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Simultaneous purification of fucoxanthin isomers from brown seaweeds by open-column and high-performance liquid chromatography

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ABSTRACT

Simultaneous purification of fucoxanthin isomers from brown seaweeds by two steps of open-column chromatography (OCC) and reversed-phase (RP)-high-performance liquid chromatography (HPLC) is described. Analysis and identification of fucoxanthin isomers were performed by chromatographic and spectrophotometric properties such as retention time (t_R), spectral shape, maximal absorption wavelength (λ_{max}), Q-ratio, and mass spectrometry (MS) data including the ratio of fragment ions. The optimal conditions for a simultaneous separation and purification were examined by changing several parameters of HPLC, i.e., mobile phase composition, equilibration time, and column oven temperature. The purification procedure consisted of the following two steps: first, highly purified fucoxanthin fraction was obtained by a silica-gel OCC. Then, four major fucoxanthin isomers, all-*trans*, 13'-*cis*, 13-*cis*, and 9'-*cis*, were simultaneously separated and purified by RP-HPLC with an analytical C_{30} column and gradient elution in a mixture of water, methanol, and methyl *tert*-butyl ether. The purity of fucoxanthin isomers purified was >95% for all-*trans* and 9'-*cis*, 85% for 13'-*cis*, and >80% for 13-*cis*. A large-scale purification by RP-HPLC using a preparative C_{18} column was effective for the purification of all-*trans* and 9'-*cis* with a yield of 95%. This developed technique was fully applicable to analyze the enhanced production of fucoxanthin isomers by iodine-catalyzed stereomutation which composed of 9 isomer species including 9-*cis*.

1. Introduction

Fucoxanthin is a major carotenoid produced by brown seaweeds and diatoms [1–5]. Embedded in its native pigment-protein complex, fucoxanthin plays an important role to harness solar photon energy in the region, where chlorophyll (Chl) *a* and *c* absent [6]. The functional properties of carotenoids, as described by Koyama and Fujii [7], correlates with their linear conjugated polyene chain. In the case of fucoxanthin, the all-*trans* configuration shows effective for the light harvesting function [8]. Carotenoids are susceptible to isomerization against environmental factors due to their conjugated double bonds system. Likewise, fucoxanthin stereomutation induced by physical thermal stresses in both pure isolated pigments and thallus of brown seaweeds to produce fucoxanthin isomers was demonstrated.

Structurally, fucoxanthin contains an allenic bond and some oxygenic functional groups, including epoxy, hydroxyl, carbonyl, and carboxyl, which were contributed for its bioactivity potentials for human health, such as antioxidant, antidiabetic, anticancer, etc. [2,9]. The bioactivity of fucoxanthin depends on their configuration which could be different within isomers: the *cis*-isomer was known to have better bioactivity than all-*trans* form [10]. Unfortunately, little information is available on the formation, separation and purification of fucoxanthin *cis*-isomers, even though it has abundant benefits for human health and there is increasing a demand of high scale availability for the fucoxanthin market which will be expected to reach $120 million in 2022 [4,11].

The separation and purification steps must be done tediously to obtain pure fucoxanthin isomers with a high yield. The classical methods are known such as the preparative thin-layer [12] and the open-column chromatography (OCC) using silica gel [13] or Toyopearl resins [14]. A centrifugal partition chromatography has been developed to obtain higher purity of all-*trans* fucoxanthin [15,16]. Moreover, a combination

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with a flash chromatography or reversed phase (RP)-high-performance liquid chromatography (HPLC) using a C_{18} column has effectively increased the purity of the all-*trans* up to 99% [16,17]. Among the RP-HPLC columns used, C_{18} and C_{30} columns are the most used to separate and purify the carotenoids. The use of C_{18} column is more plausible for the separation of hydrophilic carotenoids, but it showed a poor ability to separate geometrical isomers of carotenoid especially xanthophyll group [18]. On the other hand, C_{30} column has higher efficiency to resolve geometrical isomers of carotenoid, including mono-*cis* or di-*cis* isomers [18–22].

It has been demonstrated that the geometrical isomers of fucoxanthin (Fig. 1), i.e., all-*trans*, 13-*cis*, 13'-*cis*, and 9'-*cis*, could be successfully separated through a C_{30} column using different programmes of the elution gradient mixtures, such as methanol (MeOH), isopropanol, and *n*-hexane mixture [23], MeOH, water (H_2O), and methyl *tert*-butyl ether (MTBE) mixture [24], and MeOH and acetonitrile (ACN) mixture [10]. The structural assignment of the geometrical isomers has been done on the basis of the absorption spectrum from calculating the absorption peak ratio including Q-ratio, and by then comparing to references. However, those purifications have been made to obtain the source material used for their studies and there are no specific and generally used purification techniques to obtain fucoxanthin isomers, except for all-*trans* which has been easily purified as a mixture of several isomers [14–16]. Accordingly, the technique for the purification and identification of geometrical isomers of fucoxanthin is still needed to gain the effective and simple purification in a short time and large amount. The aims of this study are to provide simultaneous and optimal HPLC purification procedure for simply purifying and identifying fucoxanthin isomers from algal thalli such as brown seaweeds.

We also describe the application of our technique using an optimized C_{30} column RP-HPLC to a more complex composition of fucoxanthin isomers transformed by an iodine-catalyzed stereomutation.

2. Materials and methods

2.1. Chemicals and sample material

Chemicals used in this study were ACN, MeOH, ethanol (EtOH), acetone (AC), ammonium acetate, potassium iodide (KI), MTBE, ethyl acetate, and *n*-hexane with the analytical grades, while silica gel 60 for OCC and H_2O for liquid chromatography (Merck, Darmstadt, Germany). The standards, all-*trans* fucoxanthin, *β*-carotene, violaxanthin, and Chls c_1 and c_2 were obtained from NATChrom™ (≥95% purity) (Malang, East-Java, Indonesia).

Sargassum polycystum was collected from several locations in Teluk Awur beach, Jepara, Central Java, Indonesia. The seaweeds were rinsed with fresh water then put into black plastic bags and placed in a cooling box during transportation to the laboratory. The dried brown seaweeds were prepared using an oven treatment at 50 °C for 30 h. The dried brown seaweeds were ground into small particles by blender, placed in a plastic bag, and then stored at –30 °C.

2.2. Spectrometry

2.2.1. Absorption spectrometry

Absorption spectrum of the pigments was recorded in ACN using a UV–vis spectrophotometer UV-1700 (Shimadzu, Kyoto, Japan) in the wavelength range of 300–800 nm, medium scan speed, 1 nm of data points, and cell-path length of 10 mm. The measurements were carried out at room temperature under dim light. Also, the absorption spectra of the purified fucoxanthin isomers were measured separately in EtOH, AC, and *n*-hexane.

2.2.2. Mass spectrometry (MS)

Characterization of the fucoxanthin isomers was also carried out by MS analysis using a UFLC XR Prominence coupled with an LCMS-8030 triple quadrupole mass spectrometer (Shimadzu). The analysis was conducted using an RP-18e Chromolith Performance column (100 × 4.6 mm i.d., 2 μm particle size, 130 Å pore size) (Merck) with elution using two solvents: solvent A (H_2O with 0.1% formic acid) and solvent B (90% methanol with 0.1% formic acid) at a flow rate of 0.4 mL•min^{-1}. Each run of the LC-MS/MS analysis was performed with isocratic elution with 90% solvent B for 15 min and with electrospray ionization (ESI) mode in the mass range from 400 to 700 *m/z* with the interface voltage (4.5 kV), interface current (0.1 μA), nebulizing gas (N_2) flow (3.0 L•min^{-1}), drying gas (N_2) flow (15 L•min^{-1}), desolvation temperature (250 °C),

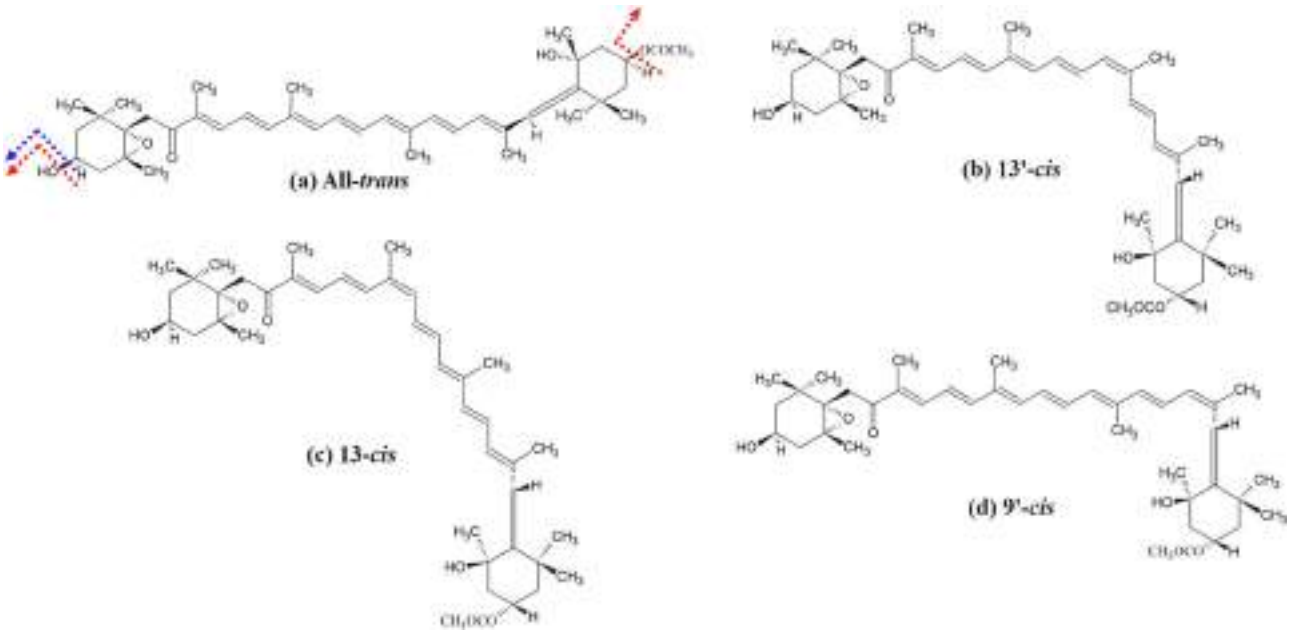

Fig. 1. Molecular structures of the major fucoxanthin isomers. The possible fragmentations of all-*trans* fucoxanthin by product ion scan are shown: blue dot line = *m/z* 641.40 $[M+H-H_2O]^+$ and red dot lines = *m/z* 581.40 $[M+H-H_2O-HOCOCH_3]^+$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and heat block temperature (400° C). The identification of the pigments by MS analysis was based on the precursor and fragment ions using a mode of Q1 scan (+), a product ion scan with –10 V of collision energy (CE), and single ion monitoring (SIM) at the *m/z* of precursor ions. The pigment was identified by comparing the recorded chromatographic and spectral data with the data on the pigment standard stored in the library using LabSolution LCMS ver. 5.4 (Shimadzu). The software compared the retention time (t_R) and aligned the MS/MS data to calculate a match factor and produce a degree of similarity between spectra. The intensity fragment ratio [25] between the fragment ions at *m/z* 641.5 $[M + H–H_2O]^+$ and at *m/z* 581.4 $[M + H–H_2O–HOCOCH_3]^+$ measured at CE = –10 V (Fig. 1) was calculated and compared to distinguish the fucoxanthin isomers.

2.3. Analysis and identification of fucoxanthin isomers

Chromatographic and spectrophotometric properties such as t_R, spectral shape, maximal absorption wavelength (λ_{max}), Q-ratio, and MS data including the ratio of fragment ions were used to characterize and identify fucoxanthin isomers. The Q ratio of fucoxanthin isomers can be calculated using a ratio between absorbance value of *cis*-band in the blue region of the spectrum (D_B) and absorbance value at λ_{max} of band II (D_{II}).

Fucoxanthin has low solubility in non-polar solvent such as *n*-hexane and toluene, but ACN, a polar aprotic solvent has good ability to dissolve fucoxanthin. Also, this solvent has a high thermal stability of fucoxanthin in ACN compared to the other polar solvents, MeOH and AC (Unpublished result). Therefore, ACN was mainly used throughout this study for dissolving and diluting fucoxanthin isomers.

2.4. Extraction of the pigments

For analytical purpose, approximately 5 g and 1 g of the fresh and dried powder seaweeds, respectively, were extracted with 10 mL MeOH which was the most efficient solvent for extracting fucoxanthin among the tested ones such as MeOH, EtOH, dimethyl ether, AC, ACN, and combinations thereof. Prior to extraction from fresh thalli, sample was frozen with a liquid N_2 and followed by grinding into small particles. For isolation and purification of fucoxanthin isomers, 100-g dried seaweed powders were suspended with 600 mL of MeOH and then pigments were extracted by gentle stirring for 1 h at room temperature. The crude pigment extract was separated from its residue through a filter paper (MN 713, Macherey-Nagel, Düren, Germany). The residue was continuously extracted with the same procedure described above until become colourless. The combined extracts were concentrated using a rotary evaporator at 40 °C for 1 h and then dried under a stream of N_2.

2.5. Analysis of pigment composition in the pigment extracts

The pigment compositions of the extracts of fresh and dried seaweeds were analyzed using an RP-HPLC according to the method of Hegazi et al. [26] with modifications. The separation of the pigments was performed on a Shim-pack VP-ODS C_{18} column (250 × 4.6 mm i.d.) (Shimadzu) with a gradient elution of ammonium acetate solution (1 M), MeOH, and AC mixture at the flow rate of 1 mL·min^{-1} at 30 °C in 70 min. The pigment extracts in AC, 20 μL, were injected to the HPLC.

2.6. Isolation of fucoxanthin by open-column chromatography (OCC)

The partially purified fucoxanthin containing its isomers was obtained by OCC using a silica gel 60 column (60 × 30 mm i.d.) with mixtures of ethyl acetate and *n*-hexane as mobile phase. Prior to the chromatography, small amounts of sea sand were layered on the top surface of the silica-gel bed (3-mm thick) to prevent from disturbance of the surface bed and also small precipitates. Then, the dried pigment extract dissolved in a mixture of 5% (v/v) of ethyl acetate in *n*-hexane (25 mL) were loaded onto the column which has been equilibrated in the sample solvent mixture and then eluted with a 100 mL of 30% (v/v) of ethyl acetate in *n*-hexane mixture until *β*-carotene, pheophytin (Pheo) *a*, and Chl *a* come out. To separate fucoxanthin, the ratio of ethyl acetate in *n*-hexane was increased to 40% (v/v) and eluted with a 150 mL of the mixture. Fucoxanthin thus obtained was concentrated using a rotary evaporator and dried using the N_2 gas and the used silica gel was discarded. Hereafter, the resulting partially purified fucoxanthin containing its isomers refers to fucoxanthin fraction. A flowchart of the pigment separation by this chromatography is shown in Fig. S1a.

2.7. Separation and purification of fucoxanthin isomers by HPLC

To isolate and purify fucoxanthin isomers from the fucoxanthin fraction, two different RP-HPLCs were performed using preparative C_{18} and analytical C_{30} columns. Before each chromatography, the fucoxanthin fraction was dissolved in ACN (1 mL) and then filtrated through a membrane syringe filter (PTFE, 0.22 μm, Shimadzu). The recovery of the separations was determined by concentrations of the isomers obtained before and after each purification step, whereas the purity was estimated by comparing the peak areas of the target isomer and sum of peak areas of the isomers emerged in the HPLC chromatograms. Fucoxanthin isomer concentrations were determined according to a linear equation of the standard curve of all-*trans* fucoxanthin ($y = 198.89x - 335.62$; $R^2 = 0.9994$, where y is the peak area detected at 450 nm ($\times 10^{-3}$) and x is fucoxanthin isomer concentration (μg·mL^{-1}).

2.7.1. HPLC using a preparative C_{18} column

To obtain a large amount of the isomers, a preparative C_{18} Zorbax column (ZODS-150 SP1, 150 × 10 mm i.d., Reading, UK) was used instead of the analytical C_{18} column which was used for the separation of the pigments extracts of algae. The flow chart of the pigment purification by preparative C_{18} column is shown in Fig. S1b. In this procedure, the filtrated fucoxanthin fraction (180 μL), which was optimized and selected by changing the injection volumes of 20–200 μL, was injected and a gradient elution of MeOH and AC mixture was used as a mobile phase by excluding the ammonium acetate solution, where its portion was added to the MeOH, and by changing the flow rate from 1 to 2 mL/min after 10 min. Thereby the running time of the preparative HPLC was reduced from 65 min to 30 min. Also, an equilibration time was examined by changing intervals of time and then optimal time was determined to be 20 min as shown in Table S2.

This procedure required at least 6 repeated injections to complete the loading of the sample obtained from the previous silica-gel chromatography. The reproducibility of purification by this system was examined by the retention time of fucoxanthin all-*trans* isomer for repeated purification. The experimental error (CV) obtained on the C_{18} column from three repetitions was less than 7.0%, demonstrating high reproducibility. The retention times tend to decrease as injection volume increased (20–200 μL), and higher than 180 μL resulted in an overlapping of peaks of 9'-*cis* and 13'- and 13-*cis* mixture, indicating that high loading volume affected the separation and purification.

Peak fractions corresponded to all-*trans* and 9'-*cis* isomers of fucoxanthin were collected and stored as the purified samples. Geometrical isomers of 13'-*cis* and 13-*cis* fucoxanthin were unable to separate and eluted as a mixture (See Table S3). However, the mixture of 13'-*cis* and 13-*cis* isomers can be clearly separated and purified by the next C_{30} HPLC system with Method IV (see Section 3.3.2, for the results).

2.7.2. HPLC using a C_{30} column

To perform complete separation and simultaneous purification, analytical RP-HPLC with a polymeric C_{30} as stationary phase was selected. The chromatography was carried using the C_{30} column (150 × 4.6 mm i.d., 3 μm particle size) (YMC, Kyoto, Japan) equipped with its guard column and gradient elution of H_2O, MeOH, and MTBE mixture as a mobile phase. In this system, optimal conditions for the simultaneous separation and purification of fucoxanthin isomers were determined by

seven different experimental parameters of the HPLC method (Methods I to VII). In these Methods, mobile phase compositions for gradient elution (solvent type and ratio), column temperature, and column-equilibration time were changed and examined, except for injection volume of 60 μL which was optimized by changing the loading volumes of 20–80 μL (Table S4). Among them, Method IV was superior to the other Methods and then this Method was selected and used hereafter. By the use of Method IV, four fucoxanthin isomers, all-*trans*, 9'-*cis*, 13'-*cis*, and 13-*cis*, were clearly separated (for flowchart, see Fig. S1c). Each isomer peak fraction was collected and stored at –30 °C to use further analysis. The reproducibility of C_{30} column was examined as similar to C_{18} column and high reproducibility was obtained by the loading volume of 60 μL for a repeated purification and the CV obtained from six repetitions was less than 3.2% for all-*trans* and 9'-*cis* and 16.8% for 13'-*cis* and 13-*cis*. For quantitative recovery, the use of high loading volume than 60 μL (20–80 μL) caused an overlapping peaks of the 13'-*cis* and 13-*cis* isomers. This system required at least 2 and 17 repeated injections to complete the loading of samples from the C_{18} and OCC systems, respectively.

2.8. Application of the optimized C_{30} column method to the transformed fucoxanthin isomers

To evaluate the efficiency of our developed technique, the separation and identification of fucoxanthin isomers formed from all-*trans* fucoxanthin by iodine-catalyzed stereomutation was applied. The physical and chemical treatments were carried out according to the modified method of Zhang et al. [27]. A solution of all-*trans* fucoxanthin (30 $\mu g \cdot mL^{-1}$) in toluene containing a saturated KI was exposed to a white light (285 $\mu mol \cdot m^{-2} \cdot s^{-1}$) through a water filter for 180 min, while stirring at room temperature. All treated samples were analyzed by HPLC using the C_{30} column with Method IV as described in 2.7.2 section.

3. Results and discussion

3.1. The pigments from brown seaweeds

Brown seaweeds are one of the most potential sources of fucoxanthin mainly because they are rich in FCP complex as light-harvesting antennas of photosystem. HPLC profiles of the pigment extracts from the fresh and dried brown seaweeds are shown in Fig. S2. Total seven pigments were separated and identified from those samples with fucoxanthin and Chl *a* as the major pigments. The fresh brown seaweed contained Chls c_1 and c_2 (peak 1), fucoxanthin (peak 2), violaxanthin (peak 3), Chl *a* (peak 4), Chl *a*' (peak 5), and *β*-carotene (peak 7) (Fig. S2a). On the other hand, the pigment composition of the dried brown seaweed was slightly different from the fresh one. Pheo *a*, a degradation product of Chl *a*, peak 6 emerged, instead violaxanthin (peak 3) disappeared (Fig. S2b). The pigment identification for the both samples is summarized in Table S1.

3.2. Isolation of the fucoxanthin by OCC

In order to purify fucoxanthin isomers, a large-scale OCC was applied (for flowchart, see Fig. S1a). In the chromatography, most of Chls and non-targeted carotenoids could be removed. Fig. 2 shows the absorption spectra of the pigment extracts before and after a single step of OCC with compared to the pure fucoxanthin.

The absorption spectrum of the pigment extracts showed absorption bands at 412 nm and 662 nm which indicate the presence of Pheo *a* and other Chls and there was no sign of fucoxanthin absorption which usually appears at 446 nm in ACN. The removal of Chl group resulted in a significant change in the absorption spectrum by decreasing absorption at 410 nm to 430 nm of the Soret band and at 662 nm of the Qy band of Chls as well as the appearance of fucoxanthin band at 446 nm as indicated by arrows. In this step, a little amount of Chls still remained in the

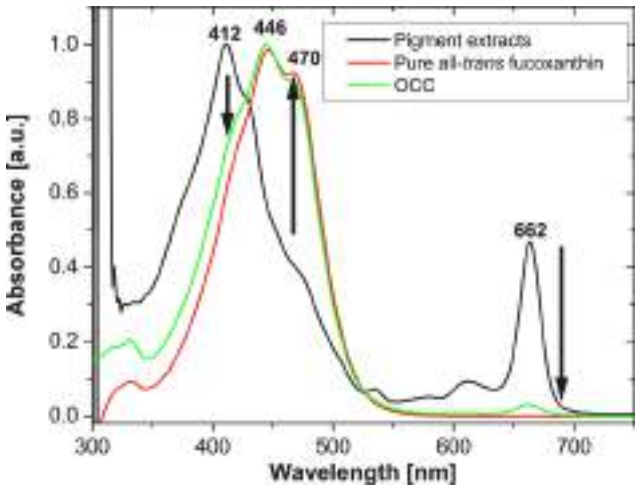

Fig. 2. Absorption spectra of the pigment extracts before and after silica-gel OCC. Absorption peaks were normalized at the highest peak. Direction of arrows indicates the absorption changes after the chromatography. Pigment extracts (black line), fucoxanthin fraction (green line), and pure all-*trans* fucoxanthin (red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

obtained sample. In addition, the spectral shape of this fucoxanthin fraction was almost similar to the pure all-*trans* fucoxanthin, although a trace amount of Chls was still detected at 662 nm. These results suggest that most of impurities were removed from the fucoxanthin fraction by OCC. The complete removal of the trace Chls, which is a critical to increase the durability of the pure fucoxanthin isomer against deterioration by light [28], is carried out by the next HPLC step together with complete purification of isomers

3.3. Purification of fucoxanthin isomers by RP-HPLC

3.3.1. Purification of all-trans and 9'-cis isomers by preparative C_{18} column

Separation and purification of fucoxanthin isomers were carried out in a preparative C_{18} Zorbax column after modification of the conditions (Table S2) to optimize the separation and purification of large amounts of samples. By this improvement, the running time of HPLC analysis was shorten to less than half of 30 min due to excluding the ammonium acetate solution from the mobile phase and increasing the flow rate from 1 mL/min to 2 mL/min after 10 min of elution time. The pigments belonging to Chl species eluted at 15–27 min were not overlapped with fucoxanthin isomers (Fig. 3). Thus, this time reduction was extremely effective for the complete removal of the Chl *a*, Pheo *a*, and *β*-carotene which have still been remained in a trace amount in the fucoxanthin fraction in the previous steps of OCC (Fig. 3b). In addition, among three equilibration times examined, 20 min was an important factor to maintain the reproducible elution result (see Table S2). Three peaks, 3–5, eluted at 7.5, 9.0, and 9.6 min are the targeted fucoxanthin isomers (Fig. 3a). Small peaks, 1 and 2, are unidentified fucoxanthin isomers which appear to be di-*cis*.

Finally, *all*-trans (peak 3) and 9'-*cis* (peak 4) isomers were purified with a high purity of above 95%. On the other hand, 13'-*cis* and 13-*cis* isomers were recovered as a mixture (peak 5), indicating that they could not be purified by this preparative method due to the close polarity of these isomers [29]. This procedure is, however, suitable for rapid and the large amounts of purification of the isomers, especially for all-*trans* and 9'-*cis*. The summary of the HPLC results of preparative C_{18} column is shown in Table S3.

3.3.2. Purification of four isomers by analytical C_{30} column

The above method used the preparative C_{18} column was effective for the purification all-*trans* and 9'-*cis* isomers, however, it was insufficient

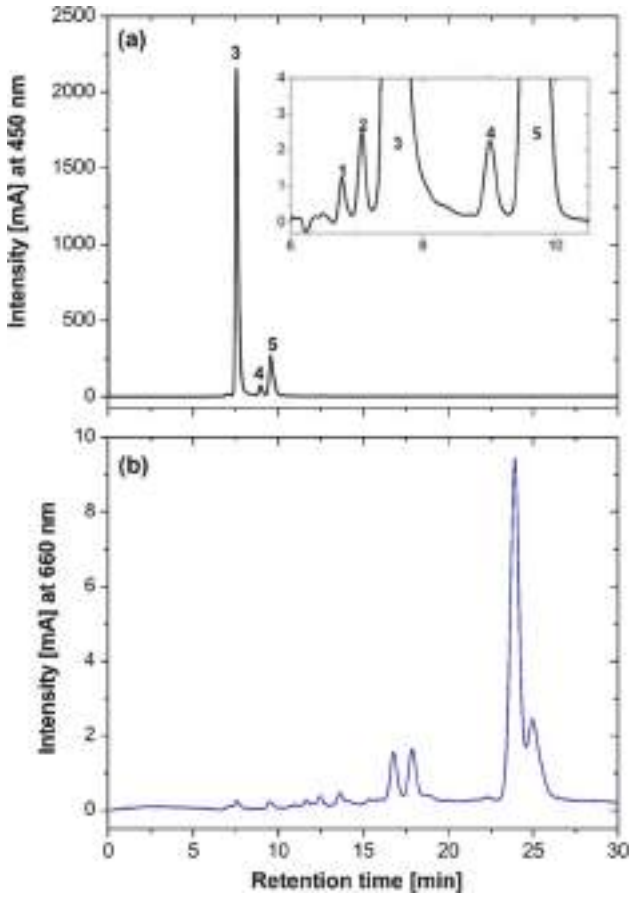

Fig. 3. HPLC elution profiles detected at 450 nm (a) and at 660 nm (b) for fucoxanthin fraction separated by a preparative C_{18} column HPLC. The peak numbers in the elution profile corresponds to those in Table S3. The insert (a) is the enlarged HPLC elution profile. Peaks: 1 and 2, unidentified isomers; 3, all-*trans*; 4, 9'-*cis*; 5, 13'-*cis*; and 13-*cis*.

to separate the other two fucoxanthin isomers of 13'-*cis* and 13-*cis*. This suggests more pronounced stationary phase is necessary to increase more interaction of solutes and stationary phase causing greater separation ability. Therefore, a polymeric C_{30} stationary phase was selected for the separation of fucoxanthin isomers. Unfortunately at present, preparative YMC C_{30} column and bulk resin were not commercially available, accordingly analytical column was used throughout this study. First, previously reported HPLC method using the C_{30} column by Zhao et al. [24] was applied, except for 10-cm shorter column length. However, the separation of the isomers 13'-*cis* and 13-*cis* was insufficient and the elution order of 13'-*cis* and 13-*cis* isomers was reverse. This reverse order might be due to mix up between them, because their Q-ratios calculated from absorption spectra were consistent with those of our results.

To achieve the complete and simultaneous purification and identification of the four fucoxanthin isomers, seven methods (Method I to VII) with varied HPLC experimental parameters were examined to obtain optimal purification conditions using a fucoxanthin fraction (See Table S4). Elution profiles of the fucoxanthin isomers using the Methods of III, IV, V, and VI are shown in Fig. 4. These Methods had an ability to separate all-*trans* (peak 5), 13'-*cis* (peak 6), and 13-*cis* (peak 7) compared to the Methods I, II, and VII (Fig. S3). Table 1 shows a summary of chromatographic properties, t_R, capacity factor (k'), selectivity (α), and resolution (R_s) for targeted fucoxanthin isomers using the Methods I to VII. These values obtained were estimated under our used conditions and within the range of the acceptable values of $R_s > 1.5$, $k' = 2$–10, and $\alpha > 1.50$ [30,31], although the selectivity is slightly low compared to others. Judging from these chromatographic values, the Method IV was the most effective HPLC method for the preparation of the purified fucoxanthin isomers.

By increasing of column temperature from 30 °C (Method I) to 40 °C (Method III) with the other conditions remained unchanged, evidently improved the resolution of peaks between each fucoxanthin isomer with faster retention times compared to the Method I (Table 1). Therefore, column temperature of 40 °C can efficiently resolve fucoxanthin isomers and the retention time decreases with slight changes in the selectivity. The Method IV, which has a slightly lower MeOH ratio than the Method III, observed a similar temperature effect the Method III. This improvement is probably due to a van Deemter equation about band broadening theory [32]. In parallel with the increasing in temperature, the decreasing in mobile phase viscosity and the increasing of diffusion coefficient led to increase column efficiency, causing greater interaction between fucoxanthin isomers and stationary phase. Moreover, the equilibration time was decreased according to the decrease in the mobile phase viscosity. In this study, the equilibration time taken was only 5 min even though it is usually taken 10 min. Another effort was to change a mobile phase composition from a polar protic (MeOH) to polar aprotic (ACN) as in the Method II. However, this method produced a poor separation, indicating that a hydroxide site of MeOH has good interaction with fucoxanthin isomers due to its extensiveness of hydrogen bond. Other Methods, III, V, VI, and VII, which have slight different gradient programs of H_2O, MeOH, and MTBE mixture (Table S4), did not show an improvement as expected for fucoxanthin isomer separation.

The reducing initial ratio of MeOH was beneficial to the separation (Methods IV, VI, and VII), but a high MeOH ratio (Methods I, III, and V) did not show a good separation, especially for 13'-*cis* and 13-*cis* isomers. The elution conditions for Methods IV and VI were almost similar, but in middle to final runs, Method VI had slightly higher MTBE and lower MeOH ratios than those of Method IV (Table S4). As shown in Fig. 4, Method VI appears to have a similar separation profile to that of Method IV, however, the Method VI showed slightly poor separation for peaks between 13'-*cis* and 13-*cis* with compared to that of Method IV as evident from their values of the resolution (Table 1). This indicates that an appropriate proportion of MTBE is needed to optimize elution conditions as reported previously [33,34]. Similar to the C_{18} column, the reproducibility of C_{30} column was carried out and high reproducibility was obtained with a loading volume of 60 μL for a series of repeated purification (See Section 2.7.2).

The elution order on C_{30} column was inconsistent with that of C_{18} column (cf. Fig. 3 (Table S3) and Fig. 4 (Table 2), although our elution order on the C_{18} column was in agreement with that of the previous reports [10,34,35]. The reason for the reverse order between C_{18} and C_{30} is probably caused by stronger interaction in C_{30} ligands compared to that of the C_{18} ligands [20,36]. C_{30} provides thicker film ligands that enhance interaction with long-chained molecules such as carotenoids. In contrast, the C_{18} column is thinner and interacts more strongly with a shorter isomer of 13'/13-*cis* compared to 9'-*cis*. This fact indicates a stronger interaction between molecular film thickness and size that is decisive for retention time compared to polarity. Therefore, for the C_{18} column, 9'-*cis* requires less time.

The summary of the HPLC results of optimized C_{30} column with the Method IV is shown in Table 2. The absorption spectra of those purified fucoxanthin isomers in ACN are presented in Fig. S4. The purity of 13'-*cis* and 13-*cis* was relatively low of 85% and 80%, respectively, compared to all-trans and 9'-*cis* of 95%, although it can be increased slightly using a high concentration of the samples. A flow chart of the overall procedure for simultaneous purification of the major fucoxanthin isomers by optimized C_{30} column HPLC is presented in Fig. 5 including large scale purification by C_{18} column.

Next, this optimized technique by C_{30} column HPLC was applied to the separation and purification of the 13'-*cis* and 13-*cis* mixture which has been obtained from the preparative C_{18} column HPLC. As expected, these isomers were clearly separated and their spectral and

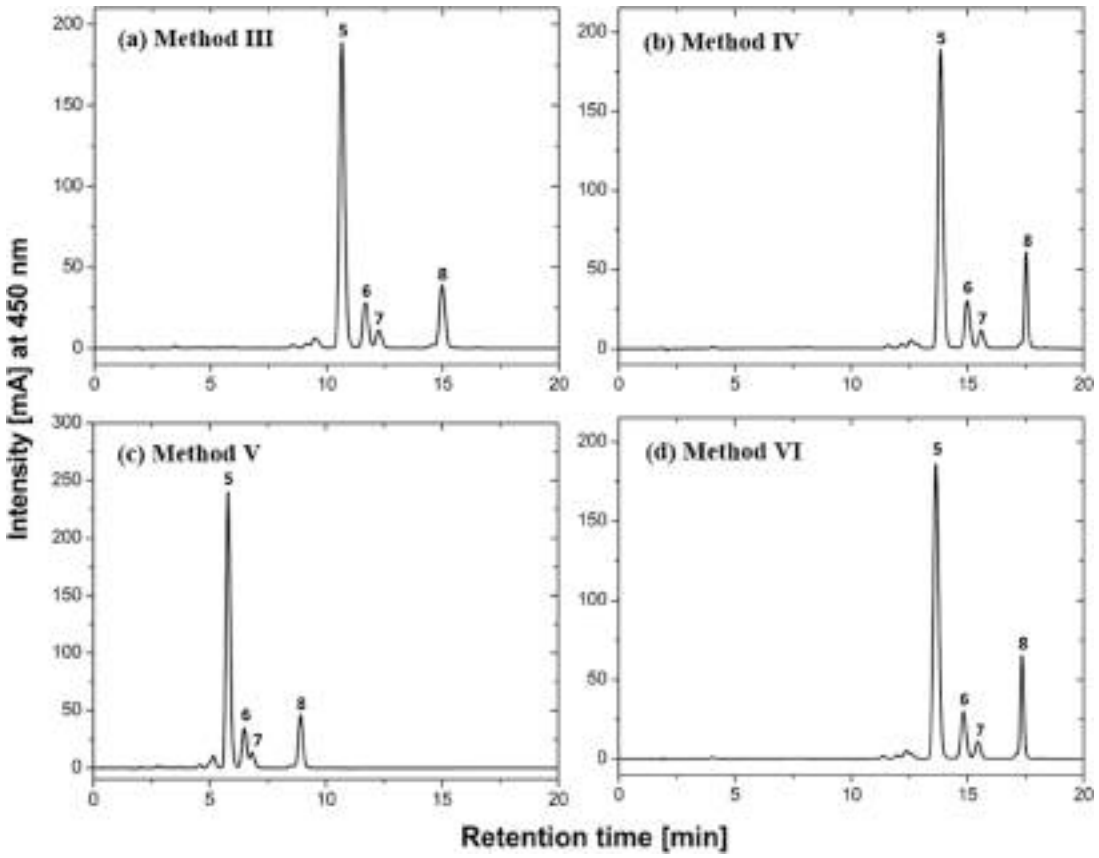

Fig. 4. Chromatograms of fucoxanthin isomers separated by RP-HPLC on a YMC C_{30} column under the conditions of Methods III to VI shown in Table S4. The peak numbers in the elution profile corresponds to the Table 2.

Table 1
Separation parameters obtained after eluting under the different chromatographic conditions, Methods I to VII described in Table S4, using a C_{30} column.

Method	Fucoxanthin isomer	t_R (min)	% area	Separation parameters ± SE		
				Capacity factor (k')	Selectivity (α)	Resolution (R_s)
I	all-*trans*	13.25	64.92	6.14 ± 0.91	1.15 ± 0.01	1.86 ± 0.04
	13′-*cis*	13.96	11.39	6.52 ± 0.95	1.06 ± 0.00	1.03 ± 0.03
	13-*cis*	14.78	4.23	6.97 ± 1.00	1.07 ± 0.00	1.17 ± 0.06
	9′-*cis*	17.52	14.57	8.44 ± 1.17	1.21 ± 0.01	4.88 ± 0.15
II	all-*trans*	11.27	74.48	5.08 ± 0.03	1.12 ± 0.04	1.09 ± 0.49
	13′-*cis*					
	13-*cis*	12.33	5.02	5.65 ± 0.03	1.11 ± 0.00	2.32 ± 0.02
	9′-*cis*	14.31	13.54	6.72 ± 0.03	1.04 ± 0.00	0.98 ± 0.07
III	all-*trans*	10.64	64.82	5.07 ± 0.40	1.15 ± 0.00	2.27 ± 0.04
	13′-*cis*	11.66	11.02	5.66 ± 0.43	1.11 ± 0.00	2.31 ± 0.04
	13-*cis*	12.23	4.05	5.98 ± 0.45	1.06 ± 0.00	1.25 ± 0.00
	9′-*cis*	14.95	14.38	7.54 ± 0.56	1.03 ± 0.00	0.37 ± 0.08
IV	all-*trans*	13.68	64.82	6.64 ± 1.35	1.10 ± 0.04	2.03 ± 1.42
	13′-*cis*	14.85	11.05	7.29 ± 1.44	1.10 ± 0.01	2.77 ± 0.09
	13-*cis*	15.47	4.05	7.64 ± 1.48	1.05 ± 0.00	1.42 ± 0.05
	9′-*cis*	17.43	14.90	8.73 ± 1.60	1.06 ± 0.00	2.83 ± 0.33
V	all-*trans*	5.78	63.56	2.04 ± 0.03	1.20 ± 0.00	1.79 ± 0.05
	13′-*cis*	6.49	10.87	2.41 ± 0.03	1.18 ± 0.00	1.91 ± 0.03
	13-*cis*	6.83	4.13	2.59 ± 0.04	1.07 ± 0.00	0.78 ± 0.02
	9′-*cis*	8.89	14.63	3.67 ± 0.06	1.18 ± 0.11	2.58 ± 1.70
VI	all-*trans*	13.65	64.98	7.09 ± 0.60	1.12 ± 0.00	2.59 ± 0.11
	13′-*cis*	14.83	11.00	7.78 ± 0.64	1.10 ± 0.00	2.66 ± 0.11
	13-*cis*	15.45	4.07	8.15 ± 0.67	1.05 ± 0.00	1.36 ± 0.03
	9′-*cis*	17.36	14.96	9.28 ± 0.76	1.05 ± 0.00	2.59 ± 0.21
VII	all-*trans*	11.53	64.90	5.57 ± 0.36	1.07 ± 0.01	1.69 ± 0.13
	13′-*cis*	12.17	10.79	5.93 ± 0.36	1.07 ± 0.00	2.52 ± 0.02
	13-*cis*	12.42	4.21	6.07 ± 0.36	1.02 ± 0.00	0.90 ± 0.07
	9′-*cis*	13.27	14.99	6.56 ± 0.32	1.05 ± 0.01	1.76 ± 0.64

All separation parameters were obtained directly from LCsolution version 1.21 SP1 (Shimadzu). Capacity factor, selectivity, and resolution of fucoxanthin isomers were calculated between the previous peak and each fucoxanthin isomer peak. The values are averages of three replications. The values of standard error are calculated by using a confidence level value of 95%.

Table 2
Properties of simultaneously purified fucoxanthin isomers by optimized HPLC with a C_{30} column and Method IV.

Fucoxanthin isomer	Peak No.[a]	t_R (min)	Purity[b] (%)	Yield[b] (%)	λ_{max} (nm)	Q-ratio		Precursor ion (*m/z*)	Main fragment ions (*m/z*)	Intensity ratio of main fragment ion (% ± SD)[c]
						This study	Ref. [37]			
all-*trans*	5	13.72	94.3	95.2	334,-,451,-	0.08	0.07	659.5 $[M + H]^+$	641.5 $[M + H–H_2O]^+$	65.8 ± 0.4
13'-*cis*	6	14.88	73.2	57.4	333,-,445,-	0.48	0.52		581.4 $[M + H–H_2O–HOCOCH_3]^+$	69.7 ± 0.5
13-*cis*	7	15.55	72.2	51.7	332,-,440,-	0.43	0.45			56.1 ± 3.2
9'-*cis*	8	17.44	92.6	56.9	332,-,448,-	0.12	0.12			17.5

[a] Peak No. are assigned for convenience. They are the same as in Table 3, Fig. 4 and Fig. 7.
[b] The values are averages of 9 to 12 replications of 3 to 4 independent experiments.
[c] $I_{fragment\ ratio}\ (\%) = I_{[m/z\ 641.5]}/I_{([m/z\ 641.5]\ +\ [m/z\ 581.4])} \times 100$.

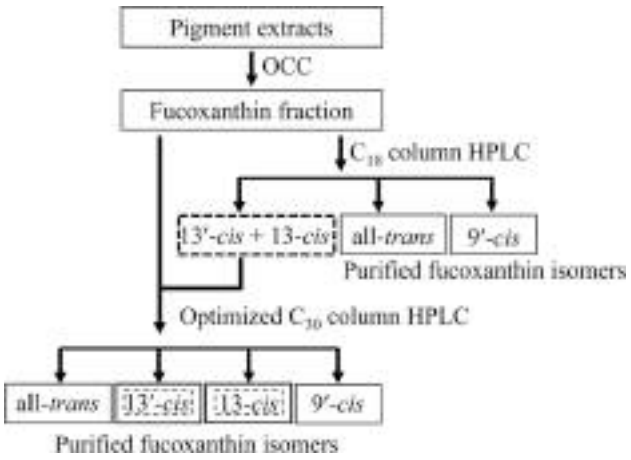

Fig. 5. A flowchart of the overall procedure for simultaneous purification of the major fucoxanthin isomers by optimized C_{30} column HPLC. For details, see text and Fig. S1a–c.

chromatographic properties were coincided to those obtained from the simultaneous separation of the fucoxanthin fraction. The purity of all-*trans* and 9'-*cis* obtained by C_{18} column HPLC was 93.7% and 80.3%, respectively. The purity of all-*trans* was almost comparable, but 9'-*cis* was slightly lower than that of the C_{30} column, despite that both yields were higher in these isomers. Accordingly, to obtain large quantity of these main isomers rapidly, use of combined column system is recommended.

3.4. Verification of fucoxanthin isomer identification

Four fucoxanthin isomers, all-*trans*, 13'-*cis*, 13-*cis*, and 9'-*cis*, were purified and identified from the extracts of the dried brown seaweed. The verification procedures included an analysis of Q-ratio [37], a ratio of absorbance values between the *cis*-band at 331–334 nm and the main absorption band (D_{II}) at 440–451 nm [38], and the calculation of the intensity fragment ratio [25]. Fig. 6 illustrates plots between the number of C sp2 bond in the shorter arm and the Q-ratio values calculated from this study in addition to the values previously reported [37].

As can be seen, those values obtained in this study agreed with the

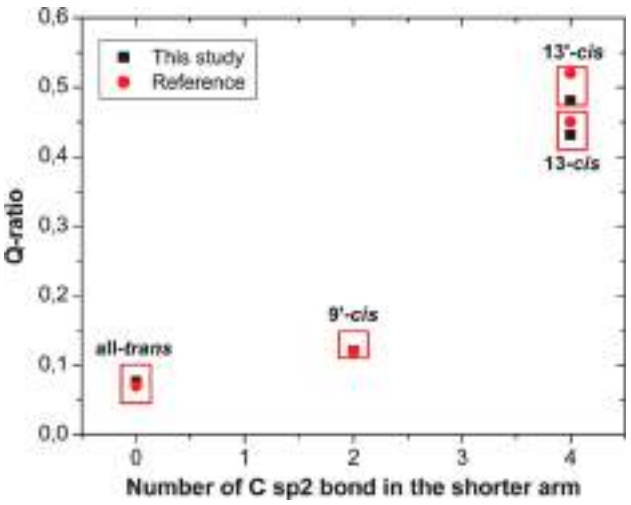

Fig. 6. Plots between Q-ratio and the number of carbon double bond in the shorter arm of fucoxanthin isomers. Black square, this study and red circle, values cited from the report by Haugan and Liaaen-Jensen [37]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reported values. Another new ratio, fragment-ion ratio, obtained by MS analysis was also applied. The fragment ions obtained peaks at *m/z* 641.5 and 581.4 were common peaks among fucoxanthin isomers as shown in Table 2 and Fig. S5, indicating that they are the base peaks resulted by product-ion scan and therefore applicable to the estimation of fragment-ion ratio for these isomers. The ratios obtained were well separated to differentiate types of fucoxanthin isomers (Table 2). To our knowledge, this is the first report of the fucoxanthin isomers, thereby there are no data to compare. From these findings, it is likely to conclude that each fucoxanthin isomer can be purified by the present method and its identification is confirmed.

3.5. Application of the optimized C_{30} column RP-HPLC to transformed isomers of fucoxanthin

Some physical and chemical treatments are known to promote the transformation of fucoxanthin isomers. To verify the ability of our developed C_{30} column method, fucoxanthin isomers transformed by the iodine-catalyzed stereomutation were applied and analyzed. As shown in Fig. 7, HPLC elution profile of the iodine-catalyzed reaction gave diverse products of fucoxanthin isomers than those of the fucoxanthin fraction used in this study. A total of 8 peaks were separated, but 9 species of isomers were identified by subsequent several spectroscopic analyses. Peaks 1 and 4 to 8 were identified as di-*cis*, 13,9'-di-*cis*, all-*trans*, 13'-*cis*, 13-*cis*, and 9'-*cis* in that order, while peaks 2 and 3 were a mixture of di-cis_1 and 9',13'-di-*cis*, and the rest peak 9 was 9-*cis* isomer. In this treatment, the transformation of isomers was enhanced 1.79-folds and 9-*cis* was newly found in this study. The summary of the results are presented in Table 3. As can be seen from the elution profile, six isomers were able to simultaneously separate and purify as similar to the main four isomers, especially, later eluted hydrophobic components such as 9'-*cis* (peak 8) and 9-*cis* (peak 9) are likely to be much superior. However, di-*cis* isomers, 9',13'-di-*cis* and 13,9'-di-*cis*, could not be separated by this method. This technique was also applicable for the physical treatments by heat and irradiation which formed 4 and 8 species of isomers, respectively. Accordingly, our method is applicable to a wide variety of structural and geometrical isomers of fucoxanthin and is versatile for the separation and simultaneous purification of the fucoxanthin isomers.

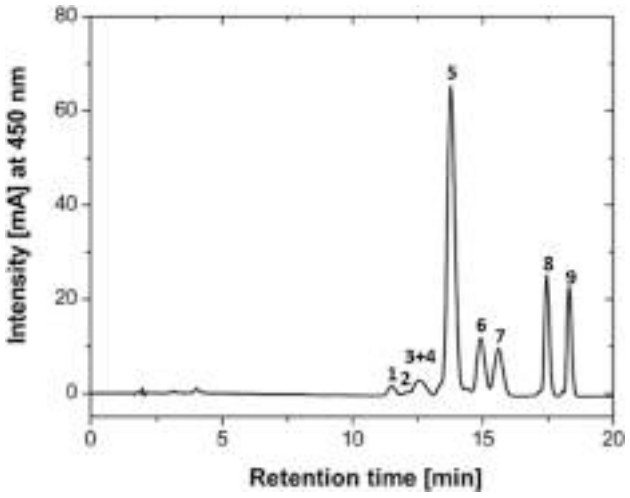

Fig. 7. HPLC elution profile of fucoxanthin isomers produced by iodine-catalysed stereomutation and separated by YMC C_{30} column with optimized Method IV (Table S4). The peak numbers in the elution profile corresponds to the Table 3.

Table 3
Summary of the separation and identification of fucoxanthin isomers after KI-catalyzed stereomutation by optimized HPLC using a C_{30} column and Method IV.

Peak No.	t_R (min)	λ_{max} (nm)	Fucoxanthin isomer	Q-ratio	
				This study	Reference [37]
1	11.44	335,-,435,-	di-*cis*	0.12	–
2	11.79	334,-,442,-	di-cis_1	0.49	–
3	12.19	334,-,439,-	9',13'-di-*cis*	0.35	0.34
4	12.50	334,-,437,-	13,9'-di-*cis*	0.38	0.40
5	13.72	334,-,451,-	all-*trans*	0.08	0.07
6	14.88	333,-,445,-	13'-*cis*	0.48	0.52
7	15.55	332,-,440,-	13-*cis*	0.43	0.45
8	17.44	332,-,448,-	9'-*cis*	0.12	0.12
9	18.30	333,-,447,-	9-*cis*	0.10	–

Q-ratios were estimated from in-line spectra.

4. Conclusion

In this study, we developed conventional purification technique for the isomers of fucoxanthin with the silica gel OCC and optimized C_{30} column HPLC. The purification procedures described in this study allows the rapid and simultaneous purification of major fucoxanthin isomers with high purity and efficiency. Also, used optimized C_{30} method is a versatile and applicable to the separation and identification of a wide range of isomers of fucoxanthin. This technique is being used routinely in our laboratory to study of fucoxanthin isomer transformation. This procedure assists the research and provides better understanding on the mechanism of fucoxanthin transformation and fucoxanthin study.

CRediT authorship contribution statement

Arif Agung Wibowo: Formal analysis, Investigation, Writing – original draft, Visualization. **Heriyanto:** Methodology, Formal analysis, Investigation, Writing – original draft. **Yuzo Shioi:** Conceptualization, Methodology, Writing – original draft, Supervision. **Leenawaty Limantara:** Project administration. **Tatas Hardo Panintingjati Brotosudarmo:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2022.123166.

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