Extraction and Determination of Astaxanthin Pigment From *Haematococcus pluvialis* Microalgae

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Abstract

Astaxanthin and β-Carotene are well-known carotenoids globally, covering more than half of the market demand for carotenoids. *Haematococcus pluvialis* microalgae are one of the most important sources of natural astaxanthin, consisting of up to 4% of its dry weight. The most critical challenge for this microalgae is the breakdown of the wall and the extraction of the pigment. In this study, chemical methods, including acid, acetone, and ionic solution, and physical processes such as ultrasound waves and magnetic stirrer, were used to break down the cell wall and meas-ure total astaxanthin in *H. pluvialis*, respectively. Due to the rapid oxidation of the pigment, in the next step, to extract and store astaxanthin from damaged cells, use olive oil. A spectrophotometer examined astaxanthin, monoester, and diester derivatives, and their amount was determined by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The results showed that using acid treatment, ultrasound waves, and extraction by acetone is the best method to measure the amount of astaxanthin in the algae. The HPLC results also showed that the amount of astaxanthin monoester (88.44%) was higher than the free forms (3.76%) and diester (7.82%) in the total content of extracted astaxanthin. In addition, the amount of total astaxanthin in the *H. pluvialis* was about 1.6% of the dry weight of the algae.

Keywords: *H. pluvialis*, Astaxanthin, Cell Disruption, Microalgae, Ultrasound Waves

Introduction

Microalgae are microorganisms that can grow in both freshwater and saltwater. Already, research on microalgae was related to the production of biofuels. Still, now they are used in the large scale production of unique and more valuable materials in large quantities, which can be helpful in the Pharmaceutical, Food, and Cosmetics Industries (Borowitzka, 2013; Koller et al., 2014; Panis and Carreon, 2016). Astaxanthin and β-Carotene are well known carotenoids globally, covering more than half of the market demand for carotenoids. Microalgae can produce valuable bio-

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chem-icals, including carotenoids, and act as a natural pool for making these substances (Suseela and Toppo, 2006). Astaxanthin is one of the most valuable carotenoids, and its global market is expected to reach 3.5 billion$ by 2026 (Basiony et al., 2022). The oxidative capacity of astaxanthin is ten times higher than other carotenoids. So, the antioxidant properties of astaxanthin make it a suitable candidate for application in aquaculture and food industries. (Reyes et al., 2014). *H. pluvialis* is the richest source of astaxanthin (1.5-4.5% dry weight of the algae) (Machmudah et al., 2006). Noroozi et al. (2012) showed two steps of cultivation are needed to produce astaxanthin. The first step is related to the green phase (bio-mass production), and the second step is associated with the red phase (astaxanthin production) (Noroozi et al., 2012). The algae cyst stored astaxanthin in a thick, rigid cell wall (Keykha akhar et al., 2021). Different methods are used to destroy the cyst cell wall to extract astaxanthin. However, the temperature and chemical stresses caused by some physicochemical extraction methods can change the chemical structure of astaxanthin, destroy astaxanthin isomers, and directly affect the quality and value of astaxanthin. The chemical composition and nature of microalgae cell walls are essential factors that should be considered to select the appropriate destruction method for recovering valuable intracellular products such as carotenoids (Cheng et al., 2015). Methods of cell wall destruction are divided into four groups: chemical, physical, physicochemical, and biological processes (Kim et al., 2016). This study extracted astaxanthin pigment from *H.pluvialis* microalgae native to Iran. After determining astaxanthin percentages in algae, also measured the amount of the oil phase (Li et al., 2012; Kim et al., 2016; Kang and Sim, 2008; Liu et al., 2018).

Material and methods

H. pluvialis microalgae powder in the red phase was prepared from Qeshm Microalgae Biorefinery. Compounds included standard Astaxanthin and 3-methyl-3-butyl imidazolium chloride (Sigma Com.) as an ionic solution, hydrochloric acid, acetone, hexane, methanol, ethyl acetate, dimethyl sulfoxide (DMSO), and Merck TLC 60 (from TLC Silica gel 60 F from MERCK), olive oil, and devices included ultrasonic cleaner bath SB-103 D (made in Korea), hot water bath (bain-marie), GFL - Gesellschaft für Labortechnik (made in Germany), HERMLE Labortechnik centrifuge (made in Germany), the HPLC, Knauer Series Wellchrom solvent organizer (K-1500) (made in Germany), and the Bio-Tek microplate reader Model Epoch (made in the USA).

Determination of astaxanthin percentage

Extraction methods with ionic solution, acid, acetone, and combination were used to determine the percentage of astaxanthin in *H. pluvialis*.

Ionic solution

Recently, ionic solutions have been used for the green extraction of carotenoids, which

methyl-3-butyl imidazolium chloride is one of the best ionic solutions with the highest efficiency (Saini and Keum, 2018, Kim et al., 2016, Desai et al., 2016) First, 10 mg of *H. pluvialis* powder was transferred to two Falcon tubes, then, 1 ml of 40% and 60% ionic solutions were added to it, and then it was placed in an ultrasonic bath (one hour), after that, the ionic solution was separated by centrifugation at a rate of 12000 g (10 minutes), and then it was washed twice with water after that 4 ml of acetone were added to it, and then the solution was placed on a shaker (1 hour) and separated in acetone by centrifugation at 12000 g (10 minutes). This operation was repeated twice, and finally, the acetone was brought to the desired volume in a 10 ml volumetric flask, and the absorption spectrum of the solution was recorded in the range of 350 to 700 nm (Figure 1). This experiment was repeated three times.

Ionic solution and ultrasound

First, 10 mg of *H. pluvialis* powder was transferred to three falcon tubes, then 1 ml of 40% ionic solution was added to it, and after that, it was placed in an ultrasonic bath (30, 60, and 120 minutes). The ionic solution was then separated, and the precipitates were washed twice with dis-tilled water. Finally, they were incubated twice (one hour), each time with 4 ml of acetone, and the acetone was brought to the desired volume in a 10 ml volumetric flask, and the absorption spectrum of the solution was recorded in the range of 350 to 700 nm (Figure 2). This experiment was repeated three times.

Ionic/acid solution/ultrasound

First, 10 mg of *H. pluvialis* powder was added to two Falcon tubes, then one ml of 40% ionic solution was added to one pellet and placed in a 60 °C water bath (one hour). Then, 1 ml of 4 M HCl was added to the other pellet, and it was placed in an ultrasonic bath at 70 °C (30 minutes). After that, the sample was centrifuged, the acid was removed, and then it was washed, and one milliliter of 40% ionic solution was added to the pellet, and then it was placed a 60 °C water bath (one hour). After that, the sample was centrifuged, separated, and washed. Then, acetone was added twice

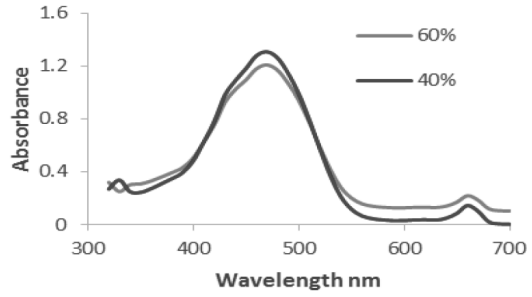

Fig. 1. UV-Vis absorption spectra of astaxanthin extracted by 40 and 60% ionic liquid

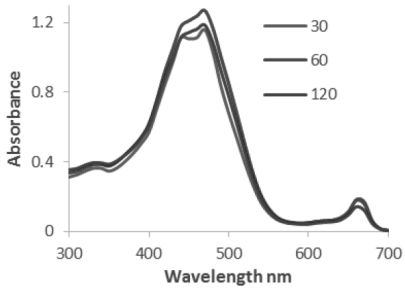

Fig. 2. UV-Vis absorption spectra of astaxanthin extracted by ionic liquid treatment and ultrasound waves at times of 30, 60 and 120 minutes

to the remaining pellets, 4 ml each time, and then it was placed in the darkness (one hour), and finally, the acetone was separated by centrifugation. It was brought to the desired volume in a 10 ml volumetric flask, and the absorption spectrum of the solution was recorded in the range of 350 to 700 nm (Figure 3). This experiment was repeated three times.

Acid and acetone

One of the high efficiency extraction methods is to use acid to break down the cell wall of *H. pluvialis* and acetone to extract astaxanthin.

First, 10 mg of *H. pluvialis* was added to two containers. Then, 1 ml of 4 M HCl acid was added to the first container, and it was placed in a water bath (1 hour) at 70° C, and 1 ml of 4 M HCl acid was added to the second container, and it was placed in a 70° C bath on a magnetic stirrer. The acid was then removed by centrifugation, and the remaining pellets were washed with water. Acetone was added to the remaining pellets twice, 4 ml each time, and then it was placed in the darkness (one hour), and finally, the acetone was separated by centrifugation. It was brought to the desired volume in a 10 ml volumetric flask, and the absorption spectrum of the solution was recorded in the range of 350 to 700 nm (Figure 4). This experiment was repeated three times.

Acid, acetone/water bath, and glass bead sterilizers

Three microtubules containing 10 mg of *H. pluvialis* and 1 ml of 4 M HCl were prepared. The first sample was placed in a water bath at 70 °C (one hour). The second sample was first rubbed with glass bead sterilizers in a mortar, and then it was placed in the water bath (one hour). The third sample was placed in an ultrasonic bath at 70 °C (half an hour), and then it was placed in a water bath (half an hour). After one hour, the supernatant of all three centrifuged samples was discarded, and the pellets were washed twice with distilled water. Acetone was added to all samples in two stages, and after separation, they were brought to the desired volume in a 10 ml

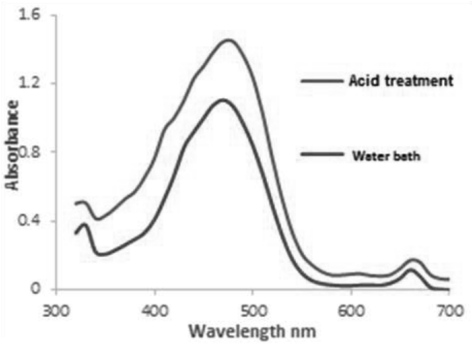

Fig. 3. UV-Vis absorption spectra of astaxanthin extracted using ionic liquid with acidic treatments and water bath

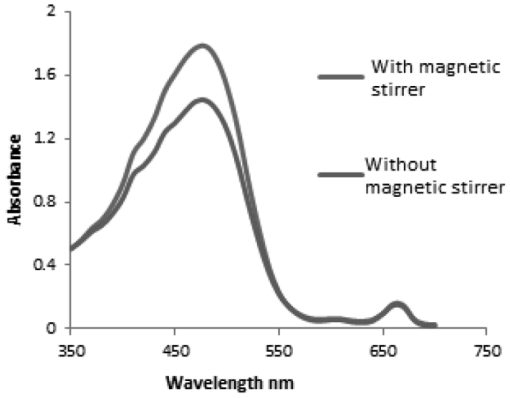

Fig. 4. UV-Vis absorption spectra of astaxanthin extracted by two methods using magnetic stirrer and without magnetic stirrer

volumetric flask. The absorption spectrum was recorded at 300 to 700 nm (Figure 5). This exper-iment was repeated three times.

Acid, acetone, and ultrasound

Two treatments (30 and 45 minutes) were examined in this test. In this test, 10 mg *H. pluvialis* powder was used for each sample. In the first case, with three replications, 1 ml of 4 M HCl was added to *H. pluvialis* powder, and then it was placed in an ultrasonic bath at 70 °C (30 minutes), and then it was placed in a water bath at 70 °C (30 minutes). In the second case, the sample was placed in an ultrasonic bath (45 minutes), and then it was placed in a water bath (15 minutes). After that, both samples were centrifuged, separated, and washed. Then acetone was added to each in two steps, and finally, it was brought to the volume of 10 ml. The absorption spectrum was recorded in the wavelength range of 300 to 700 nm (Figure 6).

Extraction of astaxanthin by olive oil with acid pretreatment and ultrasound waves

First, 100 mg of the *H. pluvialis* powder and 5 ml of 4 M HCl were added to it, and it was placed in an ultrasonic bath at 70° C (30 minutes). After completion of the ultrasound, the acid was separated from the sample by centrifugation, and then it was washed twice with distilled water. Then the remaining pellet was made up of 30 ml of distilled water, and 30 ml of olive oil was added to it and then placed on a magnetic stirrer. The absorption spectrum of the oil was recorded at a wavelength of 300 to 700 nm at 24 and 48 hours (Figure 7). After 48 hours, the sample was taken out of the water, and the sediments were separated and kept in a vial.

The extraction of olive oil with acid/bath-water pretreatment

First, 100 mg of *H. pluvialis* powder with 5 ml of 4 M HCl was placed in bath water at 70° C (one hour). After that, the acid was separated from the sample, and then it was washed with distilled water in two steps. After centrifugation, 30 ml of distilled water and 30 ml of olive oil were added to the remaining pellet, and an Erlenmeyer flask

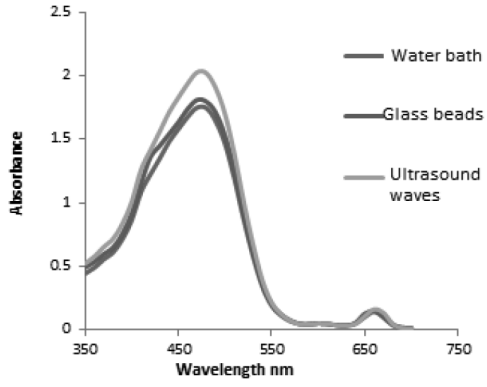

Fig. 5. UV-Vis absorption spectra of astaxanthin extracted by three methods using water bath, glass beads and ultrasound waves

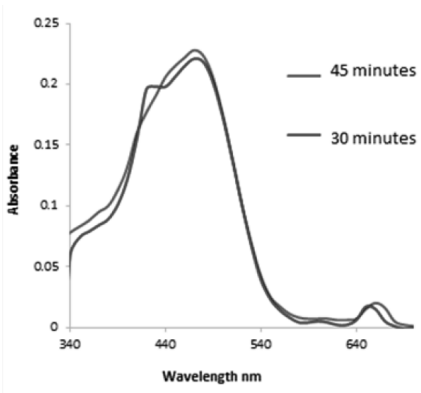

Fig. 6. UV-Vis absorption spectra of astaxanthin extracted by ultrasound pretreatment at 30 and 45 minutes

was placed on the stirrer. Then the absorption spectrum was recorded at 24 and 48 hours (Figure 8). This experiment was repeated three times.

Standard curve

To draw the standard curve of astaxanthin in acetone, first, a solution with the concentration of 1.61×10^{-4} M was prepared, and then five solutions with concentrations of 1.60×10^{-6}, 3.21×10^{-6}, 4.82×10^{-6}, 6.43×10^{-6}, and 9.65×10^{-6} were designed. In the next step, the maximum adsorption of each solution was measured with a spectrophotometer, and the adsorption curve was drawn in front of the concentration. Then the line equation was obtained, and the solutions obtained from the algae extraction were used to determine the concentration (Figure 9). To draw the standard curve of astaxanthin in olive oil, five solutions with concentrations of 8.05×10^{-7}, 1.61×10^{-6}, 4.83×10^{-6}, 9.66×10^{-6}, and 1.93×10^{-5} were also prepared. Then, their absorption spectrum was recorded at 300 to 700 nm (Figure 10).

Chromatography

Thin layer chromatography (TLC) and High performance liquid chromatography (HPLC) were used to identify and measure astaxanthin and its derivatives.

The use of thin-layer *chromatography to*

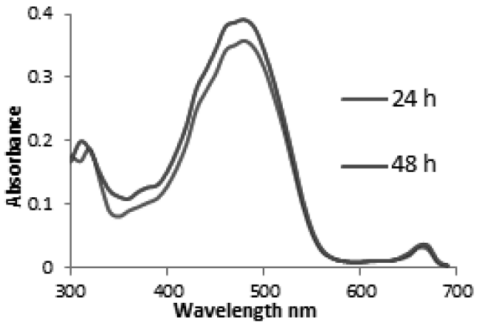

Fig. 7. UV-Vis absorption spectra of astaxanthin extracted by olive oil after 24 and 48 hours

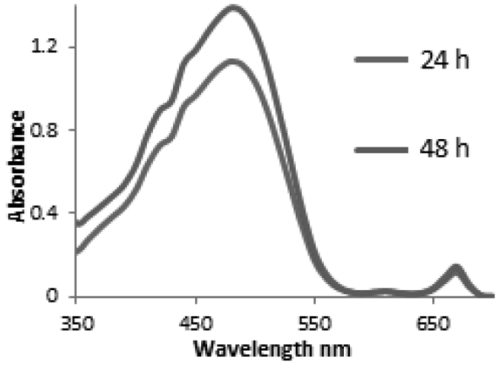

Fig. 8. UV-Vis absorption spectra of astaxanthin extracted by olive oil with acid pretreatment / water bath

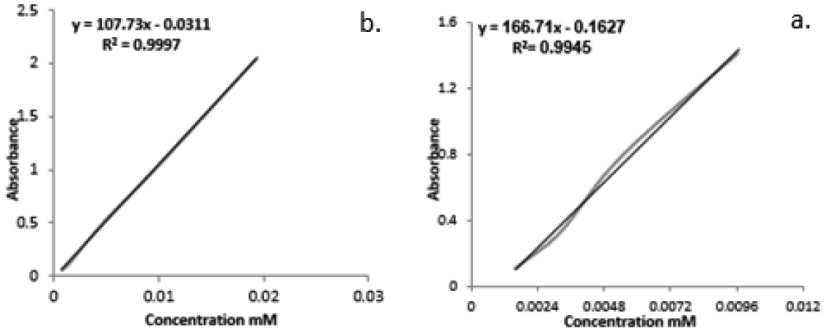

Fig. 9. Standard curve for astaxanthin in a) acetone and b) olive oil

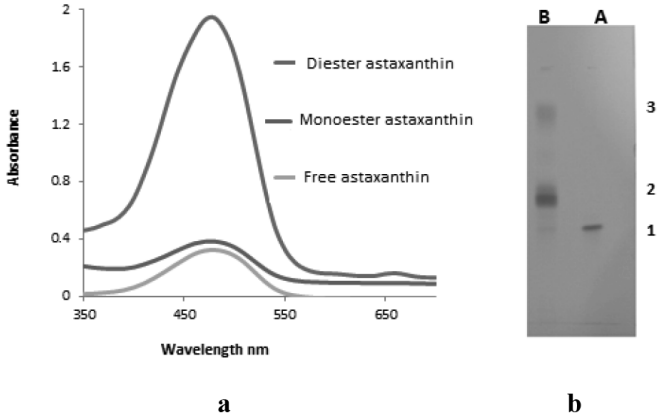

Fig. 10. a) Isolation of astaxanthin forms by TLC: A sample of standard astaxanthin, B substance extracted from *H. pulvialis,* **b**) Absorption spectrum of astaxanthin and monoester and diester derivatives isolated by TLC

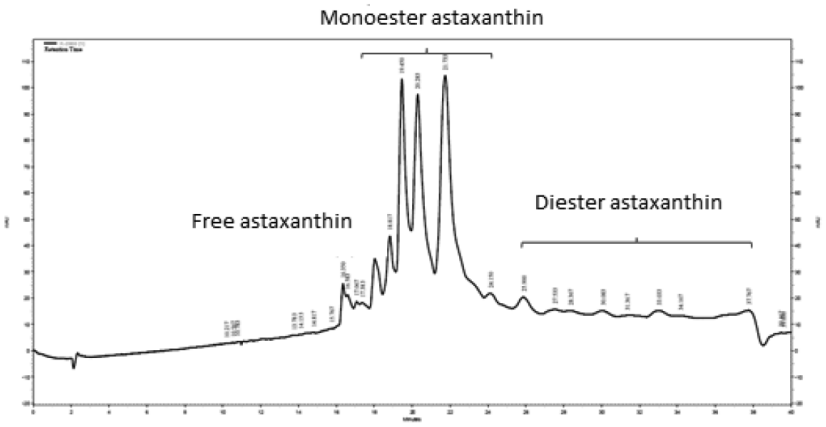

Fig. 11. Chromatogram of the extract was determined by acid-ultrasound-acetone method, which showed peaks related to free astaxanthin, monoester astaxanthin and diester astaxanthin

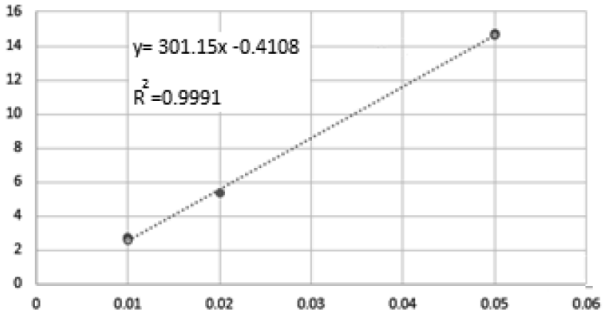

Fig. 12. Standard curve for astaxanthin by HPLC

evaluate astaxanthin extracted from H. pluvialis

The acetonic extract of *H. pluvialis* was used for further analysis, and the presence of astaxanthin and its monoester and diester derivatives was investigated by thin layer chromatography. TLC test was performed using thin layer chromatography paper (TLC Silica gel 60 F from MERCK) in a special tank in a closed environment with hexane/acetone mobile phase in the ratio of 7:3 (Orona-Navar et al., 2017) (Figure 10-a). The bands associated with free astaxanthin, astaxanthin monoester, and astaxanthin diester were isolated from TLC and extracted from silica by acetone. The absorption spectra were recorded at 350 to 700 nm (Figure 10b).

The use of high-performance liquid chromatography to evaluate astaxanthin extracted from H. pluvialis

Astaxanthin and its derivatives were investigated in the extract from *H.pluvialis* by HPLC from Knauer Well Chrom Solvent Organizer (k-1500), with pump K-1001 and UV Detector K-2600 using column C18 (10×4.6 mm) at ambient temperature, and the wavelength of 480 nm, and the flow rate of 0.8 ml/min. Run time was 40 minutes, and the mobile phase was a combination of water, and methanol, which in the first 5 minutes, isocratic with the ratio of 50:50, to 35 minutes, the ratio of two solvents as a gradient changed from 20:80 to a percentage of 0:100, and in the final 5 minutes the static phase ratio remained 50:50. Several different peaks are observed, indi-cating various derivatives of astaxanthin.

10 mg extract of *H. pluvialis* was prepared by acid ultrasound and acetone to evaluate the contribution of each of the astaxanthin derivatives and measured by chromatography (HPLC). The chromatogram spectrum of the total extract is shown in Figure (11). Free astaxanthin was inject-ed into the system to determine the peaks related to free astaxanthin, standard, and according to the exit point of the sample, the peak of free astaxanthin was identified in the chromatogram of the total extract. The whole extract was first separated by TLC to identify astaxanthin derivatives, and the bands related to the monoester and diester derivatives were separated and sepa-rately injected into the HPLC. Their peaks on the main chromatogram were determined. The relevant software calculated the area below the peaks. Then, standard concentrations of pure astaxanthin were prepared, and a standard diagram was drawn (Figure 12). The percentage of free astaxanthin and its derivatives was calculated in the sample of *H. pluvialis* by the standard diagram line equation and the area below the peaks of the tested sample and the average molar mass of the monoester and diester derivatives.

Results

Investigation of ionic solution

The results of using ionic solutions with 40% and 60% concentrations are listed in Table (1). As seen, the ionic solution with a concentration of 40% was more efficient.

According to the results for time treatments in ionic solution (Table 2), no significant difference was observed between 60, and 120 times in compare to difference between 30 and 60 minutes. So, 60 minutes treatment was considered as the best.

Determination of astaxanthin using ionic/acid solution/ultrasound

According to the results of Table (3), it can be seen that the acid/ultrasound pretreatment was more efficient.

The use of magnets for astaxanthin extraction

In this method, small magnets were used to stir the sample in the microtube. According to Figure (4), after a few repetitions, the extraction results were better when a water bath was used alone than the solution was stirred.

The Comparison of the water bath, glass bead, and ultrasound waves

In this experiment, some methods were used that are suitable for increasing the extraction per-centage, such as ultrasound waves and rubbing with glass bead sterilizers. The results (Figure 5) indicated that using a combination of ultrasound waves and a water bath showed better results than the other two methods.

Extraction of astaxanthin by acid, ultrasound, and acetone

The water bath/ultrasound method was used in 30, and 45-minute intervals and a small percent-age of the extracted astaxanthin was calculated by the standard diagram line equation (Table 4).

Extraction of astaxanthin by oil with acid pretreatment and ultrasound waves

According to Figure (7), there is no significant difference between 24 and 48 hours, and the percentage of astaxanthin was calculated to be about 1.2%.

Treatment with acid and olive oil

In this experiment, olive oil was added to

Table 1. Percentage of astaxanthin extracted with 40 and 60% ionic liquids

Extraction	Pretreatment
0.366%	40% ionic liquid
0.315%	60% ionic liquid

Table 2. Percentage of astaxanthin extracted by 40% ionic liquids at 30, 60 and 120 minutes

Treatment	Extraction
30 minutes	0.42 %
60 minutes	0.48 %
120 minutes	0.50 %

Table 3. Percentage of astaxanthin extraction using ionic liquids / acid / ultrasound

Pretreatment	Extraction
Acid/ water bath	1.00 %
Acid/ ultrasound	1.12 %

Table 4. Percentage of astaxanthin extraction with ultrasound and acetone pretreatment

Pretreatment	Extraction
Water bath/ 30 minutes ultrasound	1.321 %
Water bath/ 45 minutes ultrasound	1.328 %

the mixture in three steps, and with acid treatment, the extracted volume was estimated at 1% (Figure 8).

y=27.932x-0.1627 (Eq. 1)

Thin-layer chromatography (TLC)

As seen in Figure (11), different color bands are seen on chromatography paper related to carote-noids and chlorophyll (Elumalai et al., 2014; Orona-Navar et al., 2017). Astaxanthin with three structures, including free astaxanthin, astaxanthin monoester, and astaxanthin diester, is present in the algae extract. Given the degree of polarity of the solvent and the degree of polarity of the compounds, the astaxanthin diester moves faster on paper, followed by the astaxanthin monoes-ter, and finally, free astaxanthin (which is more polar than the other two compounds). The R_f of the three structures of free astaxanthin, astaxanthin monoester, and astaxanthin diester for *H. pluvialis*is 0.386, 0.502, and 0.817, respectively. As seen in Figure (10-b), the absorption spectra obtained from the two isolated bands of astaxanthin monoester and astaxanthin diester are similar to the absorption spectrum of astaxanthin and overlap entirely with it.

The use of high-performance liquid chromatography (HPLC)

In UV-Vis spectroscopy, as the standard di-

Table 5. The amount of free astaxanthin, monoster and diester in total extract of *H. pluvialis*

Astaxanthin forms	Free	Monoster	Diester
Peak area	0.17	3.99	0.35
Percentages of Astaxanthin forms	3.76	88.42	7.82

agram of the standard material is required to be drawn, and the absorption spectra of the three forms of astaxanthin overlap (Figure 12), the measurement is done based on the molar mass of free astaxanthin. Therefore, given the higher molar mass of mono-and diester forms, the results do not show the actual weight percentage of astaxanthin in *H. pluvialis*. Thus, HPLC should be used for a more accurate determination of astaxanthin. HPLC was used to reduce the effect of this problem by determining the actual percentage of total astaxanthin. After selecting the peak of free astaxanthin, and its derivatives, the area under the curve of each of the products, was calculated. The percentage of each of them was determined in the total extract of the algae, which is according to Table (5).

Average molar mass was used to calculate the weight percentage of each astaxanthin derivative. Since the fatty acids in astaxanthin derivatives are generally $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{18:4}$, $C_{16:0}$, and $C_{16:1}$, the average molar mass of each of the mono and diester derivatives was calculated to be 849,284, and 1101,728 g/mol, respectively.

As seen in Figure (12), by drawing a standard diagram of astaxanthin concentration based on the molar mass, free astaxanthin in the extract was determined to be 1.32%, given the average molar mass of astaxanthin mono and diester derivatives. Their constituent percentages, the percentage of total astaxanthin in *H. pluvialis*, was calculated to be 1.6%.

Discussion

The microalgae cell wall composition could be changed under different environmental and nutritional conditions. The composition of the microalgae cell wall is a critical factor that should be considered for metabolites extraction (Cheng et al., 2015). Many attempts have been reported to obtain effective methods for cell wall degradation to extract valuable compounds like carotenoids from microalgae. A main part of *H. pluvialis* red biomass is related to cell wall (Shah et al., 2016Y Kim et al., 2016). So, we attempted to disrupt the cell wall and extract astaxanthin.

Chemical methods, including acid, acetone, ionic solution, and physical processes such as ultrasound waves and magnetic stirrer were used to measure astaxanthin in *H. pluvialis*. According to the results, using each of these methods alone did not have good efficiency. Our results showed that the best outcome was related to the acid treatment with 30 minutes of ultrasound waves at 70 °C.

Molino et al., 2018 reported among different solvents (acid and base, organic and ionic solvents, etc.) without using physical methods and with one extraction step, Chloroform /methanol (1: 1 ratio) had the highest extraction percentage. However, in the case of physical and chemical pretreatment, Aceton acts more effectively (Molino et al., 2018). A similar result was observed after chemical and biological pretreatment in our experiment (Mendes-Pinto et al., 2001). Also, based on the results of using an

ionic solution, 40% showed better efficiency. Still, due to the significant difference in the results of using ultrasound waves in the extraction period of 60 minutes, it is significantly different from the time of 30 minutes, while there is no significant difference with the time of 120 minutes. We obtained 60 minutes treatment as the best for astaxanthin extraction, as Choi et al. (2019) reported. Also, Ruenngam et al. (2010) and Molino et al. (2018) mentioned the negative effect of time elongation on astaxanthin extraction. Choi et al. (2019) showed high extraction by ionic solution while we didn't get a good efficiency by ionic solution. So a combination of ionic solution with acid and ultrasound wave is proposed for astaxanthin extraction. The use of 40% ionic solution, contrary to what is stated in some sources, did not have good re-sults alone, and acid treatment was used to improve the results. Due to the rapid oxidation of the pigment, olive oil was used to extract and store astaxanthin from damaged cells. According to the results, acid treatment and then extraction by olive oil led to the best yield of about 95% of the total astaxanthin in *H. pluvialis*. A spectrophotometer examined astaxanthin, monoester, and diester derivatives, and their amount was determined by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). The results showed that the best method to measure the amount of astaxanthin in *H. pluvialis* is acid treatment, ultrasound waves, and extraction by acetone. In addition, the results of HPLC showed that the amount of astaxanthin monoester (88.44%) was higher than the free forms (3.76%) and diester (7.82%) in the total content of extracted astaxanthin. The amount of total astaxanthin in the studied *H. pluvialis* was calculated to be about 1.6% of the dry weight of the algae.

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