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Fucoxanthin from marine microalga *Isochrysis galbana*: optimization of extraction methods with organic solvents

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Abstract
Fucoxanthin was the carotenoid studied from the marine microalga *Isochrysis galbana* for its importance in preventing obesity and diabetes. In this manner, seven solvents were used to fucoxanthin extraction, highlighting methanol and ethanol with 6.282 and 4.187 mg/g, respectively. However, petroleum ether and n-hexane were the worst solvents for fucoxanthin extraction, obtaining approximately 12-folds less content. Extraction time was another relevant parameter in improve fucoxanthin extraction where 10 min was the best time reaching 7.464 mg/g under 100% ethanol. Finally, we propose the use of *I. galbana* as natural source of fucoxanthin, a bioactive compound useful for food industry.

Keywords: microalgae; *Isochrysis galbana;* carotenoids; bioactive compounds; extraction solvents; extraction time.

Fucoxantina desde la microalga *Isochrysis galbana:* optimización de métodos de extracción con solventes orgánicos

Resumen
Fucoxantina fue el carotenoide estudiado desde la microalga marina *Isochrysis galbana* por su importancia en la prevención de la obesidad y la diabetes. De esta manera, siete solventes fueron usados para extraer fucoxantina destacando metanol y etanol con 6.282 and 4.187 mg/g, respectivamente. Sin embargo, éter de petróleo y n-hexano fueron los peores solventes para la extracción de fucoxantina, obteniendo aproximadamente 12 veces menos contenido. El tiempo de extracción fue otro parámetro relevante para mejorar la extracción de fucoxantina, donde 10 minutos fue el mejor tiempo para alcanzar 7.464 mg / g con 100% etanol. Finalmente, proponemos el uso de *I. galbana* como fuente natural de fucoxantina, un compuesto bioactivo útil para la industria alimentaria.

Palabras clave: microalga; *Isochrysis galbana;* carotenoides; compuestos bioactivos; solventes de extracción; tiempo de extracción.

1. Introduction

The interest in generating functional foods has increased in the last years in the market because it offers more benefits to human health. Nowadays, the industry is focused to provide healthy food from natural origins for balanced diets due to important issues in supercharged populations [1]. Therefore, recent research reports that natural biomass such as microalgae are source of bioactive compounds which are key in healthy food industry [1,2]. Particularly, carotenoids are a group with around 600 biomolecules, biosynthesized mainly by higher plants, but also by some yeasts, fungi, algae, and bacteria from isoprene units [3].

More recent studies indicate that carotenoids are composed of more than 700 structurally different compounds, typically consist of C-40 hydrocarbon backbone, and often produce cyclic and acyclic xanthophylls by modification with various oxygen containing functional groups [4]. Significant diversity of carotenoids exists in plants, animals, fungi, and micro-organisms, and obtaining

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and maintaining pure carotenoid standards is one major problem in quantification [5]. Besides, carotenoids have an important role in photosynthesis, forming pigment-protein complexes, where they harvest light energy and this energy goes to the chlorophyll by singlet-singlet excitation transfer [6,7]. In addition, carotenoids have an important role as food colorants and antioxidants with different benefits for human health. It is demonstrated that they are able to decrease stress oxidative levels by neutralizing free radicals, and preventing some types of cancer, chronic diseases, cardiovascular and eye diseases [1,8].

Due to numerous applications, there are increasing demands for carotenoids in the international market [9]. Biomolecules as astaxanthin, fucoxanthin, and lutein among others are part of broad family of carotenoids [1]. In particular, fucoxanthin showed in Fig. 1 will be the focus of this work. It is a molecule with presence of oxygen, which are known as xanthophylls. This oxygen atom can be present in different forms such as lutein, canthaxanthin, astaxanthin or as esters of alcohol being fucoxanthin [10]. Fucoxanthin has been described for its particular bioactivity in preventing obesity and diabetes [11]. These properties are due to this xanthophyll's ability to induce the activation of protein (UPC1) producing the oxidation of fatty acids in the abdominal white tissue [8,12]. Besides, fucoxanthin is reported to absorb itself better in the metabolism than lutein or astaxanthin [2].

This carotenoid is typically found in algae such as *Eisenia bicyclis, Laminaria japonica,* and *Undaria pinnatifid*a but also in microalgae such as *Phaeodactylum tricornutum* and *Odontella aurita* [13]. Particularly, *Isochrysis galbana* is a marine microalga studied for its high content in fucoxanthin compared to other species [11-14]. Moreover, *I. galbana* is known to contain other bioactive compounds such as PUFAs (polyunsaturated fatty acids) as docosahexaenoic acid (DHA) with important commercial interest [15]. In spite of the fucoxanthin market being provided from the algae industry, novel studies demonstrate that microalgae can be considered a potential source of fucoxanthin such as *I. galbana* or diatoms [16,17] adding other bioactive compounds.

Multiple reviews have been published on various extraction methods to improve the extraction efficiency from biomasses diversity. For instance, the highlighted methods could be divided into: (i) the atmospheric liquid extraction with Soxhlet (maceration, microwave or ultrasound), (ii) pressurized liquid extraction, (iii) enzyme-assisted extraction, (iv) supercritical fluid extraction, which is often based on the use of supercritical carbon dioxide (SC-CO_2) [10,18].

This preliminary study will assess the importance of contact solvent-biomass based on extraction time and the use of polar/non-polar solvent to obtain a potential functional ingredient such as fucoxanthin. Therefore, in this work will perform a previous optimization of fucoxanthin extraction methods from *I. galbana* as from: (i) solvents selection to extract and (ii) extraction time. Finally, fucoxanthin will be quantified though analytical standard methods to use *I. galbana* as the source of this potential bioactive compound for the food industry. In the future, we evaluated other green extraction technologies to quantify this bioactive compound.

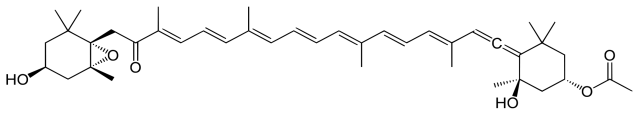

Figure 1. Chemical structure of fucoxanthin with esters of alcohol.
Source: adapted of Woo et al. [10]

2. Methodology

2.1. Materials

The marine microalga biomass of *Isochrysis galbana* was provided by "Laboratorio de Microalgas y Compuestos Bioactivos" (University of Antofagasta, Chile) where was cultured under control condition and was harvested in its exponential phase of growth. Then, this biomass was lyophilized in a freeze-dry system (Labconco Freezone 2.5L Benchtop Freeze Dry System, USA). All chemicals used in this study were of chromatographic purity. Particularly, carotenoid standards such as fucoxanthin was provided by Sigma-Aldrich with ≥ 98% purity.

2.2. UV-vis spectrum and quantification of Fucoxanthin

Fig. 2 shows the fucoxanthin UV-vis spectrum though spectrophotometer Uv-vis (Shimadzu UV- 1280, Japan) using fucoxanthin standard. This was performed to determine the maximum absorption wavelength (λ max) of this pigment. Then, a calibration curve using fucoxanthin standard (≥98% purity, Sigma-Aldrich) was carried out with concentration from 0.1 to 20 ppm measuring under λ max (447.4 nm).

Fig. 3 represents the standard calibrated curve of fucoxanthin under λ max at 447.4 nm described by the formula (1) where fucoxanthin content is calculated as mg/g.

$$\text{Fx (mg/g)} = (\text{A } 447.4 \cdot 8.665 \cdot \text{DF} \cdot \text{V})/\text{biomass} \qquad (1)$$

Where Fx had fucoxanthin content, A447.4 was the absorbance of the sample at this λ max, 8.665 was the specific slope of the standard curve, DF was dilution factor of solvent, V was the solvent volume used in mL and finally, the biomass of *I. galbana* weighted in mg.

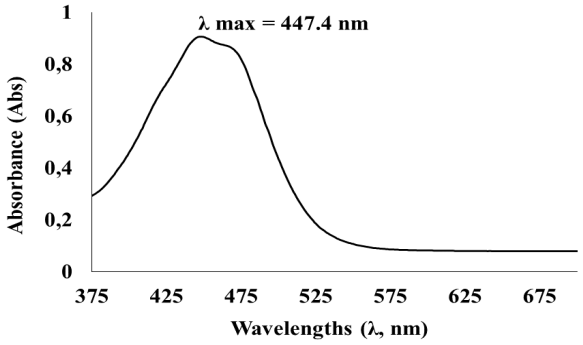

Figure 2. Fucoxanthin Uv-vis spectrum from fucoxanthin standard from 375nm to 675nm.
Source: The Authors.

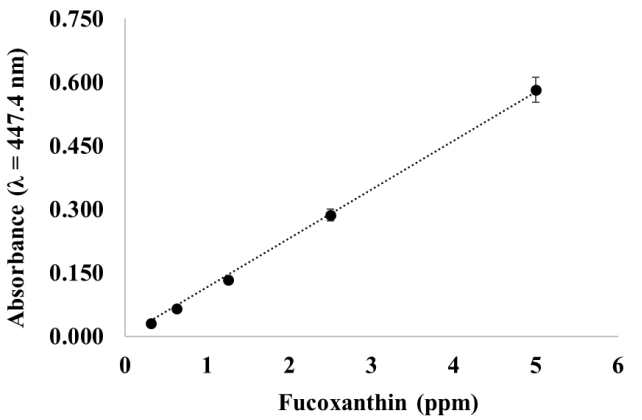

Figure 3. Fucoxanthin standard calibrate curve. This was performed with standard samples between 0.1-20 ppm of fucoxanthin. There is only showed the linear response of this calibrate curve (R=0.9988). All results are expressed as mean values from three independent experiments.
Source: The Authors.

2.3. Extraction solvents of fucoxanthin from I. galbana

Seven solvents were used under the experimental conditions for fucoxantin extraction. Solvents with different polarities were: methanol, ethanol, acetone, ethyl acetate, chloroform, n-hexane and petroleum ether. For every extraction experiment 10 mg lyophilized biomass of *I. galbana* was added with 5 mL of every solvent. Samples were put in an ultrasonic bath (Biobase, Digital Ultrasonic Cleaner, China) for 10 min at room temperature. After that, the samples were stored at 4°C and mixed at 300 rpm for 50 min in shaker incubator (Thermo Shaker Incubator MSC100). Finally, they were centrifuged at 4400 rpm for 5 min (Eppendorf 5702, Germany). This protocol was repeated in triplicate and under darkness. Finally, in this section the first extraction was only measured (*I. galbana* biomass was coloured).

2.4. Extraction times of fucoxanthin from I. galbana

The selection of extraction times was needed to determine the period of contact between biomass-solvent to reach the best fucoxanthin extraction. All samples under 5 mL selected extract solvent were put in an ultrasonic bath for 10 min at room temperature. Then, they were incubated at 4°C and mixed at 300 rpm in shaker incubator for: 5, 10 and 30 min and 1, 2, 6, 12 and 24 h. Finally, they were centrifuged at 4400 rpm for 5 min. This protocol was repeated in triplicate and under darkness until *I. galbana* biomass was colourless.

2.5. Statistics

All data was determined from three independent experiments. The selection of extractant solvents were based on theirs polarities. Mean values and standard deviations were calculated with Microsoft Excel software, and the data is expressed as the means ± standard deviation (SD). Every data was ± SD ≤ 5%. To determine the statistical differences between the mean values of the fucoxanthin concentration (mg/g) by extraction method with ethanol at different times the Analysis of Variance (ANOVA) was applied followed by the Multiple Ranges Test (MRT) of Duncan as a post hoc test to measure specific differences between the mean values. All statistical analyses employed Statgraphics Centurion XVI.1® software (StatPoint Technologies, Inc., Warrenton, VA, USA).

3. Results and discussion

The selection of accurate solvent is one of the most critical factors for efficient extraction of carotenoids. Several reports study the use of organic solvents, such as acetone, chloroform, hexane, isopropanol, methanol, methylene chloride and diethyl ether [19]. Consequently, Fig. 4 shows fucoxanthin content obtained using seven independent solvents. The best solvent to extract fucoxanthin from *I. galbana* biomass was methanol, followed by ethanol with values as 6.282 and 4.187 mg/g respectively.

A broad diversity of solvent combinations has also been used, which provides a synergistic effect on extraction of carotenoids [19]. The functional group (polarity) and chain length of the existing carotenoids are important factor to decide the extract solvent. Particularly, fucoxanthin increases the polarity due to addition of polar functional groups, such as violaxanthin and neoxanthin or lutein and zeaxanthin among others. Usually, polar solvents should be used for extraction of polar carotenoids, such as methanol or acetone.

Considering that most of the solvents possess problems with the environment, health, and safety hazards such as chronic acute toxicity and irritation [19], it is necessary to select the ideal chemical. Thus, ethanol is preferred solvent compared to hexane, methanol and chloroform which are generally used for extraction of polar and non-polar fraction molecules [19,20]. Therefore, ethanol was selected as extraction solvent of fucoxanthin from *I. galbana* in spite of methanol reached better results. It is less toxic tan methanol, allowing use this biomolecule in food industry [1,11,21]. In the case of polar solvents as acetone, ethyl acetate showed similar results than semi-polar as chloroform. Conversely,

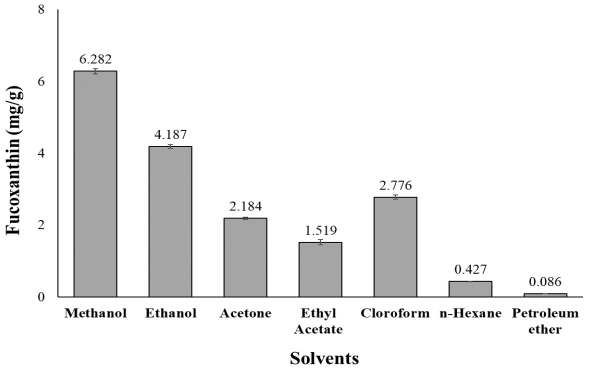

Figure 4. Fucoxanthin content (mg/g) under seven extraction solvents from freeze-dried *I. galbana.* The extracted solutions were performed as described in the Methodology section and they were analysed by spectrophotometer. Fucoxanthin contents are expressed as mean values from three independent experiments.
Source: The Authors.

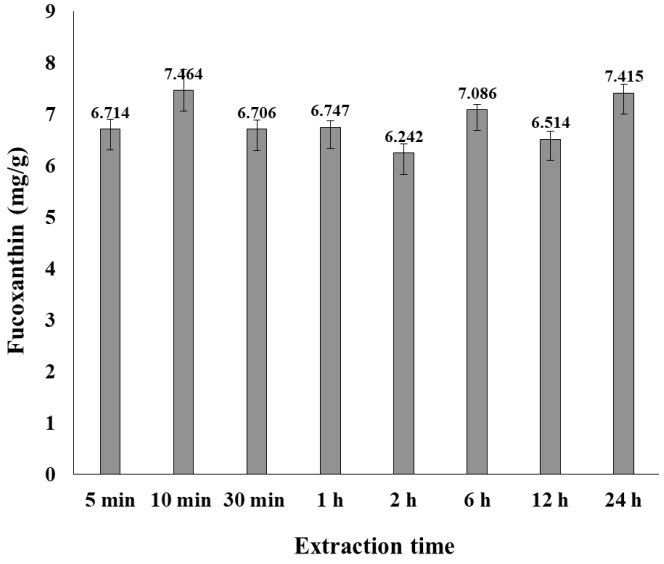

Figure 5. Concentration of fucoxanthin (mg/g) under different extraction time from freeze-dried *I. galbana.* The extracted solutions were performed as described in the Methodology section and they were analysed by spectrophotometer. Fucoxanthin contents are expressed as mean values from three independent experiments. Results in graphical form of the application of the Duncan's Multiple Range Test.
Source: The Authors.

non-polar solvents such as n-hexane and petroleum ether were the worst to extract the xanthophyll (0.427 and 0.086 mg/g, respectively). This was due to fucoxanthin being a polar carotenoid because its chemical structure contains polar functional groups [12].

On the other hand, this research also studied the extraction time necessary to obtain better fucoxanthin from *I. galbana* biomass. Fig. 5 shows the periods of time to improve the fucoxanthin extraction under ethanol solvent. Particularly, 10 min and 24 h were times without significant difference regarding fucoxanthin content (7.464 and 7.415 mg/g, respectively). Despite the fucoxanthin content being similar in both cases, the one with the least time (10 min of extraction time) was selected, because it demonstrated that the biomass-solvent contact should be less likely to degrade the pigment [11].

4. Conclusion

In conclusion, this initial research was performed to determine the best solvent for fucoxanthin extraction from *I. galbana* biomass. Ethanol was selected in favour of methanol for not being a toxic solvent for the food industry. Moreover, extraction time was a relevant parameter to improve in fucoxanthin extraction from microalga. Here, 10 min was the time selected for this under 100% ethanol reaching 7.464 mg/g of fucoxanthin. Besides, future research will be done with several fucoxanthin extractions from *I. galbana* using green extraction technologies such as super and subcritical fluid extractions. Finally, *I. galbana* could be described as natural source of potential functional ingredients useful for food industry.

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