


## Green ultrasound-assisted extraction of astaxanthin from fermented rebon shrimp (cincalok) using vegetable oils as solvents<sup>☆</sup>

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Cincalok is a typical food from West Kalimantan made from fermented rebon shrimp containing astaxanthin, the most potent antioxidant in nature. This study investigated an efficient method for extracting astaxanthin from the cincalok using vegetable oils as solvents. Olive, sesame, grape seeds, coconut, and virgin coconut oil were used as alternative solvents. The effect of various parameters on extraction yield was also studied. N-hexane and acetone were also used for comparison. Amplitude level and extraction time were the factors investigated concerning extraction yield. Comparative studies between traditional extraction methods and extraction assisted by ultrasonication have also been carried out. The astaxanthin content as total carotenoids in oil extract was analyzed using a UV-vis spectrophotometer with a standard external method. The optimum ultrasound-assisted extraction condition of astaxanthin from cincalok was 40% amplitude for 3 minutes, with 100.62  $\mu\text{g/g}$  of astaxanthin extraction yield when used virgin coconut oil as a solvent. In this way, oils enriched with astaxanthin are produced.

**Keywords:** astaxanthin / fermented shrimp / virgin coconut oil / ultrasonication / extraction

**Résumé – Extraction verte assistée par ultrasons de l’astaxanthine de la crevette rebon fermentée (cincalok) à l’aide d’huiles végétales comme solvants.** Le cincalok est un aliment typique du Kalimantan occidental (île de Bornéo, Indonésie) fait de crevettes rebon fermentées contenant de l’astaxanthine, l’antioxydant le plus puissant de la nature. Cette étude a examiné une méthode efficace pour extraire l’astaxanthine du cincalok en utilisant des huiles végétales comme solvants. L’huile d’olive, de sésame, de pépins de raisin, de noix de coco et l’huile de noix de coco vierge ont été utilisées comme solvants alternatifs. L’effet de divers paramètres sur le rendement d’extraction a également été étudié. Le N-hexane et l’acétone ont également été utilisés à des fins de comparaison. Le rendement d’extraction a été jugé sur la base de deux facteurs : l’amplitude des ondes acoustiques et le temps d’extraction. Des études comparatives entre les méthodes d’extraction traditionnelles et l’extraction assistée par ultrasons ont également été réalisées. La teneur en astaxanthine comme caroténoïdes totaux dans l’extrait d’huile a été analysée à l’aide d’un spectrophotomètre UV-vis avec une méthode externe standard. La condition optimale d’extraction assistée par ultrasons de l’astaxanthine du cincalok était une amplitude de 40 % pendant 3 minutes, avec un rendement d’extraction de 100,62  $\mu\text{g/g}$  d’astaxanthine lors de l’utilisation d’huile de coco vierge comme solvant. Ce procès permet la production d’huiles enrichies en astaxanthine.

**Mots clés :** astaxanthine / crevette fermentée / huile de coco vierge / ultrasonication / extraction

<sup>☆</sup> Contribution to the Topical Issue “Lipids from aquatic environments / Lipides issus des milieux aquatiques”.

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## 1 Introduction

Cincalok (Fig. 1) is a traditional fermented planktonic shrimp (*Acetes* sp.) widely produced in the coastal areas of West Kalimantan. Cincalok product is made from a mixture of shrimp, salt, and sugar in various compositions. Fermentation usually proceeds at ambient temperature for eight days in a closed container. Fermentation is completed when the shrimp paste becomes bright pink and develops a strong, sour, and savory flavor. A mixture of red and orange colors appears during shrimp fermentation.

Pigments in the group of carotenoids influence the color change of shrimp. Generally, carotenoid pigments in the shrimp species are astaxanthin, canthaxanthin, zeaxanthin, lutein,  $\beta$ -carotene, and other xanthophylls (Niamnuy *et al.*, 2008; Rodríguez *et al.*, 2017). However, since the amount of astaxanthin in shrimp varies between 64% and 98% of the total carotenoids, astaxanthin is usually regarded as the most important carotenoid in shrimp (Rodríguez *et al.*, 2017; Su *et al.*, 2018).

Astaxanthin (3,3'-dihydroxy- $\beta$ , $\beta$ -carotene-4,4'-dione, Fig. 2) has been reported to have many health functions. It is even more bioactive than other carotenoids such as zeaxanthin, lutein, and  $\beta$ -carotene; this is mainly due to the keto- and hydroxyl groups at each end of the molecule. Because of its molecular structure, astaxanthin has unique features that support its potential to promote human health, delaying or preventing degenerative diseases, arteriosclerosis, cataracts, and cancer. In particular, the polar end groups quench free radicals, while the double bonds of its middle segment remove high-energy electrons. These unique chemical properties explain some of its features, notably a higher antioxidant activity than other carotenoids (Ishikawa *et al.*, 2015; Kishimoto *et al.*, 2016; Davinelli *et al.*, 2018; Brotosudarmo *et al.*, 2020).

Several methods of extracting astaxanthin from shrimp have been studied such as maceration using petroleum solvent mixture (Dong *et al.*, 2014), microwave-assisted (Zhao *et al.*, 2009), and supercritical carbon dioxide extraction method (Bauer and Minceva, 2019). In the extraction cases, cell disruption has been reported as a necessary process for obtaining a higher extraction efficiency of astaxanthin (Cheong *et al.*, 2014). Generally, two types of cell disruption can be adopted: (1) the non-mechanical approach includes chemical treatment, osmotic shock, and enzymatic treatment; (2) the mechanical approach involves a high-pressure homogenizer, bead milling, grinding, and ultrasonication treatment. The advantage of using the ultrasonication approach is the relatively lower energy consumption, which is beneficial for reducing the operation cost, faster extraction, and greener processing. The principle of cell disruption by ultrasound is due to the high shear forces arising from the cavitation bubbles of ultrasonic waves and mechanical shearing, promoting the cell disruption of shrimp shells (Fabre *et al.*, 2015; Gulzar *et al.*, 2020; Vallejo-Domínguez *et al.*, 2021).

Generally, different solvents have different extraction efficiency. The majority of solvents have petroleum origins. The intricate processing steps involved in the industrial extraction cycle make it increasingly difficult to predict the overall environmental impact. The ideal alternative solvents

suitable for green extraction should have high solvency, high flash points with low toxicity and low environmental impacts, be readily biodegradable, and be obtained from renewable (non-petrochemical) resources at a reasonable price. They should be easy to recycle without any deleterious effect on the environment (Chemat *et al.*, 2019). Due to astaxanthin solubility in the oil (Ambati *et al.*, 2014), one promising alternative to the traditional processes is to substitute organic solvents with vegetable oils. Apart from being environmentally friendly and a sustainable extraction method, oil plays a barrier against oxygen and consequently retards the astaxanthin extract's oxidation time and degradation rate.

The appropriate extraction method is a critical factor affecting the final yield and physicochemical properties of astaxanthin obtained. Therefore, in this study, ultrasound-assisted extraction of astaxanthin from cincalok using variations of vegetable oil as solvents has been done to develop greener, sustainable, and viable industries.

## 2 Materials and method

### 2.1 Preparation of Cincalok

Cincalok was made using fresh *Acetes* shrimp directly taken from fishers. Cincalok was prepared by the traditional method with some modifications. The shrimp was cleaned with saltwater and sorted from other sea animals. Sugar and salt were added to the shrimp by a ratio of 10:1:1 (w/w/w), respectively, then appropriately mixed. The mixture was kept in a closed plastic container and maintained at room temperature without light for eight days. Cincalok produced was filtered, and the residue was dried at 50 °C for 3 hours using vacuum drying. Dry cincalok was ground using a screw press and ready for further treatment.

### 2.2 Extraction of astaxanthin from cincalok using ultrasonication methods

Ultrasound-assisted extraction was carried out by a Vibra Cell-750 HV (20 kHz, probe 13 mm) ultrasonic processor. A sample of 2 g of dry cincalok was dispersed in 10 mL of different treatments of vegetable oil and inserted into a 20 mL plastic vessel. The probe was immersed to a depth of 3 cm. Ultrasonication total time was varied for 1 to 5 minutes, with 5 seconds of ultrasonication and 5 seconds of resting time to prevent overheating the sample. Separation was conducted by filtration using a syringe filter (0.43  $\mu$ m), and the supernatant was analyzed for astaxanthin yield. This extraction method was also performed by varying amplitude from 20, 30, and 40 %. The mixture was filtered, the filtrate was analyzed using UV-Vis spectrophotometers. Each treatment was carried out in the darkroom for three replications. The data presented in this paper are average values.

### 2.3 Extraction of astaxanthin from cincalok using conventional methods

Two grams of dried cincalok was added into 10 mL of vegetable oil. The mixture was blended for 30 minutes, macerated for 24 hours, and filtered. Each treatment was



**Fig. 1.** Cincalok.

carried out in the darkroom for three replications. Each filtrate that contains astaxanthin was measured by a UV-Vis spectrophotometer. The data presented in this paper are average values.

#### 2.4 Determination of astaxanthin as total carotenoid

The astaxanthin as total carotenoid concentration extracted in each oil was measured spectrophotometrically using modified form the procedure by [Goula \*et al.\* \(2017\)](#) and [Corbu \*et al.\* \(2019\)](#). For the first experimental step, the astaxanthin analytical standard was dissolved in acetone to prepare standard solutions at concentrations of 0.5 until 7.0 mg/L. The analyses were carried out by measuring the absorbance in the range 350–650 nm using a UV-Vis Spectrophotometer U-1800 SHIMADZU. The spectrum was used to identify the wavelengths for the maximum absorbance of astaxanthin. Linear regression was applied to calculate the standard curve. The coefficients of determination ( $R^2$ ) value (0.9996) revealed good linearity over the wavelength of 477 nm for astaxanthin was shown in [Figure 3](#).

Each extracted filtrate was pipetted as much as 1 mL and put into a 5 mL volumetric flask, then acetone was added until the mark. The mixture was shaken until homogeneous, the absorbance was read at the wavelength obtained previously from the standard astaxanthin. Pure vegetable oil before extraction was also given the same treatment to see their absorption spectra in acetone in the wavelength range of 300–700 nm. The absorbance of the extracted filtrate at the maximum wavelength obtained is then reduced by the absorbance value of vegetable oil at the same wavelength (if there was absorption) as a correction factor, then converted in units of concentration through the resulting equation from the standard curve obtained.

### 3 Results and discussion

Fermentation continued by an extraction process using green solvents is an eco-friendly and straightforward method to recover astaxanthin from shrimp ([Cheong \*et al.\*, 2014](#); [El-Bialy and El-Khalek, 2020](#)). An essential issue in the recovery of astaxanthin from shrimp shells is the initial water content ([Hu \*et al.\*, 2019](#)). The high-water content of food sources is considered a negative factor for an efficient carotenoid extraction. Thus, the superiority of the recovering yield from dry solid samples over submerged ones could be

attributed to the extraction efficiency, not to the fermentation process itself. For this reason, in this work, before the extraction process, cincalok as fermented rebon shrimp was dried at 50 °C in vacuum drying for eliminating the water content up to 3.8%.

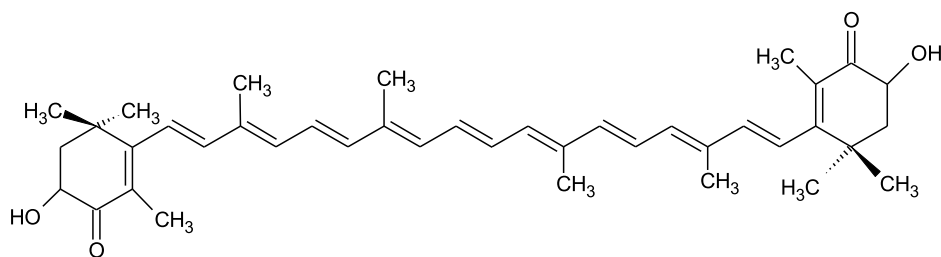
As shown in [Figure 4](#), the oil turns reddish during the extraction process, indicating that the astaxanthin is penetrated into the oils. The reddish color of astaxanthin is due to a conjugated polyene structure. [Li \*et al.\* \(2013\)](#) reported that the endogenous amphiphilic content of vegetable oils and the polyunsaturation degree of triglycerides play an important role in utilizing green solvents for recovering natural pigments such as astaxanthin. The astaxanthin inside the cell was extracted into the vegetable oil phase by hydrophobic interactions.

#### 3.1 Effect of time on the astaxanthin extraction yield

The effect of extraction time on the astaxanthin yield was investigated. In this work, extraction was carried out at amplitudes of 30%. [Figure 5](#) shows the astaxanthin extraction yield was time-dependent and increased with extended ultrasonic times, especially from 1 to 3 minutes, but slowly from 3 to 5 minutes. Thus, the efficient extraction period for achieving the maximum yield of astaxanthin was about 3 minutes. This can be attributed to the fact that extraction presents two stages. The first stage is characterized by a rapid rate, involves the solvent's penetration into the cellular structure followed by the dissolution of soluble constituents in the solvent. In contrast, the second one involves the external diffusion of soluble constituents through the porous structure of the residual solids and its transfer from the solution in contact with the particles to the solution's bulk ([Goula \*et al.\*, 2017](#)).

A similar trend was reported by [Li \*et al.\*, \(2013\)](#), [Zhou \*et al.\* \(2013\)](#), and [Goula \*et al.\* \(2017\)](#), who extracted  $\beta$ -carotene from sunflower oil, astaxanthin from *Haematococcus Pluvialis*, and carotenoids from pomegranate wastes, respectively. They attributed this observation that ultrasonic waves could disrupt the cell walls, so a larger contact area between solvent and material was created and more oil appeared on the surface. However, this effect would be increasingly weak on the inner cell walls as the distance is increased. Thus, the ultrasonic waves affect the mass transfer rate mainly in the solvent penetration stage.

In this work, after 3 minutes of extraction, the astaxanthin concentrations obtained depending on the applied extraction solvent were 32.54, 53.46, 26.57, 46.34, and 100.62  $\mu\text{g}$  astaxanthin/g of dry cincalok using olive, sesame, grape seeds, coconut, and virgin coconut oil respectively ([Fig. 5](#)). A low solvent viscosity is usually associated with an improved solvent migration through the matrix to increase extraction efficiency during an extraction process. Thus, the highest extraction yield obtained for virgin coconut oil may be attributed to its lowest viscosity. The viscous medium prevents the distribution of ultrasonic waves and the formation of cavitation bubbles ([Challis and Pinfield, 2014](#)), thus decreasing the mass transfer and extraction yield, as seen during the present study. Some previous studies supported this phenomenon while extracting astaxanthin using natural deep eutectic solvent ([Zhang \*et al.\*, 2014](#); [Roy \*et al.\*, 2021](#)). [Table 1](#)



**Fig. 2.** Chemical structure of astaxanthin.

shows the viscosity values of oils used in this work, which was determined using an Ostwald viscometer.

The higher astaxanthin extracted in sesame oil than grape seeds and coconut oil may be attributed to its higher free fatty acid value (as shown in [Tab. 1](#)). The extraction yield of astaxanthin increased with the presence of acid. Regarding organic solvents, there was no significant difference between acetone and n-hexane. The recovery of astaxanthin in these solvents was not higher than using virgin coconut oil as a solvent. The reasons were probably that the polarity of acetone and n-hexane is lower than the vegetable oil used in this study, so astaxanthin is easier to extract using vegetable oil, which contains long-chain fatty acids.

### 3.2 Effect of amplitude on the astaxanthin extraction yield

The effect of ultrasound amplitude on the astaxanthin yield was also investigated. In this study, extraction was carried out at different amplitudes while extraction time parameters were constant (3 minutes). The system temperatures for 20, 30, and 40% amplitudes were recorded at 45, 60, and 90 °C, respectively. The extraction yield improved with an increased amplitude level up to values 40% ([Fig. 6](#)). This effect may be due to ultrasound's improved cavitation and mechanical effect, which increased the contact surface area between solid and liquid surfaces and caused greater penetration of the solvent into the peel matrix. At high amplitude levels, cavitation bubble collapse is more violent. According to [Zhang \*et al.\* \(2014\)](#), since the temperature and pressure were very high inside the bubbles and the collapse of bubbles occurred over a very short time, a violent shock wave and a high-speed jet were generated, which could enhance the penetration of the solvent into the cell tissues and accelerate the intracellular product release into the solvent by disrupting the cell walls. The astaxanthin concentrations obtained at amplitude level 40% were 46.31, 67.33, 34.60, 61.68, 100.56, 55.61, and 53.52 µg astaxanthin/g of dry cincalok using olive, sesame, grape seeds, coconut, virgin coconut oil, acetone, and n-hexane, respectively.

An increase in amplitude accompanies the increase in temperature. Temperature is also an important factor in the extraction of heat-sensitive compounds. Along with the increase of temperature, the solvent diffusion rate and the mass transfer intensification result in the dissolution of objective components. In this work, the system temperatures for amplitudes of 20, 30, and 40 % were recorded at 45, 60, and

90 °C, respectively. As shown in [Figure 6](#), the effect of temperature on the astaxanthin content extracted by vegetable oils was significant. Increasing extraction temperature from 45 °C to 90 °C causes increasing extraction yield. The phenomenon may be attributed to the fact that an increase in temperature increases solubility and diffusion coefficients of the compounds to be extracted and decreases the solvent's viscosity, thus facilitating its passage through the solid substrate mass. Under the conditions of this study, no lipid oxidation was observed. This is supported by several studies which reported that lipid oxidation occurred above 100 °C ([Gertz \*et al.\*, 2014](#); [Vaskova and Buckova, 2015](#)).

### 3.3 Comparative studies with the conventional method

The extraction method by maceration at room temperature for 24 hours was also carried out as a comparative study. In all experiments, the conventional method showed lower astaxanthin extraction yields than the ultrasonication method performed at 30% amplitude for 3 minutes (see [Fig. 7](#)). The enhancement in extraction obtained by using ultrasound is mainly attributed to the effect of acoustic cavitation produced in the solvent by the passage of an ultrasound wave. Ultrasound also exerts a mechanical effect, allowing greater solvent penetration into the tissue and increasing the contact surface between the solid and liquid phases. As a result, the solute quickly diffuses from the solid phase to the solvent. Based on the experiments that have been done, virgin coconut oil shows the best astaxanthin extraction ability.

### 3.4 Before and after treatment fatty acid profile

The gas chromatography analysis has been carried out to determine the fatty acid profile of virgin coconut oil before and after extraction. The results are presented in [Table 2](#). Although astaxanthin's lipid nature, the result showed no significant difference between the fatty acid profile of virgin coconut oil before and after astaxanthin extraction from cincalok using the ultrasonic method. A similar trend was reported by [Gutte \*et al.\* \(2015\)](#), who showed no significant effects on flaxseed fatty acid profile by ultrasonic-assisted extraction. [Chemat \*et al.\* \(2004a, 2004b\)](#) also reported no significant change in the lipid profile of sunflower oil due to the ultrasonication process in a relatively short time and low temperature. This extraction condition could prevent the oil oxidation effect from the metal horn ([Pingret \*et al.\*, 2012](#)).

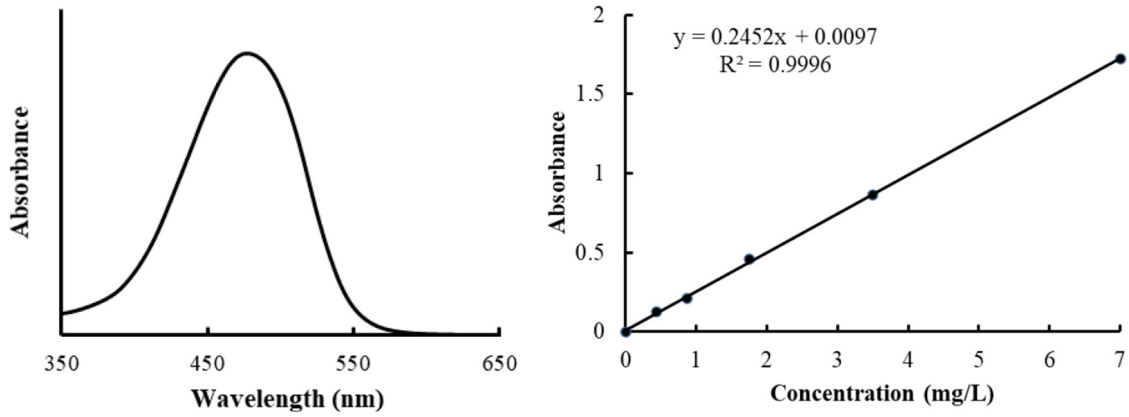


Fig. 3. Spectrum and a calibration curve of astaxanthin.

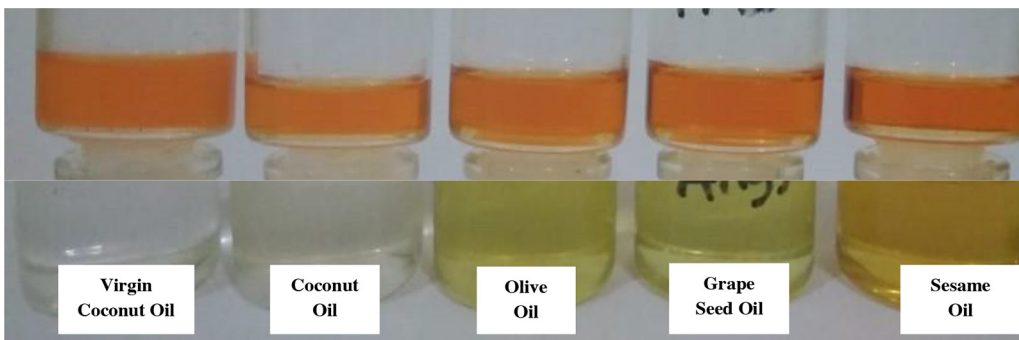


Fig. 4. Vegetable oil used (bottom) and supernatant extracted using ultrasonication (top).

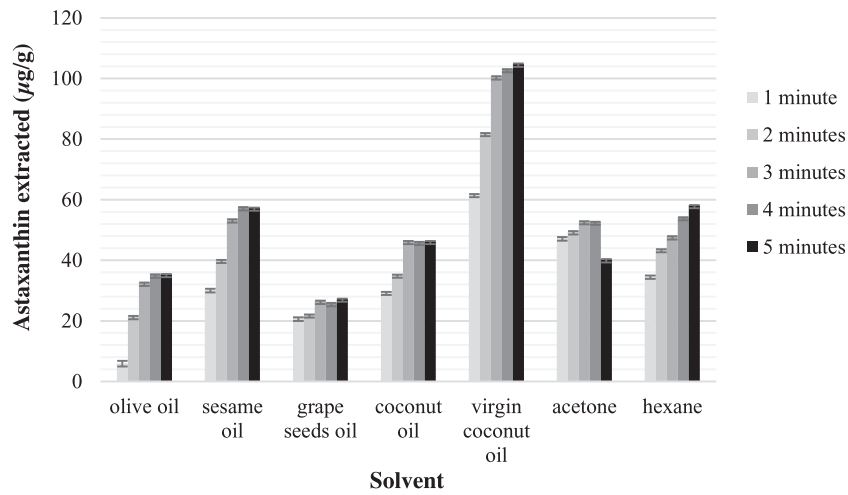


Fig. 5. Effect of ultrasonic times on the astaxanthin extraction yield.

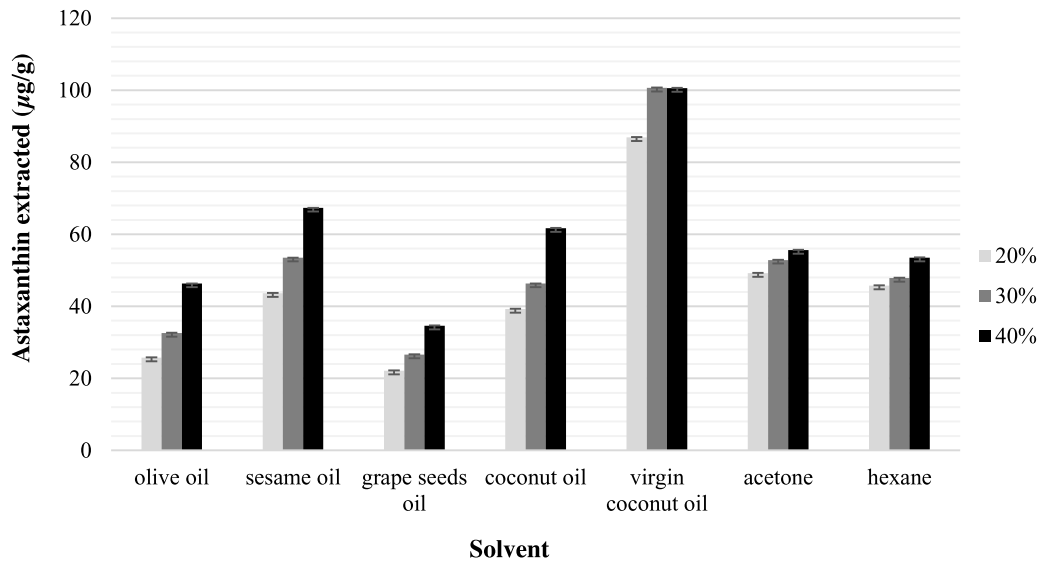


Fig. 6. Effect of ultrasonic amplitude on the astaxanthin extraction yield.

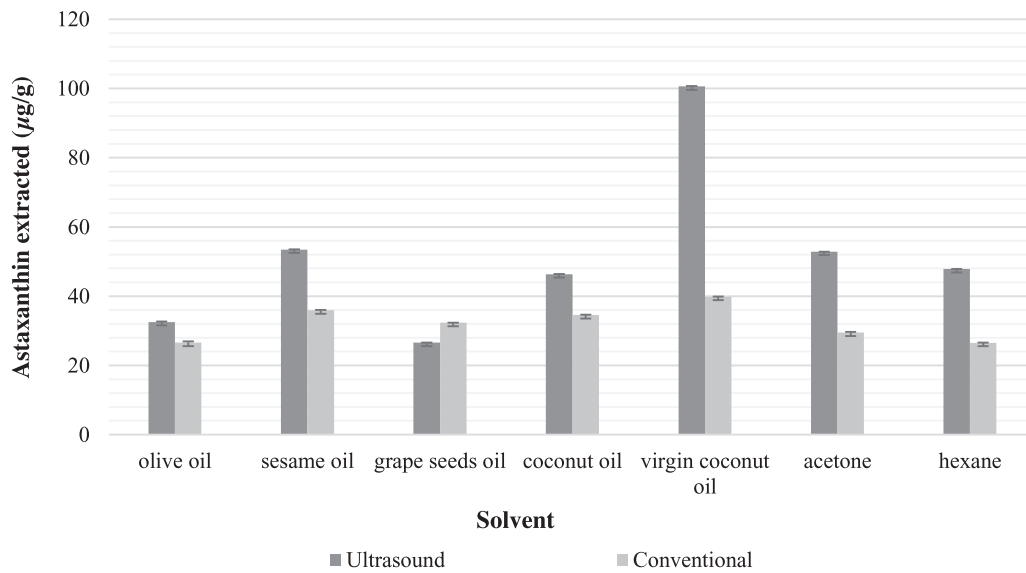


Fig. 7. Comparative studies with the conventional method.

Table 1. Physico-chemical properties of oils used.

Oil	Viscosity (cSt) at room temperature	Free Fatty Acid Value (%)
Olive	47.85	0.4096 a
Sesame	46.09	0.5632 b
Grape seeds	36.36	0.2812 b
Coconut	30.78	0.3072 c
Virgin coconut	27.67	0.3584 c
Acetone	0.37	–
n-hexane	0.49	–

FFA values were expressed in oleic acid (a), linoleic acid (b), lauric acid (c).

**Table 2.** Fatty acid profile of virgin coconut oil before and after astaxanthin extraction from cinalok using the ultrasonic method.

Fatty acid	Virgin coconut oil before astaxanthin extraction (%)	Virgin coconut oil after astaxanthin extraction (%)
Butyric acid (C4:0)	1.93	2.13
Hexanoic acid (C6:0)	0.40	0.41
Octanoic acid (C8:0)	7.34	7.21
Decanoic acid (C10:0)	6.29	6.14
Lauric acid (C12:0)	48.49	47.75
Tetradecanoic acid (C14:0)	17.49	17.32
Palmitic acid (C16:0)	8.39	8.67
<i>Trans</i> -9 elaidic acid (C18:1)	2.48	2.62
<i>Cis</i> -9-oleic acid (C18:1)	5.97	6.37
Linoleic acid (C18:2)	1.21	1.26
<i>Cis</i> -13-16-docosadienoic acid (C22:2n-6,9)	-	0.12

## 4 Conclusion

Ultrasound-assisted extraction has been developed and demonstrated to be a green bio-refining technique to extract astaxanthin from cinalok with various advantages in terms of time and yield. Virgin coconut oil has been proven to be a “green” substitute for petroleum-based solvents that can be safely used in extraction. The best extraction results were achieved in 3 minutes with an amplitude of 30%. In addition, there is no need for subsequent separation of oil and astaxanthin since the pigmented oil can find use as an astaxanthin source in different products. However, the perspective of a Response Surface Methodology (RSM) to better understand the action of each parameter with the indication of the oil characteristics can be applied for future investigation.

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