid extraction from plants or marine materials, in the present study, ethanol was used as the extraction solvent for the screening considering its cost, generality, and toxicity (Kim et al., 2012). Fig. 1 shows the HPLC chromatograms of the ethanol extracts from the five microalgae recorded at 455 nm. In all chromatograms (Fig. 1A–E), a common peak (peak **1**) was detected at 4.9 min and tentatively identified as fucoxanthin based on the fragment patterns at *m/z* 641, 659, and 581 corresponding to $[M+H-H_2O]^+$, $[M+H]^+$, and $[M+H-H_2O\text{-}AcOH]^+$, respectively (Fig. 1F) (Maoka et al., 2002). The fucoxanthin contents ranged from 2.24 mg/g dry weight (DW) sample in *C. gracilis* to 18.23 mg/g DW sample in *I.* aff. *galbana* (Table 1). In the case of *P. tricornutum*, 8.55 mg/g DW sample of fucoxanthin was extracted, whereas 15.42–16.52 mg/g DW sample of fucoxanthin was found in our previous study (Kim et al., 2012). The constituents of microalgae can change readily according to the cultivation conditions and origin of the microalgae, which may explain the

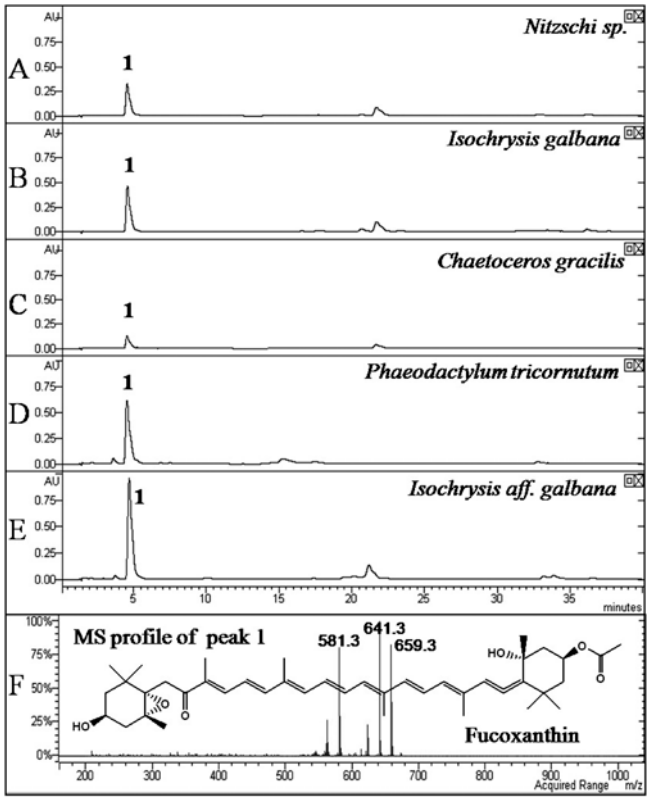

Fig. 1 HPLC chromatograms of ethanol extracts of fucoxanthin-containing microalgae (A–E) and mass data of fucoxanthin (F). The UV chromatogram was detected at 445 nm for carotenoid compounds, and the mass fragment of peak 1 at 4.9 min was identified as fucoxanthin in the positive mode of atmospheric pressure chemical ionization in the LC-MS analysis.

Table 1 Fucoxanthin contents in fucoxanthin-containing microalgae. Extraction was conducted for 1 h at room temperature with freeze-dried microalgae, and the extracted solutions were analyzed by HPLC for quantification. Fucoxanthin contents are expressed as mean values from three independent experiments

Microalgae	Fucoxanthin contents (mg/g DW sample)
Phaeodactylum tricornutum	8.55±1.89
Chaetoceros gracilis	2.24±0.01
Isochrysis galbana	6.04±0.28
Isochrysis aff. *galbana*	18.23±0.54
Nitzschia sp.	4.92±0.11

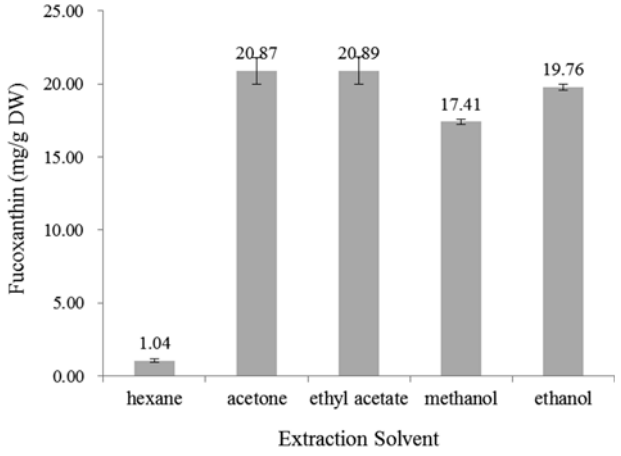

Fig. 2 Effect of solvents on fucoxanthin extraction efficiency from freeze-dried *Isochrysis* aff. *galbana*. Extraction was conducted for 1 h at room temperature with each solvent, and the extracted solutions were analyzed by high-performance liquid chromatography for quantification. Fucoxanthin contents are expressed as mean values from three independent experiments.

Table 2 Effect of extraction number on fucoxanthin extraction from freeze-dried *Isochrysis* aff. *galbana*. Each extraction was conducted for 1 h at room temperature, and the extracted solutions were analyzed by high-performance liquid chromatography for quantification. Fucoxanthin contents are expressed as mean values from three independent experiments

Extraction number	Fucoxanthin contents (mg/g DW sample)
First extraction	19.57±0.32
Second extraction	1.26±0.11
Third extraction	0.13±0.02

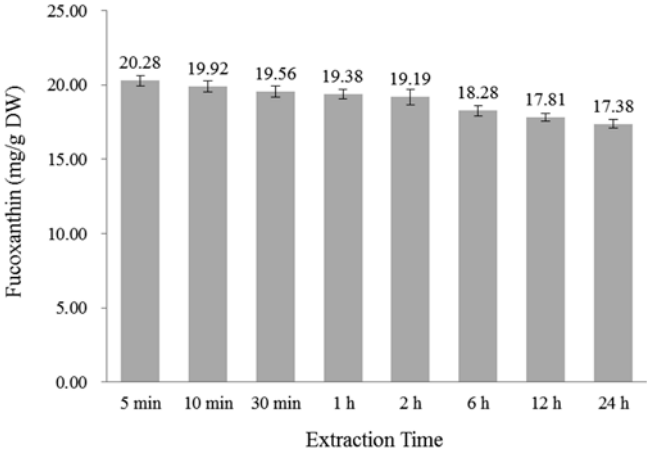

Fig. 3 Effect of extraction time on fucoxanthin extraction efficiency from freeze-dried *Isochrysis* aff. *galbana*. Extraction was conducted for 24 h using 100% ethanol at room temperature, and the extracted solutions were collected in the indicated time and analyzed by high-performance liquid chromatography for quantification. Fucoxanthin contents are expressed as mean values from three independent experiments.

difference in results among studies. Clearly, the results for the five microalgae show that *I.* aff. *galbana* possesses more fucoxanthin than does *P. tricornutum*, suggesting that *I.* aff. *galbana* has potential as a commercial source of fucoxanthin.

Extraction properties of fucoxanthin in *I.* aff. *galbana*. Use of the microalgae for commercial purposes such as the production of lipids or other functional ingredients, it is important to understand the extraction properties of target compounds. Only a few reports have evaluated the characteristics of *I.* aff. *galbana* in terms of the extraction properties of fucoxanthin and carotenoids. Furthermore, only one research group has demonstrated carotenoid extraction for commercial use from this microalga, which revealed that carotenoid content accounted for 2% of the DW (Bai et al., 2011). However, their study used only a methanol and *n*-hexane two-phase system in the same volume ratio as an extraction solvent, and fucoxanthin was not identified from the extract.

In the present study, the properties of *I.* aff. *galbana* was investigated in regard to fucoxanthin extraction using different solvents, numbers of extractions, and extraction times. Unlike *P. tricornutum*, which showed a significant difference in the fucoxanthin extraction efficiency according to solvent (Kim et al., 2012), *I.* aff. *galbana* demonstrated similar extraction efficiency with all tested conventional solvents except for *n*-hexane (Fig. 2). The fucoxanthin contents extracted with these solvents ranged from 17.41 to 20.89 mg/g DW sample. However, only 1.04 mg fucoxanthin was extracted with *n*-hexane from 1 g dried *I.* aff. *galbana*. Thus, effects of the number of extraction and time were examined with ethanol for the reasons described above. Table 2 shows the amount of fucoxanthin extracted in 1 h at room temperature in each extraction step. Most of the fucoxanthin (over 95% of the total extracted amount) was extracted in the first extraction. Furthermore, a similar extraction level could be accomplished within 5 min, indicating that fucoxanthin extraction occurred in a very short time (Fig. 3). In contrast, at least 24 h of extraction time is usually required for the extraction of lipids from microalgae. The fucoxanthin appeared to degrade gradually during the long extraction period. After 24 h, the remaining amount of fucoxanthin was 17.38 mg/g DW sample, which equaled to 85.7% of the fucoxanthin after 5 min. Taken together these results indicate that *I.* aff. *galbana* possesses a large amount of fucoxanthin that can be easily extracted with a general solvent in a short time.

The *I.* aff. *galbana* used in the present study was isolated from an aquaculture pond at the Centre National pour l'Exploitation des Oceans (CNEXO) in Tahiti by K. Haines; it is a small unicellular (~5–7 μm) phytoplankton without a cell wall (Wikfors and Patterson, 1994). The phytoplankton cell wall has a substantial effect on solvent extraction. Generally, cell wall disruption using chemical or physical processing is required for the extraction of chemical constituents from microalgae, as described in a number of reports (Mendes-Pinto et al., 2001; Lee et al., 2010; Prabakaran and Ravindran, 2011). Hence, an effective method for cell wall

Table 3 Partition coefficient (*K*) values of fucoxanthin in two-phase solvent systems of *n*-hexane-ethanol-water. Fucoxanthin (1 mg) was dissolved in the two-phase solvent systems with different ratios and equilibrated for 10 min after vigorous mixing. Fucoxanthin in each phase was analyzed by high-performance liquid chromatography and the *K* value was calculated as the peak area of fucoxanthin in the upper phase divided by that in the lower phase

Solvent ratio (*n*-hexane-ethanol-water, v/v/v)	10:9:1	10:7:3	10:5:5	10:3:7	10:1:9
K value	0.01	0.02	1.25	0.89	6.09

disruption is considered important in the extraction or analysis of chemical constituents in microalgae. However, *I.* aff. *galbana* does not have a cell wall, which may help explain its easy extraction using conventional solvents. Furthermore, fucoxanthin could be extracted easily with water without any cell disruption; however, in this case, the extraction solution caused a difficulty in the filtration process owing to its colloidal state (data not shown). Further work is needed to investigate the unique extraction property of this microalga.

Spontaneous production of crude fucoxanthin and lipids from *I.* aff. *galbana*. Marine microalgae have been reported to be rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). *I.* aff. *galbana* is also a well-known source of such fatty acids and has been used extensively as a food source for aquaculture (Wikfors and Patterson, 1994). Therefore, research on this microalga has mostly focused on the effects of various environmental factors including temperature, light conditions, media composition, and salinity on lipids (Burgess et al., 1993; Saoudi-Helis et al., 1994; Tzovenis et al., 1997). Such investigations have led to *I.* aff. *galbana* becoming a commonly used bioresource not only in the aquaculture industry but also in neutral lipid production for biodiesel (Demirbas and Demirbas, 2011). Compared to lipids, fucoxanthin in *I.* aff. *galbana* has not received as much attention as a biofunctional ingredient.

In the present study, a method for the spontaneous production of crude fucoxanthin and lipids from *I.* aff. *galbana* was investigated to increase the value of this microalga in the aquaculture industry. The method used was based on a new method of lipid extraction from *P. tricornutum*, with slight modifications (Ramírez Fajardo et al., 2007). In *P. tricornutum*, 80% of crude lipids in the ethanol extract were recovered from HX in the *n*-hexane-hydroethanol two-phase solvent system. The optimal water content in the HA phase for the highest lipid recovery was 40% in a volume ratio. However, in our experience, the amount of lipid in HX is not significantly affected by the water content of HA (within a two-fold change) compared to the amount of fucoxanthin in HA, which changes dramatically depending on water content. Thus, the optimal water content for the separation of fucoxanthin from lipids in the *n*-hexane-ethanol-water two-phase solvent system was examined, and the result was expressed as a *K* value of fucoxanthin as mentioned in the Methods section. The *K* values for fucoxanthin showed an increasing trend with increasing amount of water, indicating that more fucoxanthin can be dissolved in HX than in HA (Table 3). Because lipids and fucoxanthin have to be present in different phases for separation, it could be concluded that the water content in HA should be less

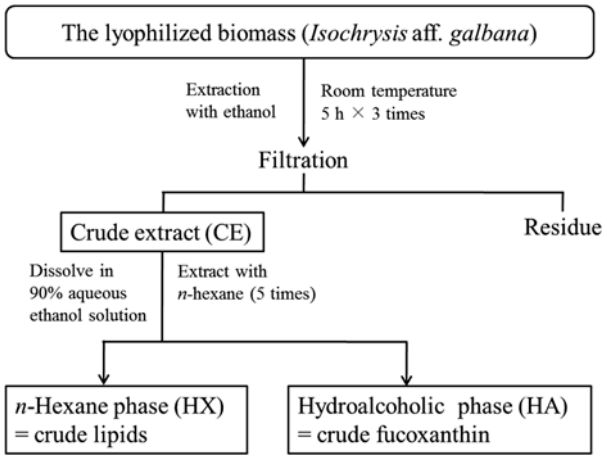

Fig. 4 Process for the preparation of crude fucoxanthin and lipids from the microalga *Isochrysis* aff. *galbana*.

than 10% in the volume ratio. In a 10:9:1 of *n*-hexane-ethanol-water system, the *K* value of fucoxanthin determined with pure compounds was 0.01, indicating that only 1% of fucoxanthin in the extract is present in HX. Therefore, this condition was selected as the optimal system for spontaneous production of crude lipids and fucoxanthin. Each crude sample was prepared for fucoxanthin quantification (Fig. 4). Table 4 shows the extraction efficiency of the CE and its HX and HA. In addition, the amount of fucoxanthin in each sample was quantified by HPLC. The extraction efficiency of CE was approximately 44.33% of the dry sample by three extractions with ethanol, and the CE was divided into HX and HA, which had 11.08 and 32.44% of the extraction efficiency of the dry sample, respectively. The concentrations of fucoxanthin in CE, HX, and HA were 3.61, 0.97, and 3.31 mg/100 mg of each extract sample, respectively. Finally, total amounts of fucoxanthin in CE, HX, and HA were calculated as 14.31, 1.07, and 10.73 mg/g of dry microalgae, respectively. Hence, 75% of fucoxanthin in CE was concentrated into HA, and only 7% of fucoxanthin in CE was present in HX, indicating that the method used in the present study is feasible for the industrial production of lipids and fucoxanthin simultaneously, even though complete separation (over 99%) was not achieved. On the other hand, total amount of fucoxanthin in CE was lower than that from extraction test (Figs. 2 and 3), and the sum of fucoxanthin in HX and HA was not equal to that in CE. In addition, the concentration of fucoxanthin in HA was lower than that in CE even though lipid compounds were removed from CE. These results could have resulted from the degradation of fucoxanthin during the experimental process. Thus,

Table 4 Extraction efficiency and fucoxanthin contents of the crude extract (CE), *n*-hexane phase (HX), and hydroalcohol phase (HA) of freeze-dried *Isochrysis* aff. *galbana*. The extraction efficiency was calculated by measuring the weight of each sample. The fucoxanthin content in each sample was analyzed by high-performance liquid chromatography

Sample	Crude extract (CE)	Hexane phase (HX)	Hydroalcohol phase (HA)
Extraction efficiency	44.33±3.38 mg/100 mg DW sample	11.08±0.51 mg/100 mg DW sample	32.44±2.45 mg/100 mg DW sample
Fucoxanthin concentration	3.61±0.64 mg/100 mg CE	0.97±0.03 mg/100 mg HX	3.31±0.04 mg/100 mg HA
Total amount of fucoxanthin	14.31 mg/g DW sample	1.07 mg/g DW sample	10.73 mg/g DW sample

the effect of antioxidants such as vitamin C in the purification step should be evaluated in further study. In addition, when fucoxanthin with high purity is required for pharmaceutical and cosmetic applications, the HA can be further concentrated by using several purification techniques such as centrifugal partition chromatography or silica gel chromatography after lipid separation (Kim et al., 2004; 2011).

In conclusion, fucoxanthin was screened in five fucoxanthin-containing microalgae and identified *I.* aff. *galbana* as a potential commercial source of fucoxanthin in addition to lipids. The extraction properties of freeze-dried *I.* aff. *galbana* were characterized in terms of the extraction solvent, number of extractions, and extraction time. In addition, a new method for the simultaneous production of crude fucoxanthin and lipids was developed from the crude ethanol extract to improve the value of *I.* aff. *galbana* as an industrial bioresource. Our results may aid the commercial development of this microalga for large-scale fucoxanthin production.

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