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Characterization and Analysis of Fucoxanthin and its Isomers

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ABSTRACT:

Fucoxanthin (FUCO) is extremely instable and occur in nature as different isomers which may differ considerably in their biological effectiveness. Therefore, the aim of this work was to identify the FUCO isomers in oil and powder samples by LC-MS and NMR analyses and then to develop a precise, reliable, and rapid HPLC-DAD method for quantifying them; moreover, the effect of heating, basic and acid medium and light on their stability was measured. FUCO and isomers were identified as all-trans and 9'-cis fucoxanthin with MS and NMR spectra. Different chromatographic parameters were carefully optimized and the method was specific, linear (1-40 µg mL−1; r>0.99), precise (standard deviation values <0.7%), accurate (mean recovery = 103.25%), low limit of quantification (0.1 µg mL−1). Moreover, the robustness was investigated using the Box–Behnken experimental design and factors studied simultaneously were: pH of mobile phase, wavelength and column temperature, each at three levels, producing results within the acceptable range. This proposed method was successfully applied the analysis of FUCO, emphasizing thus the advantages of the method: high efficiency, reliable and rapid analysis. Our results suggested that method can be used for the quality control of FUCO and its isomers.

KEY WORDS: Marine carotenoids, fucoxanthin, isomers, study stability, validation, HPLC.

1. INTRODUCTION

Carotenoids are isoprenoid molecules, which are some of the first naturally occurring pigments and are responsible for the bright color of various fruits, vegetables, insects, and marine animals [1-3]. They are supposedly potential agents for the treatment of cancer, diabetes, and cardiovascular diseases [4-7].

Fucoxanthin (FUCO) (3'-acetoxy-5,6-epoxy-3,5'-dihydroxy-6',7'-didehydro-5,6,7,8,5',6'-hexahydro-β,β-caroten-8-one) is a marine carotenoid present in the chloroplasts of numerous classes of microalgae and brown macroalgae, such as wakame (*Undaria pinnatifida*), kombu (*Laminaria japonica*), hijiki (*Hijikia fusiformis*), arame (*Eisena bicyclis*), and *Sargassum fulvellum*. It is the most abundant of all carotenoids, accounting for more than 10% of the estimated total natural production [8-10].Currently there is great interest in isolating novel bioactive compounds from the marine environment, which contains rich natural resources, such as algae [1].

The complete structure of FUCO including chirality was determined by Englert and co-workers [11]. This carotenoid has a distinctive structure with an uncommon allenic bond and 5,6-monoepoxide in its molecule and further includes oxygenic functional groups, such as hydroxyl, epoxy, carbonyl, and carboxyl groups in addition to its polyene chain (Figure 1) [12-13].

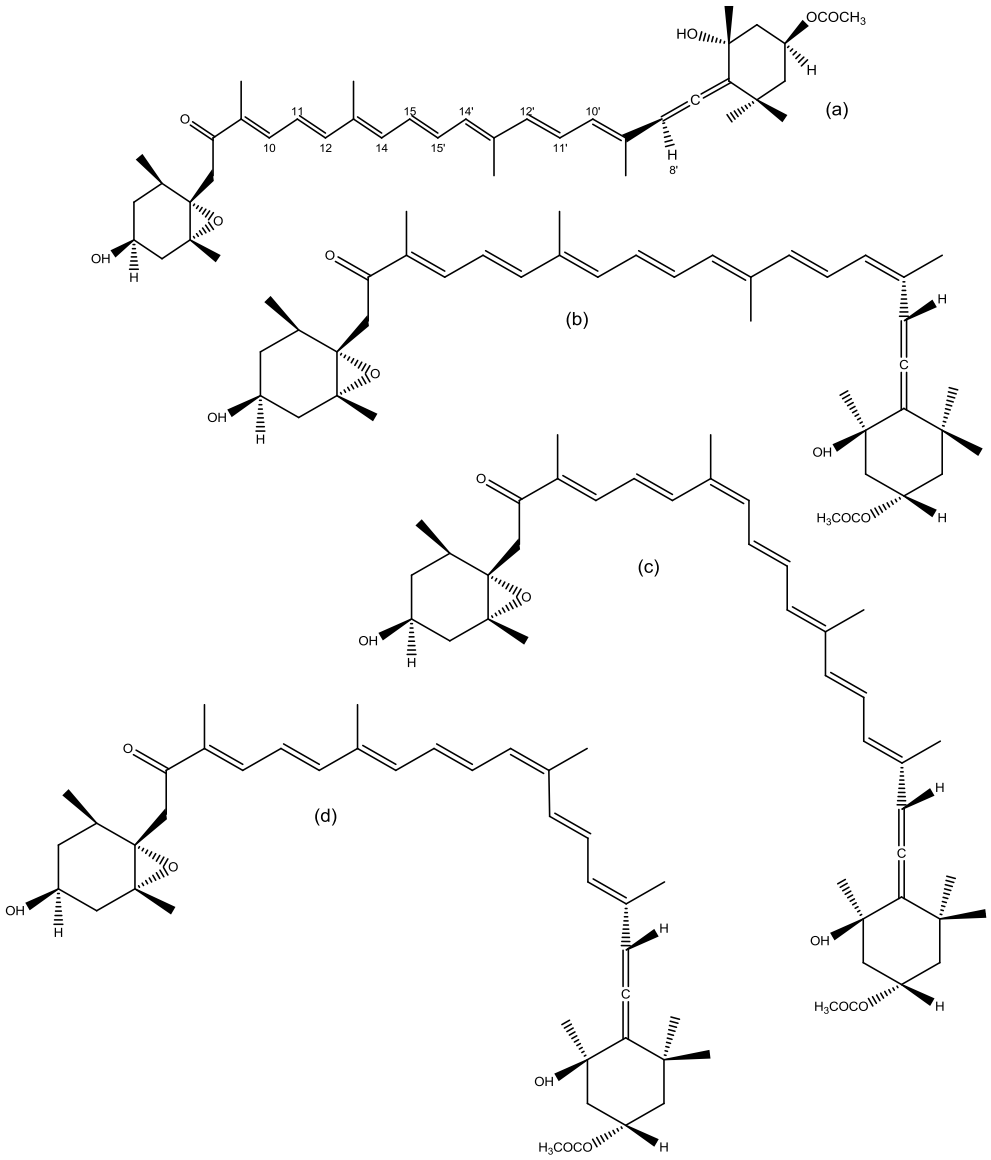

Figure 1. Structure of (a) all-*trans* fucoxanthin, (b) 9'-*cis* fucoxanthin (c) 13-*cis* fucoxanthin, and (d) 13'-*cis* fucoxanthin ($C_{42}H_{58}O_6$).

FUCO rich brown seaweeds have traditionally been used in the Southeast and is a promising option for the prevention and treatment of cancer and a wide variety of related pathologies, including obesity and diabetes [14-17].Cancer preventing influences of FUCO are mediated through different signaling pathways, as the caspases, different proteins and enzymes. Furthermore, other molecules are involved in cell cycle arrest, apoptosis and anti-angiogenesis or inhibition of metastasis [18-21].

This carotenoid is extremely instable and occur in nature as different isomers which may differ considerably in their biological effectiveness [2]. Therefore, the aim of this work was to identify the FUCO isomers in oil and powder samples by LC-MS and NMR analyses and then to develop a precise, reliable, and rapid HPLC-DAD method for quantifying them; moreover, the effect of heating, basic and acid medium and light on their stability was measured.

2. MATERIALS AND METHODS

2.1 Materials

A reference standard of FUCO ($C_{42}H_{58}O_6$) with purity of >95%, was supplied by Cayman Chemical Company® (Ann Arbor, MI, USA). Samples of FUCO in powder and oil were assigned a content of 30% and 2%, respectively, were kindly donated by Shaanxi Jiahe Phytochem Co., Ltd. (Xian, China). All chemicals used were

of pharmaceutical or analytical grade. Ultrapure water was obtained from a Milli-Q apparatus (Millipore®, USA).

2.2 Identification of fucoxanthin in oil and powder

2.2.1 Liquid chromatography coupled to mass spectrometry (LC-MS/MS)

LC-MS/MS measurements were carried out using an Agilent 1100 Series chromatographic system coupled to an AB Sciex API 5000 triple quadrupole mass spectrometer with an electrospray source in positive ionization mode (ESI+). The conditions used were collision energy 10 eV, declustering potential 100 eV and source temperature 300 °C. Instrument control and data processing were carried out by means of Analyst 1.6.1 software. Separation was achieved in a Symmetry C18 LC column (75 x 4.6 mm; 3.5 mm particle diameter) from Waters. The injection volume was 6 µL, with flow rate adopted was 0.4 µL min^{-1} and mobile phase is composed by acetonitrile and water (9.5:0.5 *v/v*). Separately, FUCO in oil and powder were prepared in acetonitrile at 5.0 µg mL^{-1}.

2.2.2 Nuclear magnetic resonance (NMR) analysis

Previously, several solvents (methanol, ethanol, acetone, and n-hexane), solvent volume and extraction temperature were tested to determine the optimal conditions for FUCO extraction. Every procedure was performed under dark conditions to prevent pigment degradation. Separately, approximately 10 mg of FUCO in oil and powder were spiked with 2 mL of n-hexane. The mixtures were centrifuged at 3,000 rpm for 5 min (Centrifuge Excelsa®). Supernatants were separated and the extraction procedure was repeated twice using 1 mL of n-hexane. The n-hexane was evaporated to dryness at room temperature. The residue of FUCO was dissolved in 1 mL deuterated methanol (CD3OD) and used for NMR spectroscopy. The proton NMR (^{1}H NMR) and two-dimensional COSY spectra of FUCO in oil and powder were recorded on a Bruker® AVANCE600 (Rheinstetten, Germany). Chemical shifts were adjusted with δ (ppm) referring to the solvent peak δH 3.31 for CD3OD. Data were processed with the MestRe Nova program, interpreted and compared with data in the literature.

2.2.3 Gas Chromatography

For preparation of FUCO, into 0.5 g oil containing FUCO were added and then shaken 5.0 mL methanol and 0.1 mL methanolic potassium hydroxide. The mixture was heated at 100 ºC for 15 min in a water bath. After cooling, 2.5 mL hexane were added and occasionally shaken; 5.0 mL saturated sodium chloride solution were added and shaken vigorously. The organic layer was separated and analyzed. The chemical composition was analyzed by gas chromatography–mass spectrometry (GC–MS) (Shimadzu®, quadrupole MS system QP5000, at 70 eV). A silica capillary column of VF-23 Varian® (30 m x 0.25mm x 0.25 m, coated with DB-5) was used to separate constituents. Injector and detector temperature were set at 220 °C and 250 °C, respectively; the oven temperature was programmed as 50-260 ºC at 3 °C min^{-1}, using helium as carrier gas at a flow rate of 1.0 mL^{-1}. The injection volume was 1.0 µl (splitless mode). The identification of compounds was based on the comparison of retention indices and their mass spectra, with authentic standards and with data taken from the literature [22] or by comparison with mass spectra recorded in the database. Relative amounts of the components were calculated based on GC peak areas.

2.3 HPLC-DAD system

An HPLC Agilent® model 1200 UV/LC system (Santa Clara, USA), consisting of a quaternary pump, a degasser, a column thermostat, a diode array detector (DAD) and a thermostated auto sampler was used. System control and data analysis were performed using ChemStation software. Chromatographic identification and quantification of FUCO was achieved on an Agilent® Eclipse Plus C18 analytical column (4.6 mm x 150 mm, 5.0 µm). All separations were obtained in isocratic mode using acetonitrile and water, pH adjusted to 7.0 with sodium hydroxide 0.1 *M* (NaOH) (9.5:0.5 *v/v*). The system was programmed at 40 ºC for the column oven, 450 nm for the detection wavelength, flow rate was 1.0 mL min^{-1} and 50 µL for the injection volume.

The stock solutions of reference standard and sample solutions of FUCO in oil were prepared in order to optimize the experimental procedure and decrease the chance of analytical errors. The solutions were prepared with acetonitrile and the dilutions were done with acetonitrile and water, pH adjusted to 7.0 with NaOH 0.1 *M* (9:1 *v/v*). All analyses were performed under dark conditions.

2.4 Validation of HPLC-DAD method for fucoxanthin in oil

The method was validated based on the International Council on Harmonization (ICH) guidelines

[23-24] and United States Pharmacopeia (USP) requirements [25], following the validation parameters: specificity, linearity, precision, accuracy, limit of detection, limit of quantification, and robustness. A Box-Behnken design with three factors was planned for robustness evaluation.

Specificity. Forced degradation studies were performed in order to provide an indication of stability. FUCO in acetonitrile, at 30.0 µg mL^{-1}, was subjected to degradation under acidic (hydrochloric acid - HCl 0.01 *M*), basic (NaOH 0.01 *M*), photolytic (UV-A radiation - 352 nm and UV-C radiation - 254 nm), oxidative (hydrogen peroxide - H_2O_2 0.3%), and thermal (65 °C) stress conditions.

The acidic and basic conditions were carried out at room temperature for 4 h and the solutions were neutralized with base or acid before injection. The oxidation reaction with H_2O_2 0.3% was performed out at room temperature for 3 h before injection. For the photolytic degradation study, aliquots of FUCO were transferred to a 1 cm quartz cell, for 4 h. The cells were exposed, positioning them horizontally to have the maximum exposure area, under the direct incidence of UV-A radiation (Blacklight blue lamp – Orion, 352 nm/ 30 W) and UV-C radiation (Light Express lamp LE UV, 254 nm/30 W), in a glass chamber that is mirrored internally (100 × 16 × 16 cm). For the thermal degradation study, FUCO was placed at a constant temperature of 65 ºC for 1 h. All samples were analyzed by HPLC-DAD to ensure the purity of the FUCO peak in all the stressed sample solutions.

Linearity. Linearity was determined by three analytical curves (peak area versus concentration), each one with nine concentrations for reference standard of FUCO, in the range of 1.0-40.0 µg mL^{-1} (1.0; 3.0; 5.0; 10.0; 15.0; 20.0; 25.0; 30.0 and 40.0 µg mL^{-1}).

Precision. The precision of the method was evaluated by injection of six preparations of FUCO at a concentration of 30.0 µg mL^{-1}, quantified against a reference standard. The results were expressed by repeatability (intra-day) and intermediate precision (inter-day) and the relative standard deviation (RSD, %) was calculated.

Accuracy. The accuracy of the method was evaluated in triplicate by adding standard solution at three concentration levels, low, medium, and high (5.0, 20.0, and 40.0 µg mL^{-1}, respectively) by the standard addition technique. The percentage of recovery was calculated at each level.

Limit of detection (LOD) and limit of quantification (LOQ). The limits were obtained based on the signal-to-noise by the ChemStation software. The LOD and LOQ for each sample were estimated at a concentration level in which the signal-to-noise ratio reached 3:1 and 10:1, respectively. The precision of LOQ was evaluated and the RSD were calculated.

Robustness. The robustness was investigated using the Box–Behnken experimental design. Three experimental parameters range in three levels were tested, pH range +/- 0.2 pH unit; temperature column +/- 2 ºC, and wavelength +/- 2 nm. Three replicates were used in the experiment and Minitab® software was used for modeling.

2.5 Kinetic degradation of fucoxanthin in oil

Degradation kinetics study was carried out under oxidative (H_2O_2 0.3%), stress conditions at room temperature, at pre-established times (0, 15, 30, 45, 60, 90, 120, 150 and 180 min). In this study, kinetic rate of FUCO degradation in oil was determined by plotting the drug concentration (zero order process), the log (first-order process), and the reciprocal (second-order process) of concentration versus time. The determination coefficients (R^2) were obtained and the best observed fit indicated the reaction order. The kinetic parameters, such as the apparent order degradation rate constant (*k*), half-life time ($t_{1/2}$), and $t_{90\%}$ (time where 90% of original concentration of the drug is left), were obtained.

2.6 Analysis using HPLC-DAD method of *fucoxanthin* in powder

After identifying FUCO in powder, the sample was analyzed by HPLC-DAD using the method previously described in item 2.3. Stability studies were performed according to described forced degradation studies for FUCO in oil.

2.7 Statistical analysis

Data were analyzed by one-way ANOVA, a value of $p<0.05$ was considered statistically significant. The experimental designs was performed by the Minitab 14 (Minitab Inc, State College, PA, USA) data analysis software system.

Table 1. ^{1}H NMR chemical shifts of the olefinic protons (in ppm, CD3OD) of fucoxanthin.

	H10	H11	H12	H14	H15	H15'	H14'	H12'	H11'	H10'	H8'	H19'
Oil	7.5	6.7	6.98	6.63	6.8	7.38	6.41	6.5	6.74	6.19	6.1	1.87
Powder	7.49	6.68	6.98	6.63	6.82	7.34	6.4	6.43	7.03	6.15	7	4.75

3. RESULTS AND DISCUSSION

3.1 Identification of fucoxanthin in oil and powder

Several studies were conducted for the extraction and purification for FUCO from algae employing the use of different methods, modifying the solvent type, extraction time, temperature and extraction technology (maceration, ultrasound-assisted extraction, Soxhlet extraction, and pressurized liquid extraction). These methods, however, can induce oxidation, producing degradation products and isomers [26-28]. The correct identification of isomers is a fundamental point for the development of a stability indicating HPLC-DAD method, because accurate analysis is a prerequisite, since isomers differ substantially in their biochemical activity [29-30].

In the present study, FUCO isomers were identified by LC-MS/MS. Daughter ions obtained from protonated molecular ions of FUCO in oil ([M + H] 659.2 *m/z*) included main ions from each compound at *m/z* 641.6, 581.4, 429.4, 411.5 and 213.3. In powder, daughter ions obtained from protonated molecular ions ([M + H] 659.3 *m/z*) included main ions from each compound at *m/z* 641.3, 581.4, 497.7, 297.4 and 213.3. The mode scanning at *m/z* 641, 659, and 581 corresponding to $[M + H - nH_2O]^+$, $[M + H]^+$ and $[M + H - H_2O\text{-}AcOH]^+$, respectively. These three fragments ions and dehydrated product ions from protonated molecules [M + H - nH2O]+ are observed for all hydroxylated carotenoids, such FUCO. Moreover, intensities of the dehydrated product ions may vary and reveal the structural characteristics of the hydroxylated end groups of isomeric carotenoids, allowing the distinction between structural isomers. FUCO and its isomers possess the same molecular formula, $C_{42}H_{58}O_6$, but the results showed differences in the fragments ions in LC-MS spectra [9-31].

The pure compounds from the extractions were subjected to ^{1}H NMR and two-dimensional COSY analysis. Chemical shift values for each FUCO isomer corroborated with previously reported values (Table 1). Furthermore, a detailed comparison of these data, particularly focusing on the olefinic protons in the COSY spectra (Figure 2), revealed that strong cross peaks between H8' and H11' and between H19' and H10', proved the 9'-*cis* isomer. The purified FUCO in oil was identified as all-*trans* fucoxanthin and FUCO in powder was identified 9'-*cis* fucoxanthin [11-32,33].

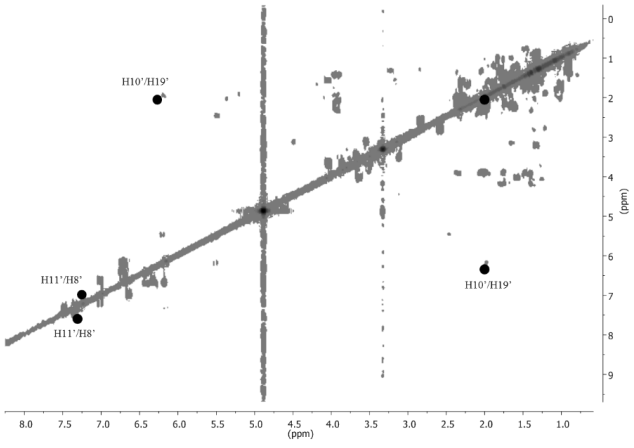

Figure 2. COSY spectrum of fucoxanthin in powder. Only relevant cross-peaks were assigned, between H8'/H11' and H19'/H10'.

In order to identify the components of oil by GC–MS, a representative chromatogram of FUCO in oil is provided in Figure 3. Seven integratable peaks were observed, four compounds were identified like as fatty acid, caprilic acid (49.4% in 13.1 min) and capric acid (36.1% in 20.5 min) were largest components. Although present in relatively small amounts are palmitic (37.6 min) and γ-linolenic acid (46.8 min). Three peaks were observed as fragments of FUCO, 13.4% of total peak area in 51.4, 57.4, and 60.2 min. Identification of fragments were made by comparison with mass spectrum with data taken from the literature.

The information provided by MS is of great help since it allows differentiation of carotenoids, because give rise to molecular weight and characteristic fragmentation patterns. On the other hand, carotenoids and its isomers are very similar in their chemical composition, having the same molecular weight and differences in the fragments ions [34]. In these cases, the information provided by and NMR and HPLC-DAD analyses contributes to unequivocal

identification. In conclusion, FUCO in oil was identified as all-*trans* fucoxanthin and FUCO in powder was identified 9'-*cis* fucoxanthin. In addition, the oil has been identified like as fatty acid.

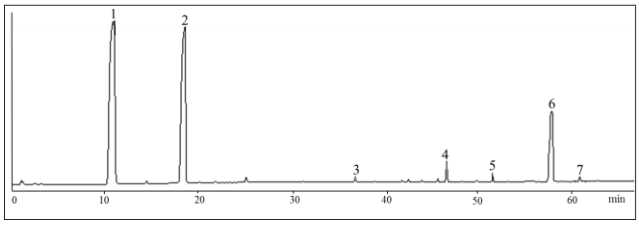

Figure 3. GC chromatogram of the FUCO in oil: (1) caprilic acid, (2) capric acid, (3) palmitic acid, (4) γ-linolenic acid and (5-7) fragments of FUCO.

3.3 Optimization of chromatographic conditions

The main problem in carotenoid analysis arises from their instability. This means precautionary to avoid quantitative losses, such as completion of the analysis within the shortest possible time, protection from light, avoiding high temperature and contact with acid [3].

The physical and chemical properties of FUCO, such as polarity and solubility will be considered for determination by HPLC. Likewise, various experimental conditions were tested and optimized by using different column temperature, flow rate, ratios of acetonitrile/water, and pH values.

Acetonitrile was selected because FUCO is insoluble in water and soluble in organic solvents and DMSO. The flow rate was altered between 0.9, 1.0, and 1.2 mL min^{-1} to shorten the time of analysis, but it was observed that the resolution was not drastically affected by increasing the flow rate. The column was heated to temperatures of 40 ºC, thus reducing the pressure, because the viscosity of the mobile phase strongly depends on the temperature. It was reported that FUCO has good thermostability, between 80 – 100 °C, for 1 h [3,35].

After selection of column temperature, flow rate and ratio of acetonitrile/water, the next step is to optimize pH in the mobile phase. According to the USP requirements, system suitability tests are an integral part of methods. These tests are used to verify that the chromatographic system is adequate for the intended analysis. Parameters affecting the chromatographic response were studied, such as number of plates (column efficiency), retention time, tailing factor, retention factor, and resolution compared with different pH values, 3.0, 5.0, 7.0, and 9.0 (Figure 4). At higher pH values, separation occurred with decreased retention times, but pH 7.0 is more efficient with a high number of plates. Resolution and tailing factor do not change at different pHs.

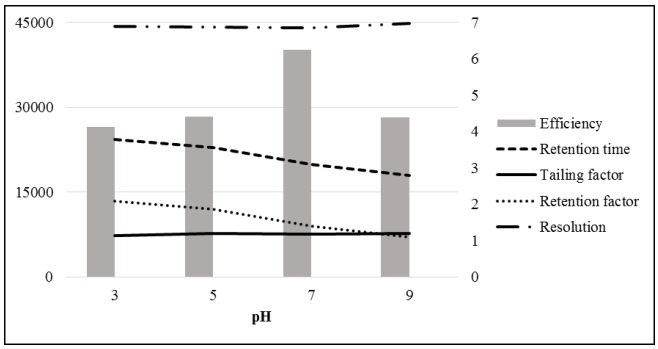

Figure 4. The effect of pH 3.0, 5.0, 7.0 and 9.0 on plates (column efficiency), retention time, tailing factor, retention factor and resolution of FUCO.

Based on the previous studies by Muthuirulappan and Francis [36] and Yip and co-workers [37], when FUCO was extracted from Malaysian brown seaweed, the stability tests showed that is most stable in pH 5.0-7.0, stored in conditions of darkness. In addition, according to Hii [38], FUCO is less stable in an acidic pH condition.

In Figure 5a shows the chromatograms obtained by HPLC-DAD and UV absorption spectra of reference standard and FUCO; FUCO eluted after 3 min, with a retention time identical to the standard reference and the purities of all the peaks were greater than 99.9%, indicating homogenous peaks. FUCO and reference standard showed an absorption spectrum with maximum at 332, 447 and 468 nm. Most carotenoids absorb maximally at three wavelengths, resulting in three peaks and the greater the number of conjugated double bonds, the higher the wavelength maximum values [3]. Other minor peak appears (retention time of 4.4 min) around the FUCO peak and the UV-VIS spectrum of this peak showed a hypochromic shift (Figure 5b).

3.3 Method validation for fucoxanthin in oil

The purpose of developing an analytical method is to provide a qualitative and quantitative result with an acceptable uncertainty level. In addition, the validation is needed to assure high quality, so that the products will be accepted by the regulatory agencies and obey the mandatory requirements for the registration of any pharmaceutical product [39,40]. The method validation

parameters have been defined in different national and international committees. However, some of the definitions vary between the different organizations and an attempt was made to harmonize them for pharmaceutical applications [24].

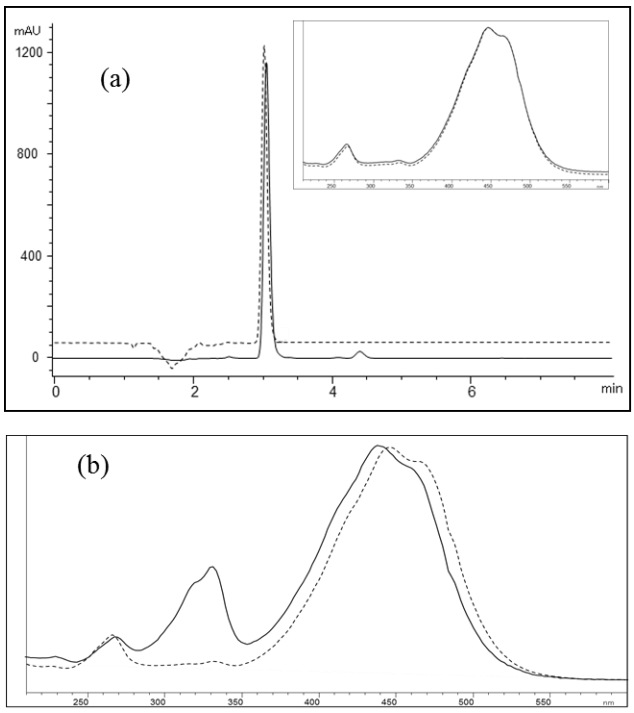

Figure 5. (a) HPLC-DAD chromatogram and UV absorption spectra (30.0 µg mL^{-1}) of reference standard (trace) and FUCO (line); (b) HPLC-DAD chromatogram and UV absorption spectra of FUCO (trace) and minor peaks (line).

Forced degradation studies

Forced degradation studies can help to identify the likely degradation products, which can in turn help establish the degradation pathways, intrinsic stability of the molecule, and presence of its potential impurities [41]. The specificity of the developed method was determined by injecting sample solutions, 30.0 µg mL^{-1}, which was prepared under stress conditions. Table 2 summarizes the forced degradation results and chromatograms of the stress tests are shown in Figure 6.

After exposure to acid and alkaline conditions, approximately 48% and 65% of FUCO were degraded, respectively. The FUCO peak was recorded at 3.2 min, under the same condition and additional peaks were seen in the chromatogram. FUCO was found to be highly labile to oxidative degradation, after 3 h, approximately 60% drug degradation was observed. Under photolytic conditions, decreases in FUCO concentration from acetonitrile solution were observed. After 4 h of exposition to UV-A irradiation approximately 26% of FUCO degradation was detected and after only 45 min of UV-C, 72% of FUCO was degraded. FUCO was found to be stable at 65 °C, indeed after 1 h, only 1.6% degraded. The minor peak (retention time of 4.4 min) maintained its area. In addition, the resolution of peaks was calculated. This

Table 2. Summary of forced degradation results of FUCO in oil subjected to degradation under acidic (HCl 0.01 *M*), basic (NaOH 0.01 *M*), photolytic (UV-A and UV-C radiation), oxidative (H_2O_2 0.3%), and thermal (65 °C) conditions.

Stress Condition	Time	Degradation	Resolution	Remarks
Reference standard	-	-	-	-
FUCO	0	-	-	-
Acidic	4 h	48.20%	6.28*	Increase area of minor peaks
Alkaline	4 h	65.20%	2.7/5.71**	Increase area of minor peaks and degradation products formed
Oxidative	3 h	59.80%	5.38*	Increase area of minor peaks
UV-A radiation	4 h	26.60%	5.52*	Increase area of minor peaks and degradation products formed
UV-C radiation	45 min	71.90%	6.03*	Significant degradation observed, increase area of minor peaks and degradation products formed
Temperature	1 h	1.60%	5.83*	-

* Resolution from the FUCO peak (3.2 min) and peak about 4.4 min

** Resolution from the FUCO peak (3.2 min) and peaks about 2.2 and 4.4 min

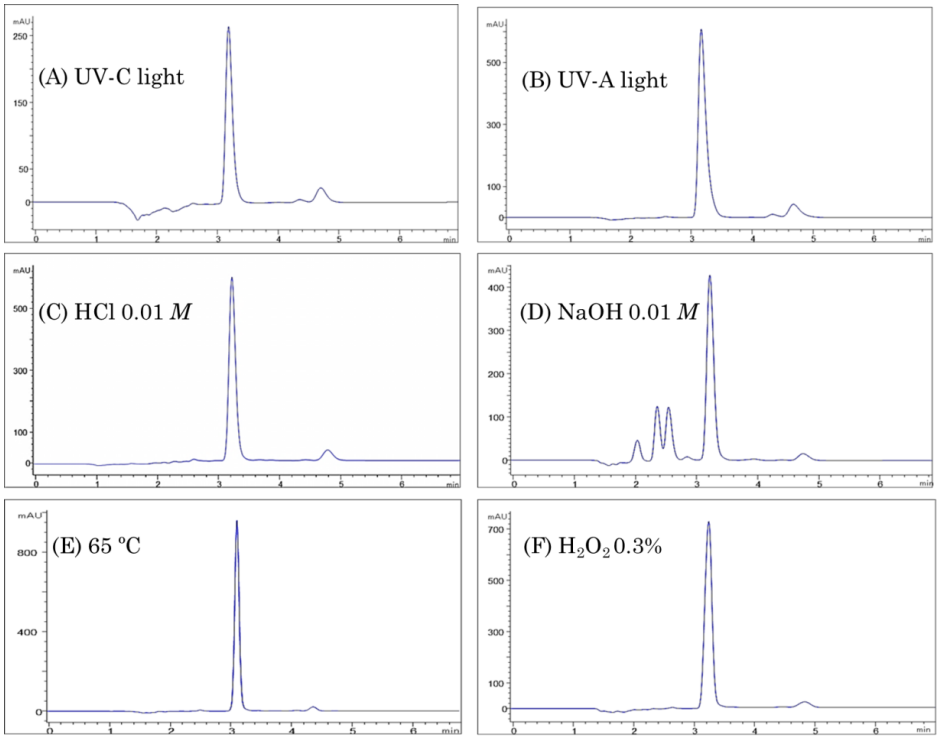

Figure 6. HPLC chromatograms obtained after forced degradation: (A) UV-C light for 45 min; (B) UV-A light for 4 h; (C) HCl 0.01 *M* for 4 h; (D) NaOH 0.01 *M* for 4 h; (E) 65 ºC during 1 hour; (F) H_2O_2 0.3% for 3 h.

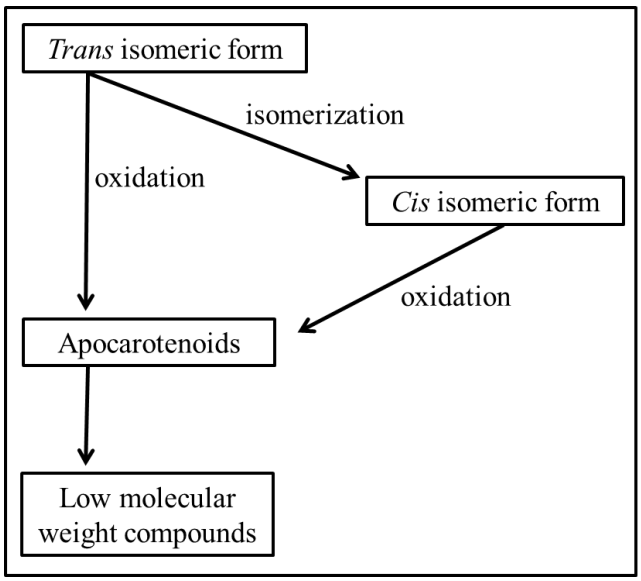

Figure 7. Possible scheme for the carotenoid degradation (Adapted from Yahia and Ornelas, [46]).

value of 2.0 or greater between two peaks will ensure that the sample may be accurately measured. Analyzing the chromatograms and values expressed in the table, all peaks were separated by this method with satisfactory resolution.

Alkaline condition and light irradiation (UV-C) were the main accelerators for the degradation of FUCO and other peaks appear in the chromatograms of the forced degradation study, demonstrating that the degradation products were detected using the optimized chromatographic conditions. During the analysis, bleaching solution of FUCO was observed under all stress conditions. Loss or change of color at any time during the analysis provides an immediate indication of degradation or structural modification [3].

Like other carotenoids, FUCO can be degraded when exposed to heat, light, oxygen and other pro-oxidant molecules, due to an extensive system of conjugated double bonds (polyene chain) [42]. The formation of some *cis* isomers by isomerization would happen which was related to treatment conditions. FUCO usually resulted in one main peak consisting of the *trans* form along with its isomers [13] and small fragments are also generated through breakdown at various carbon-carbon double bonds in the polyene chain, such as apocarotenoids (carotenoids with a shortened carbon skeleton) [43] (Figure 7).

The presence of a *cis* double bond carotenoid molecule creates greater steric hindrance between nearby hydrogen atoms and/or methyl groups, so that *cis* isomers are generally less stable thermodynamically than the *trans* form. With some double bonds, steric hindrance is

relatively small so that isomers with *cis* double bonds in these positions are relatively easily formed and relatively stable. Therefore, 9'-*cis*, 13-*cis*, and 13'-*cis* isomers were formed as main isomers during the incubation of all *trans* FUCO [30,44]. In this respect, isomerism has an effect on the absorption spectrum, with a new maximum appearing in the UV spectra (around 320–340 nm), retention time in chromatograph, melting points, solubility and molecule stability [45].

A spectrophotometric analysis of FUCO in canola oil was performed and results showed that heating caused the degradation of all-*trans* when exposed to a temperature of 25-100 ºC. By increasing the temperature, the formation of 13-*cis* and 13'-*cis* and the degradation of 9'-*cis* would also be promoted. The degradation of all-*trans* and 13-*cis* and 13'-*cis* FUCO was synergistically promoted when exposed to both air and light [47].

Linearity. Method linearity was observed over the concentrations range of 1.0 to 40.0 µg mL^{-1} at 450 nm, with significantly high coefficient correlation values (r=0.9994) for FUCO. The validity of the assay was verified by means of ANOVA, which demonstrated significant linear regression ($F_{calculated}<F_{critical}$ = 4.23; $p<0.05$) and non-significant linearity deviation ($p>0.05$), shown in Table 3.

LOD and LOQ. LOD with a signal-to-noise ratio of 3:1 was found to be 0.03 µg mL^{-1} and LOQ with a signal-to-noise ratio of 10:1 was found to be 0.10 µg mL^{-1}. These results indicate the adequate sensitivity of the method.

Precision. Precision evaluated as the repeatability (intra-day) and intermediate precision (inter-day) of the method was determined by calculating the RSD for six determinations at a concentration of 30 µg mL^{-1}. The experimental values obtained are presented in Table 3. The results indicate low RSD obtained for the repeatability and intermediate precision and showed the good precision of the method in both samples.

Accuracy. Determination of accuracy allows estimating the extent to which systematic errors affect the analytical method [40]. The accuracy of the method to quantify FUCO was calculated by percentage of recovery. The mean values were found to be 103.25% (RSD= 0.92%). These results showing satisfactory accuracy of the proposed method.

Table 3. Validation parameters of the stability-indicating HPLC-DAD method.

Parameters	Fucoxanthin
Retention time of standard (min)	3.01
Retention time of fucoxanthin (min)	3.14
Linearity	
Working range (µg mL^{-1})	1.0 – 40.0
Equation	y = 66,503x + 16,853
Coefficient correlation	0.9994
LOD (µg mL^{-1})	0.03
LOQ (µg mL^{-1})	0.1
Repeatability (RSD, %)	0.46
Accuracy (%)	103.25
Intermediate precision (RSD, %)	0.69

Robustness. The robustness refers to the signal drug's ability to remain unaffected by small and deliberate variations in method parameters. In order to evaluate the simultaneous variations of the factors in the responses considered, a multivariate approach using experimental design is recommended [40]. A response surface Box-Behnken design was applied with focus on the pH of the mobile phase, wavelength, and column temperature. According to the ANOVA of the regression model, both linear and quadratic terms were significant, with $p<0.05$ and showed a good correlation (r>0.96). There were no significant interaction effects and no evidence of inadequacy was detected by the lack-of-fit test ($p>0.05$). These results indicated that the stability indicating the HPLC-DAD method was robust since the variations applied to the experimental parameters did not produce any statistically significant effects.

3.4 Kinetic degradation

Considering preliminary stability tests, the degradation kinetics of FUCO was performed under basic conditions. FUCO degradation shows a first-order kinetics (r= 0.9918) under the experimental conditions applied. This reaction consider that the reaction velocity is directly proportional to the drug concentration. With this information, through mathematical calculations, a constant value of the decomposition of FUCO was k = 0.0363 min, the $t_{1/2}$ and $t_{90\%}$ were 19.08 and 2.92 min, respectively. The kinetics reactions were useful to determine the concentration changes as a function of

time and the kinetics run parameters. These data, applied to decomposition speed and probable reaction mechanisms involved, allow discussing the parameters related to formulation, stabilization, and administration of drugs [48].

3.5 Analysis using HPLC-DAD method of *fucoxanthin* in powder

For the HPLC-DAD analysis, the same chromatographic conditions were used, as described in item 2.3. In Figure 8 is showed the HPLC-DAD chromatogram and UV absorption spectra of reference standard and FUCO in powder (line).

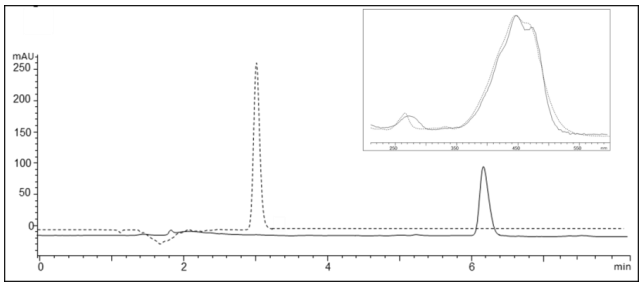

Figure 8. HPLC-DAD chromatogram and UV absorption spectra (30.0 µg mL^{-1}) of reference standard (trace) and FUCO in powder (line).

Figure 8 shows that the retention time for reference standard was 3.0 min and the retention time of FUCO in powder was 6.1 min. The maximum absorption wavelengths of both standard and FUCO in powder were similar. This peak, with retention time of 6.1 min, is believed to be *cis* isomer of FUCO, as suggested by previous studies [2,32]. Different retention times between reference standard and FUCO in powder may be due to the polarity. As reversed phase HPLC system was used, the more polar, reference standard, elute before the 9'-*cis* fucoxanthin.

It was also observed, during the analyses that the color of FUCO in powder was less intense. *Cis* isomerization of double bond causes a slight loss in color, small hypsochromic shift and hypochromic effect, accompanied by the appearance of a *cis* peak in or near the ultraviolet region. Moreover, the intensity of the *cis* band is greater as the *cis* double bond is nearer the center of the molecule [3].

Table 4 summarizes six degradations of FUCO in powder analyzed by HPLC-DAD. In the chromatograms, no additional peak was detected during the analyses. FUCO in oil, when compared with FUCO in powder, showed greater stability. Usually, *cis* isomers are thermodynamically less stable.

Table 4. Summary of forced degradation results of FUCO in powder subjected to degradation under acidic (HCl 0.01 *M*), basic (NaOH 0.01 *M*), photolytic (UV-A and UV-C radiation), oxidative (H_2O_2 0.3%), and thermal (65 °C) conditions.

Stress Condition	Time (min)	Degradation	Purity factor
FUCO in powder	-	-	99.9%
Acidic	60	85.7%	89.0%
Alkaline	60	84.5%	73.7%
Oxidation	60	75.5%	83.4%
UV-A radiation	60	43.5%	80.6%
UV-C radiation	30	90.9%	81.4%
Temperature	90	35.6%	99.8%

CONCLUSION

The purified FUCO in oil was identified as all-*trans* fucoxanthin and FUCO in powder was identified as 9'-*cis* fucoxanthin. An HPLC-DAD method has been developed for detection and quantification of FUCO. The method was validated according to current ICH guidelines and demonstrated specificity, linearity precision, accuracy, and robustness within the validated range. Furthermore, response surface and factorial designs was successfully used to test the robustness of the HPLC procedures. In forced degradation studies, all-*trans* and 9'-*cis* fucoxanthin differ substantially in their stability. The degradation kinetics of FUCO was determined, allowing kinetic parameters of basic degradation, such as rate constants (k) $t_{1/2}$ and $t_{90\%}$ to be calculated. The method is advantageous, because, besides being fast, good resolution, efficient it can be used for quantification of FUCO and its isomers. These results suggested that method can be used for the quality control of FUCO and the results will be employed of future studies in our research group for the development of nanoparticles.

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