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Comparison of Different Methods for Carotenoid Extraction from *Dunaliella Salina*

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ABSTRACT

Background: *Dunaliella salina* (*D. salina*) is a unicellular marine alga known for its carotenoid ingredient. Carotenoid has been used in food industry as a coloring additive and as an antioxidant. In addition, it has anticancer, antiaging, and immune-modulatory properties. Different methods have been used for extraction of carotenoids from algae. This study evaluated different extraction methods for carotenoid and compared the carotenoid yield and extraction time for procedures.

Methods: In an experimental study, *D. salina* was isolated from Maharloo Lake in Shiraz, Iran. *D. salina* was centrifuged, dried, and suspended in methanol for further carotenoid extraction using different methods, namely ultrasound-assisted, supercritical fluid, microwave-assisted, electromantle, magnetic stirring, and maceration extraction methods.

Results: Simultaneously, ultrasound-assisted, microwave-assisted, and electromantle extraction methods showed higher carotenoid yield and during a shorter time when compared with supercritical fluid, magnetic stirring, and maceration extraction methods. Since ultrasound-assisted extraction is a cold extraction method and ultrasound waves could release carotenoid and chlorophyll from broken cells to solvent quickly, the highest yields of carotenoid and chlorophyll were obtained during 10 min applying ultrasound-assisted extraction method. In contrast, the lowest yields of carotenoid and chlorophyll were undertaken by supercritical fluid extraction method.

Conclusion: Ultrasound-assisted extraction method was demonstrated to be a promising tool to recover higher value-added compounds from *D. salina*.

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Introduction

At present, microalgae offer great possibilities for the isolation of natural substances of significant commercial interest and added value in industries such as pharmaceuticals, alimentary and cosmetic products. *Dunaliella salina* is one of the marine algae species in particular that is able to produce a variety of substances with a range of properties. *D. salina* is found in saline environments and exhibits optimal growth at different salt concentrations with varying abilities to turn orange-red under particular culture conditions (1, 2). The main morphological characteristic that distinguishes this alga from the rest of the species is the absence of a polysaccharide cell wall. For this reason, *D. salina* can be easily digested by humans and animals (3).

Although, other microalgae and superior plants usually have a content of around 0.3% in *ß*-carotene, *D. salina* contains more than 10% of its dry weight as *ß*-carotene. In addition to *ß*-carotene, this microalga contains other pigments such as chlorophyll *a*, chlorophyll *b*, lutein, violaxanthin, antheraxanthin, and zeaxanthin (4); but the main pigments are *ß*-carotene, chlorophyll *a* and chlorophyll *b;* as a result, the ratio of carotenoid/chlorophyll is used as an indicator for carotenoid extraction. Extraction is one of the main techniques in carotenoid recovery from *D. salina* that can be used for commercial application (5).

According to the disadvantages of conventional extraction methods such as low efficiency, high solvent consumption and high extraction time, recently advanced extraction methods such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) have been introduced (6-8). Macias-Sanchez *et al*., (2009) reported carotenoid yield and carotenoid/chlorophyll ratio from *D. salina* by SFE and UAE. Their results showed that carotenoid yield and carotenoid/chlorophyll ratio obtained by SFE (15 and 27 µg/mg, respectively) were higher than UAE (14 and 2 µg/mg, respectively) (9).

In another research, Parniakov *et al*. (2015) investigated chlorophylls extraction by UAE and aqueous extraction from *Nannochloropsis spp*. The highest yield of total chlorophylls (0.043 µg/mg) was obtained by UAE, being more than 9-folds higher than those obtained for the untreated samples and aqueous extraction (0.004 µg/mg) (10). Dey and Rathod, (2013) investigated the extraction of *ß*-carotene from *Spirulina platensis* by UAE in different times. Results demonstrated that the rate of *ß*-carotene yield was very high initially till 4 min and then illustrated down reaching saturation at 8 min with the highest of 47% extraction because increasing extraction time had destructive effect on carotenoid yield (11).

Ludwig *et al.* (2021) investigated the extraction of β-carotene from *D. salina* by supercritical carbon dioxide. Results illustrated that the supercritical extraction at 500 bar, 70°C, and 10% (wt) ethanol as co-solvent yielded in the highly efficient pigment recovery of over 90% (7). Hosseini *et al.* (2016) reported the extraction of carotenoids from *D. salina* microalgae by supercritical carbon dioxide. Results demonstrated that the most appropriate operating condition to obtain the best extraction yield of carotenoids was at 400 bar and 55°C (115.43 g/g dry microalgae) (12). The objective of this study was to compare different conventional and modern methods for the extraction of carotenoid from *D. salina* in terms of efficiency, solvent consumption, and extraction time.

Materials and Methods

All chemicals and reagents were purchased from Merck Company (Darmstadt, Germany). *D. salina* was isolated from Maharloo Lake (2927N-5248E), which is a salt lake located 27 kilometers southeast of Shiraz, Iran. The spread, depth and height of Maharloo Lake from sea were 600 km², 3 m and 1460 m, respectively. The sample was taken in July 2015, while the lake's salinity was 22% and the temperature was upper 38°C in summer. Water component contained 22% NaCl, 2% $MgCl_2$ and 4% Na_2SO_4.

Regarding carotenoid extraction methods; first, lake samples containing *D. salina* were centrifuged at 5000 g for 10 min (SW14R, Froilabo, France); and then were dried in an oven (FAG, Bangalore, India) at 40°C for 24 h. after that, 0.2 g of dried *D. salina* was suspended in 200 mL of methanol in all extraction methods and the exception was in SFE that 1 g sample was suspended in 50 mL methanol. Finally, carotenoid was extracted by six different extraction methods.

Different Extraction Methods of D. salina

In the magnetic stirring extraction (MSE) method, carotenoid was extracted by magnet stirrer (L-81, Labinco Co, Netherlands) for 2 and 4 h with speed of 1000 rpm at 25°C (13).

In maceration extraction (ME) method, samples were kept for 72 h at 25°C in the absence of light (14). When the electromantle extraction (EME) method was applied, an electromantle heater (EM2000/C, 335 W, Electrothermal Engineering Ltd., Rochford, UK) was used at 300 W for 10 and 20 min (14). For MAE method, a domestic microwave oven was utilized (ME3410, Samsung Malaysia Electronics, Kuala Lumpur, Malaysia) at the microwave power level of 300 W for 10 and 20 min. The dimensions of the PTFE-coated cavity of the microwave oven were 23.9×37.5×38.6 cm (15).

Regarding the UAE method, the ultrasonic irradiation experiments were performed by UAE (Bandelin, Berlin, Germany) for 10 and 20 min, with the power of 80 W, amplitude of 50 and cycle (on/off) 20/10 at 25°C (6). In SFE method, a Suprex PrepMaster (Suprex, Pittsburgh, PA) supercritical fluid extractor was used for the carotenoid extraction at 60°C, while this extractor was equipped with a dual-piston pump for CO_2. Extraction was carried out for 30 and 60 min, at 100 and 150 bar (16).

Carotenoid and Chlorophylls Calculations

For carotenoid and chlorophyll calculations; after the extraction process, the solvent was separated by rotary and carotenoid, chlorophyll *a* and chlorophyll *b* yield of *D. salina* were then determined by the equations proposed by Macias-Sanchez *et al.* (2009) by measuring the absorbance of the samples using a UV visible spectrophotometer (Spec 1650PC, Shimadzu, Japan). The concentration of total carotenoids was calculated using the following equation (17):

Eq. (1);

where A_{470} is the absorbance at 470 nm, and Ca and Cb are the concentrations of chlorophyll *a* and *b* calculated according to the following equations: Ca (µg/mL)=$16.72A_{665.2}-9.16A_{652.4}$Eq. (2) Cb (µg/mL)=$34.09A_{652.4}-15.28A_{665.2}$ Eq. (3); where $A_{665.2}$ and $A_{652.4}$ are the absorbance values at 665.2 nm and 652.4 nm, respectively. The yields are expressed in µg of pigment per mg of the dry weight of microalgae.

All experiments were carried out in triplicates. The results were reported as the mean±standard deviation. Statistical analysis was performed through data subjection to the analysis of variance (ANOVA) using the SPSS software (version 22, IBM, New York, USA). Means were subjected to Duncan's

$$total\ carotenoids\ (x+c)\ =\frac{1000A470-1.63Ca-104.96Cb}{221}$$

multiple ranges test and a *P* value of <0.05 was considered statistically significant.

Results

Influence of Extraction Time on Carotenoid Yield

The influence of different extraction methods on carotenoid yield was presented in Figure 1a. The carotenoid yield was 0.09-2.62 µg/mg, indicating that significant differences were observed among various extraction methods. Among different extraction methods, the higher carotenoid yields were obtained when UAE, MAE and EME methods were utilized at a shorter extraction time (10 min) in comparison with SFE, MSE and ME (1, 4 and 72 h, respectively).

In UAE method, the higher carotenoid yield was achieved for 10 min. This could be due to a fast transfer of the solute that took place from the solid surface and the outer broken cells of *D. salina* to the solvent by ultrasound waves as a result, it facilitated the penetration of the methanol through the *D. salina* cell membrane, thus increasing the yield of the carotenoid presented in the raw material (18). By increasing extraction time, carotenoid yield decreased significantly from 2.62 µg/mg (10 min) to 1.83 µg/mg (20 min) (Figure 1a). So, 10 min was an optimum sonication time for obtaining the highest carotenoid yield and there was not any need to consume more energy and higher time. Although, carotenoid yield after 20 min of ultrasound was lower than that of 10 min, but still noticeable carotenoid was extracted from *D. salina*.

After UAE method as the best carotenoid extraction procedure, the higher carotenoid yield was achieved by MAE and EME, respectively. In MAE method similar to UAE, the higher carotenoid yield was achieved for 10 min. By increasing extraction time, carotenoid yield significantly decreased from 1.05 µg/mg (10 min) to 0.70 µg/mg (20 min) (Figure 1a). In EME method, carotenoid yield was similar to MAE. It increased insignificantly from 0.90 µg/mg (10 min) to 1.06 µg/mg (20 min) (Figure 1a). Higher

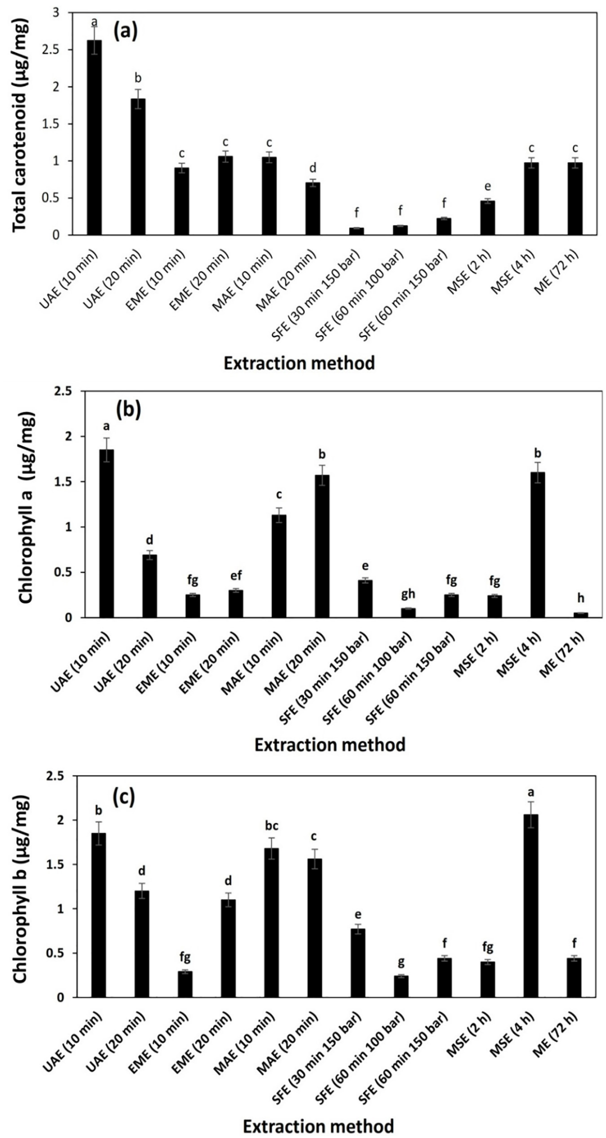

Figure 1: (a) Total carotenoid yield, (b) chlorophyll *a* yield, and (c) chlorophyll *b* yield by different extraction methods. Various superscript letters represent significant differences of mean (p<0.05). UAE: Ultrasound assisted extraction, EME: Electromantle extraction, MAE: Microwave assisted extraction, MSE: Magnetic stirring extraction, ME: Maceration extraction, SFE: Supercritical fluid extraction, methods.

extraction time helped reaccelerate the release of carotenoid compounds into the solvent through the increased diffusivity.

In SFE method, the carotenoid yield was dependent on time and pressure. By increasing extraction time and pressure, carotenoid yield increased insignificantly. The best result of carotenoid yield was achieved for 60 min at 150 bar (Figure 1a). As the time increased from 30 to 60 min, carotenoid yield increased from 0.09 µg/mg to 0.22 µg/mg. By increasing pressure, the carotenoid yield increased from 0.12 µg/mg (100 bar) to 0.22 µg/mg (150 bar). Increasing extraction pressure caused a simultaneous increase of both the SC-CO_2 density and diffusion coefficient. An increase in the density of SC-CO_2 was associated with an increase in its solvation power which caused higher carotenoid yield. In addition, an increase in the diffusion led to an increase in the interaction between the supercritical fluid and the solute contained within the matrix which caused increasing carotenoid yield.

In MSE method, by increasing extraction time, carotenoid yield increased from 0.46 µg/mg (2 h) to 0.97 µg/mg (4 h). So higher agitation and breaking down of the cell membrane in more time could increase carotenoid yield (Figure 1a). In ME method, carotenoid yield was 0.97 µg/mg for 72 h (Figure 1a). Although, the extracted carotenoid yield by ME and MSE methods were similar, but due to the lack of agitation in ME method, increased the extraction time from 4 h to 72 h. Among modern extraction methods (UAE, SFE and MAE), the carotenoid yield decreased in the order of UAE>MAE>SFE method. Carotenoid yield in conventional extraction methods (EME, MSE and ME) was similar among all methods. Generally, in our research carotenoid yield in modern extraction methods (except for SFE method) were better than conventional extraction methods. As a result, new extraction methods were more beneficial than conventional ones. In our research, UAE and MAE methods were better extraction methods. Power comparison in UAE, MAE and EME methods in spite of higher power in EME and MAE (300 W) methods, showed the carotenoid yield obtained by UAE (80 W) in lower power was higher than MAE and EME methods in equal time. This indicated a substantial saving in the extraction cost and energy consumption by UAE, when compared to EME and MAE methods.

Influence of Extraction Temperature on Carotenoid Yield

Thermal extraction methods (EME, MAE, and SFE) were performed at 60°C, but non-thermal extraction methods (UAE, MSE, and ME) were at 25°C. Carotenoids were susceptible to some reactions of oxidation and isomerization under certain conditions (light, heat, acids and oxygen). Carotenoid yield of UAE as the non-thermal method was 2.5 times higher than MAE and EME as thermal methods. Generally, in our research, the carotenoid yields of ME and MSE as non-thermal methods were similar to EME and MAE as thermal methods. Despite the optimum carotenoid yield achieved by SFE method in higher temperature and pressure, but it had the lowest carotenoid yield compared with non-thermal methods.

Carotenoid/Chlorophylls Ratio

The influence of different extraction methods showed that chlorophyll *a* yield was 0.05-1.85 µg/mg and chlorophyll *b* was 0.24-2.06 µg/mg indicating that significant differences were observed among different extraction methods. UAE, MAE, and MSE methods had the highest yields of chlorophyll *a* and *b*, whereas ME and SFE methods had the lowest yield of chlorophyll *a* and *b*, respectively (Figure 1b and Figure 1c). Changes in carotenoid and chlorophyll yields caused significant differences in the ratio of carotenoid/chlorophyll. The ratio of the yields carotenoid/chlorophyll *a* varied from 0.22 to 18.83 and the ratio of carotenoid/chlorophyll *b* varied from 0.12 to 3.08 (Table. 1).

The highest carotenoid/chlorophyll ratio was in ME method. Although, MAE method had high carotenoid yield but chlorophylls yields were considerably high, so it reduced the ratio of carotenoid/chlorophyll. In SFE method, chlorophyll yields were significantly higher than carotenoid, so it reduced the ratio of carotenoid/chlorophyll and it had the lowest carotenoid/chlorophyll ratio. In UAE method, carotenoid and chlorophylls yields were high and carotenoid/chlorophyll ratio did not change and remained high. In EME method, carotenoid yield was higher than chlorophylls, so carotenoid/chlorophyll ratio was high. In MSE method, at shorter extraction time, carotenoid yield was higher than chlorophylls, so carotenoid/chlorophyll ratio was high. Although by increasing extraction time, a downward trend in carotenoid/chlorophyll *a*, and carotenoid/chlorophyll *b* was noticed in EME, MSE and MAE methods; but, an upward trend was noted in UAE and SFE methods. This behavior could be attributed to the difference in the performance of extraction methods.

Discussion

In UAE method, 10 min was a suitable sonication time for achieving the optimum carotenoid yield. By increasing extraction time, carotenoid yield

Table 1: Ratios of carotenoid/chlorophyll *a* (Carot/Chlor *a*), carotenoid/chlorophyll *b* (Carot/Chlor *b*) and carotenoid/chlorophyll *a* and *b* (Carot/Chlors) by different extraction methods.

Extraction method	Carot/Chlor *a* ratio	Carot/Chlor *b* ratio	Carot/Chlors ratio
UAE* (10 min)	1.41±0.09^{de**}	1.41±0.10^{c}	0.70±0.01^{cd}
UAE (20 min)	2.65±0.18^{bc}	1.51±0.10^{c}	0.96±0.01^{c}
EME (10 min)	3.53±0.24^{b}	3.08±0.21^{a}	1.66±0.21^{b}
EME (20 min)	3.47±0.24^{b}	0.95±0.06^{de}	0.75±0.21^{c}
MAE (10 min)	0.91±0.06^{def}	0.72±0.05^{f}	0.37±0.18^{de}
MAE (20 min)	0.44±0.03^{ef}	0.45±0.03^{g}	0.22±0.02^{e}
SFE (30 min 150 bar)	0.22±0.01^{f}	0.12±0.01^{h}	0.07±0.01^{e}
SFE (60 min 100 bar)	1.66±0.11^{d}	0.83±0.05^{ef}	0.35±0.24^{de}
SFE (60 min 150 bar)	1.05±0.07^{def}	0.47±0.03^{g}	0.31±0.17^{e}
MSE (2 h)	1.85±0.13^{cd}	1.14±0.08^{d}	0.71±0.05^{cd}
MSE (4 h)	0.60±0.04^{ef}	0.47±0.03^{g}	0.26±0.04^{e}
ME (72 h)	18.83±0.33^{a}	2.17±0.15^{b}	1.97±0.18^{a}

*UAE: Ultrasound assisted extraction, EME: Electromantle extraction, MAE: Microwave assisted extraction, MSE: Magnetic stirring extraction, ME: Maceration extraction, SFE: Supercritical fluid extraction. **Data are expressed as Mean±SD. Different superscript letters represent significant differences of mean (p<0.05).

reduced considerably, which could be due to the destructive impact of the produced free radicals. Actually, free radicals attack the highly conjugated bond of β-carotene molecule, thus reducing the yield of carotene (11). Similar findings according to our research were reported by Dey and Rathod (2013) that used UAE method for extraction of *β*-carotene from *Spirulina platensis*. The results showed that the rate of β-carotene extraction was very high after 8 min (1.2 mg/g) (11). Also, in another research, the highest yield of carotenoid recovery (60%) was achieved by UAE method for 5 min, in 600 W (19).

In addition, In MAE method, the higher carotenoid yield was achieved for 10 min, because microwaves caused a quick rupture of the glandular walls and loosened the cell wall matrix; leading to the cells severing and release of the carotenoid (20). Reduction of carotenoid by increasing extraction time in MAE method could be due to the destructive impact of heating on the carotenoid yield, so short extraction time was sufficient for the extraction of the total carotenoid. Pasquet *et al.* (2011) reported similar results for MAE method for chlorophyll *a* from *D. tertiolecta*. Their results showed that chlorophyll *a* yield decreased from 9 µg/mg (5 min) to 5 µg/mg (15 min) (21).

In SFE method, internal diffusion increased, because there was more time available for supercritical carbon dioxide (SC-CO_2) to penetrate the matrix structure and to extract carotenoid. A similar result was reported by Macias-Sanchez et al. (2007) that used SFE method for extraction of carotenoids from *Scenedesmus almeriensis*. Their findings revealed that the highest carotenoid yield was observed at 400 bar (1.188 µg/mg) and by decreasing pressure carotenoid yield decreased (0.004-0.880µg/mg) (17). In addition, Ludwig *et al.* (2021) reported the SFE method for extraction of β-carotene from *D. salina* at 500 bar, 70°C, and 10 wt% ethanol as co-solvent yielded a highly efficient pigment recovery of over 90% (7).

In MSE method, also similar results were reported by Agbangnan *et al.* (2012) that used MSE for extraction of *Sorghum's* polyphenols. The results illustrated that the extraction rate for 2 h (0.30 g/mL) was lower than that of 4 h (0.36 g/ml) (22). In comparison to modern extraction methods, similar findings were reported by Bagherian *et al.* (2011), who extracted pectin from grapefruit by water bath, UAE and MAE methods (23). Their results showed that the highest yield was obtained by MAE (27.8%) for 25 min and UAE method (17.9%), when compared to water bath (19.1%). Actually, the application of modern extraction methods was better than traditional ones, because they are environmentally friendly, have shorter extraction time and higher extraction yield (23).

Also similar results were reported by Golmakani *et al.* (2015), who extracted essential oil from *Citrus limon* peel by MAE and other conventional hydrodistillation methods (24). Their findings demonstrated that the highest yield was noted when MAE method was used (1.36%) due to a synergy of two transfer phenomena of mass and heat acting in the same way in comparison with conventional hydrodistillation methods (1.22%). Also, similar results were noticed by Wakte *et al.* (2011), who extracted curcumin from *Curcuma longa* by Soxhlet, MAE, UAE and SFE methods. Their results indicated that MAE (90.4%) was the best method of extraction, when compared with UAE (71.4%), SFE (69.3%) and Soxhlet (2.1%) methods (25).

Carotenoid yield in non-thermal methods was higher than thermal methods. These differences could be attributed to the intensification of mass transport due to cavitation effects on the surface of the particles through disruption of the cell structure of *D. salina* matrix and carotenoid release. Although, the temperature evolution of the mixture helped to enhance the extraction of carotenoid through increased diffusivity, increased mass transfer resulting from higher solubility, decreased density, decreased viscosity of solvent and also loosened cell wall components, but the decrease in the carotenoid yield in thermal methods in comparison with non-thermal ones was due to the destructive impact of temperature on carotenoid (23, 26). In contrast to our findings, Cha *et al.* (2010) compared carotenoids yield extracted from *Chlorella vulgaris* by thermal (Pressurized liquid extraction and Soxhlet methods) and non-thermal methods (ME and UAE methods). Their results revealed that thermal methods had higher carotenoid yield than non-thermal methods (27). Among these extraction methods, pressurized liquid extraction method displayed the highest extraction yield (0.50 mg/g) in comparison with Soxhlet (0.26 mg/g), UAE (0.10 mg/g) and ME (0.08 mg/g) methods. In addition, in contrast to our findings, Pasquet *et al.* (2011) reported the *ß*-carotene extraction from *D. tertiolecta* by MAE, UAE and ME methods. Their findings showed that *ß*-carotene extraction yields were similar by all extraction methods (1.2 µg/mg) (21).

In addition, similar results for comparing the extraction yield of chlorophyll *a* and *b* were reported by Pasquet *et al.* (2011) that investigated the extraction of chlorophyll *a* and *b* from *D. tertiolecta* by MAE, UAE and ME methods (21). Their findings showed that, the chlorophylls yield by UAE method (4.8 µg/mg chlorophyll *a* and 1.5 µg/mg chlorophyll *b*) was higher than MAE and ME methods (4.3 µg/mg chlorophyll *a* and 1.2 µg/mg chlorophyll *b*). The ME method had the highest carotenoid/chlorophyll ratio, because the carotenoid and chlorophyll *b* yields were higher than chlorophyll *a*.

Totally, UAE, EME and MSE methods were suitable ones for extraction of carotenoids, because carotenoid/chlorophyll ratio and the purity were high. Whereas, SFE, MAE and ME methods had low purity and they were not suitable extraction methods. In contrast to our research, Macias-Sanchez *et al.* (2009) reported the ratio of carotenoid/chlorophyll from *D. salina* by UAE and SFE methods. Their results showed that the ratio of carotenoid/chlorophyll in SFE (24.74) was higher than UAE (5.64) method (17), which can be related to the fact that the carotenoid/chlorophyll ratio in their SFE method has been applied at different pressures (200-500 bar) and also at different temperatures (40-60 °C), which caused the highest yield of pigments.

Conclusion

These days, the society demands of products were made with additives that are natural in origin and beneficial to human health. In this sense, marine microalgae offer great possibilities as sources of these substances. Novel methods for carotenoid extraction from microalgae were substituted with the conventional ones, because of their lower efficiency and higher solvent consumption and extraction time. This work investigated the influence of different extraction methods of carotenoid from *D. salina*. The extraction yield of carotenoid by UAE method in 10 min was significantly higher than other methods, so it was a fast method and reduced extraction time. The ratio of carotenoid/chlorophyll in UAE method was higher than all other methods, except for EME and ME methods; because it had the highest yield of chlorophyll *a, b* and carotenoid. So the ratio of carotenoid/chlorophyll was high. It is an environmentally friendly, cheap, widely available, and a low energy requirement method that can also improve the efficiency. Totally, UAE method was demonstrated to be a promising tool to recover high-added value compounds from *D. salina*.

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Conflict of Interest

None declared.

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