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Optimizing the Extraction of Carotenoids and Omega Fatty Acids from Microalgae

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Abstract

Microalgae are the most significant renewable sources of natural carotenoids and omega fatty acids. These include *Dunaliella salina*, *Haematococcus pluvialis*, and *Scenedesmus obliquus*. The aim of this research is to use various polar solvents to improve the extraction efficiency of various carotenoids and omega-fatty acids from these microalgae. Our results showed that the polarity of the solvent, the polarity of the solute component, and microalgae species had a substantial effect on the extraction efficiency of the carotenoids β-carotene, lutein, zeaxanthin, and astaxanthin as well as omega fatty acids. The high-polar solvents Dichloromethane: Methanol, 3:1, v/v (DIC: MeOH), Hexane: Ethanol, 2:1, v/v (Hex: EtOH), and Hexane: Ethyl acetate,1:1, v/v (Hex: EtOAc) showed a positive effect on the extraction efficiency of lutein, zeaxanthin, and astaxanthin, the high-polar carotenoids, when compared with pure Hexane (Hex), the low-polar solvent. The solvent mixture of Hex: EtOH recovered the highest yield of omega fatty acids, especially omega-3 from *D. salina* and *H. pulvalies*, however Hex: EtOAc showed the same effect on *S. obliquus*. In conclusion, the solvent mixture of Hex: EtOH and Hex: EtOAc is strongly recommended for high extraction of carotenoids and omega fatty acids from investigated microalgae.

Keywords: astaxanthin, β-carotene, lutein, zeaxanthin, omega fatty acids, microalgae.

1. Introduction

Natural carotenoids are one of the most effective antioxidants and have prevalent attention due to their high ability to decrease the risk of a variety of human chronic diseases like cardiovascular disease, cancer, diabetes, liver Fibrosis, and osteoporosis [1–3]. Microalgae received a lot of scientific interest worldwide as a natural and renewable source of bioactive functional ingredients, including carotenoids and polyunsaturated fatty acids [4, 5]. Different carotenoids and polyunsaturated fatty acids have many different applications in the industrial formulation of nutraceutical, cosmetic, and pharmaceutical products [6]. Different studies revealed that microalgae can synthesize high amounts of bioactive carotenoids, for example, *H. pluvialis* can synthesize extreme amounts of astaxanthin and β-carotene, effective antioxidant carotenoids mostly applied in functional foods, pharmaceuticals, and cosmetics [5, 7]. *D. salina* is a leading source of natural bioactive carotenoids, including zeaxanthin, lutein, β-carotene, α-carotene, and 9-cis-β-carotene besides considerable amounts of polar lipids [8, 9]. *S. obliquus* has a significant ability to accumulate astaxanthin, β-carotene, adonixanthin, zeaxanthin, canthaxanthin, and lutein [10, 11]. Microalgae can produce different polyunsaturated fatty acids especially omega-3 and 6 including α-linolenic, docosahexaenoic, eicosapentaenoic, arachidonic, and linoleic acid, well beneficial for humans as an anti-inflammatory, cardioprotective, and neuroprotective agents [12]. The polarity of the organic solvent or solvent system used to extract lipidic ingredients (carotenoids and fatty acids) has a significant effect on the recovery efficacy of such compounds [13]. The combination of polar solvents with nonpolar solvents

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achieved efficient recovery of different carotenoids, such as the combinations of hexane/isopropanol (3:2, v/v), hexane/isopropanol (6:4, v/v), hexane/ethanol (70:30 v/v), hexane/methanol (3:2, v/v), hexane/ethanol (50:50 v/v), cyclohexane/1-butanol (9:1, v/v), acetone/water (92.5:7.5 v/v), hexane/ethanol/water (17:77:6, v/v/v), acetone/water (95:5 v/v), acetone/water (97.5:2.5 v/v), ethanol/water (96:4 v/v), and ethanol/ethyl acetate [14–16]. Correspondingly, solvent systems containing polar and non-polar solvents are suitable for extracting higher amounts of lipids [14]. Hexane/isopropanol (3:2, v/v) and chloroform/methanol (2:2.5, v/v) are the best solvent mixtures to extract fatty acids from microalgae and other materials [17]. The solubility findings of different carotenoids also explained that the more polar carotenoids namely astaxanthin, zeaxanthin, and lutein are highly dissolved in polar solvents namely methanol, ethanol, acetone, ethyl acetate, and dichloromethane, while the nonpolar carotenoids namely β-carotene, α-carotene, or carotenoids in ester form are highly dissolved in nonpolar solvents namely petroleum ether or hexane [18]. The purpose of this research is to improve the extraction efficiency of carotenoids and fatty acids from the microalgae *D. salina*, *H. pluvialis*, and *S. obliquus* by using several solvent combinations with various polarities.

2. Materials and methods

2.1. Cultivation of microalgae

Three different species of algae: *D. salina*, *H. pluvialis*, and *S. obliquus* were identified by Prof. Gamila H. Ali, Professor of Water Pollution, Environmental Research & Climate Changes Institute, National Research Centre, Cairo, Egypt. The three algal species were grown indoors in a cultivation unit made of plastic bottles with a 15 L of Bold's basal media [19] containing 100 g/L of NaCl and 0.075 g/L of urea for *D. salina*, 0.025 g/L of urea for *H. pluvialis*, and 0.3 g/L of urea for *S. obliquus*. After the algal culture had grown for 10 days, it was moved to a 4000 L fully automated and computer-controlled vertical photobioreactor (PBR). The culture was left to grow until the biomass reached 2–2.5 g/L. Algal biomass was harvested by centrifugation, dried, and then pulverized in an electrical grinding mill (FRITSCH Cross Beater Mill PULVERISETTE 16, Germany) to be a fine powder with ≥80 mesh particle size.

2.2. Preparation of algal extracts

The powder cells of *D. salina*, *H. pluvialis*, and *S. obliquus* were extracted using different solvent systems which differ in polarity index to estimate the optimal solvent system for the optimal extraction from the algae. Hexane, Hexane: Ethyl acetate (1:1, v/v), Hexane: Ethanol (2:1, v/v), and Dichloromethane: Methanol (3:1, v/v) were used for the extraction [20–22]. Concisely, the pulverized algal materials (25 g/ each alga) were soaked with each solvent system (250 mL, 3 times) in a 1000 mL conical flask and kept on an orbital shaker (Stuart, England) at 160 rpm at room temperature for 24 h. Then, each extract was centrifuged using a centrifuge (Sigma 3-18ks Centrifuge, Germany) at 5000 rpm for 20 min at 25°C to separate cell debris from the supernatant. The extraction step was repeated twice and the pooled supernatants of each solvent system per each alga were concentrated using a vacuum rotary evaporator (Heidolph Unimax 2010, Germany) at 40°C to dryness giving the crude extract of each solvent system per each alga. The dried extracts were weighted to calculate the extract yield. All the extraction steps were conducted under dim light conditions to prevent photooxidation of carotenoids [5].

2.3. Determination of carotenoids by HPLC

β-carotene, lutein, zeaxanthin, and astaxanthin were identified and quantified by HPLC-DAD in the crude extract of each solvent system of *D. salina*, *H. pluvialis*, and *S. obliquus* using an Agilent 1260 infinity series HPLC-DAD system (Agilent Technologies, Waldbronn, Germany) equipped with binary gradient Agilent 1260 prep pump (G1361A), an autosampler Agilent 1260 prep ALS (G2260A). An Agilent diode array detector 1260 DAD VL (G1315D) was employed for the detection of the separated β-carotene, lutein, zeaxanthin, and astaxanthin. The separation was performed using an Agilent normal phase (NP) silica column (ZORBAX RX-Sil, 5µm, 4.6 X 150 mm). The following solvents (A) n-hexane and (B) acetone were used at a flow rate of 1 mL/min using a gradient between solvents A and B following the method of [23] with some modifications as follows: B was run at 0 to 30% for 5 min, 30 to 50% for 15 min, 50 to 100% for 3 min, and maintaining 100% of B until the end of the separation at 30 min. The peaks were integrated at 450 nm to quantify β-carotene, lutein, and zeaxanthin, and at 476 nm to quantify astaxanthin. β-carotene, lutein, zeaxanthin,

and astaxanthin (Sigma-Aldrich Co., USA) were used as standards. β-carotene, lutein, zeaxanthin, and astaxanthin were identified and quantified in the extracts by comparing retention time and the peak area of the unknown peak with the standards. Total carotenoids were quantified using the total peak area relative to the peak area of the β-carotene standard.

2.4. Determination of omega fatty acids by GC-FID

2.4.1. Preparation of total lipid fractions

An aliquot (0.3 g, a mixture of 3 replicates) of each dried crude extract was re-dissolved in Dichloromethane: Methanol (3:1, v/v), then the lipid fraction including omega fatty acids of each extract was prepared following the method of [24]. The Dichloromethane: Methanol solution was washed with 0.2 volumes of 0.9% NaCl solution. The mixture was transferred to a separating funnel and shaken well for 5 min then kept separating two different layers, the upper phase was removed, and the lower chloroform phase containing lipid was evaporated under vacuum at 40°C using a Rota evaporator (Heidolph Unimax 2010, Germany) to give the total lipid fraction.

2.4.2. Preparation of fatty acid methyl esters

The total lipid fraction (100 mg/ solvent/ alga) was boiled with hydrochloric acid, then fatty acids were separated by petroleum ether. The petroleum ether fractions were saponified by using sodium hydroxide in methanol, then methylated with boron trifluoride in methanol, and finally extracted by heptane following the Method of [25].

2.4.3. GC-FID analysis

The heptane fractions were used for the determination of fatty acids including omega fatty acids by using a PerkinElmer Auto system XL GC (Perkin Elmer, Norwalk, Massachusetts, USA) equipped with a dual flame ionization detection (FID) system, autosampler, and Ezchrom integration system. Fatty acid separation was achieved by using fused-silica capillary columns (60 m×0.22 mm I.D., film thickness 0.25 μm). The carrier gas was Hydrogen with a flow rate of 1mL/min as a constant flow. One microliter of each sample was injected in Inlet SSL, split mode with a 1:50 ratio. The temperature program was from 60 °C to 200 °C at 2 °C/min and then held isothermally at 200 °C for 35 min. Injector and detector temperatures were held at 250 °C. Component relative concentrations were calculated based on GC peak areas.

2.5. Statistical analysis

All the tests were performed in three replicates. Data are presented as mean ± standard deviation (SD). Effects of the various solvent mixtures were evaluated for significance by one-way analysis of variance (ANOVA) using the COSTAT computer package. The least significant difference (LSD) at P≤0.05 level was calculated.

3. Results

3.1. Extract yield

Increasing the polarity index of the solvent system significantly (P≤0.05) improved the extract yield of microalgae. The extract yield efficiency depends on the polarity index of the solvent system and the microalgae species. The solvent DIC: MeOH possesses the highest extraction yield of the three microalgae followed by Hex: EtOH, Hex: EtOAc, then Hex. Microalgae *H. pluvialis* and *S. obliquus* gave the highest extract yield compared with *D. salina*, especially with the high polar solvents DIC: MeOH and Hex: EtOH (Fig. 1).

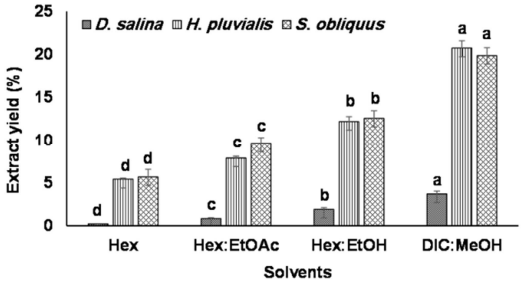

Fig.1. The effect of different solvents on the extract yield (%) of *D. salina*, *H. pluvialis*, and *S. obliquus*. Different letters in each series represent significant differences at P≤0.05.

3.2. Carotenoids content

Fig. 2 displays that total carotenoid content increases significantly (P≤0.05) depending on the polarity of the solvents and microalgae species. The solvent DIC: MeOH extracted the highest amounts of total carotenoids 1.39±0.01, 13.09±0.97, and 11.79±0.99 mg g-1 DW of *D. salina*, *H. pluvialis*, and *S. obliquus*, respectively, followed by Hex: EtOH, Hex: EtOAc, then Hex. Microalgae *H. pluvialis* and *S. obliquus* yielded the highest total carotenoids compared with *D. salina*, especially with the high polar solvents DIC: MeOH and Hex: EtOH (Fig 2).

The content of β-carotene significantly (P≤0.05) increased with the solvent polarity in the case of *D. salina* and *H. pluvialis*, the solvent DIC: MeOH extracted the highest amounts 0.057±0.001 and 0.961±0.013 mg g-1 DW of β-carotene, respectively compared with other solvents (Fig. 3). Contrariwise, in the case of *S. obliquus* the low polar solvents, Hex, Hex: EtOAc, and Hex: EtOH extracted the highest amounts of β-carotene 1.06±0.07, 1.05±0.08, and 1.04±0.06 mg g-1 DW, respectively, compared with DIC: MeOH which extracted 0.781±0.012 mg g-1 DW of β-carotene (Fig 3). Microalgae *S. obliquus* yielded the highest amount of β-carotene followed by *H. pluvialis* then *D. salina*.

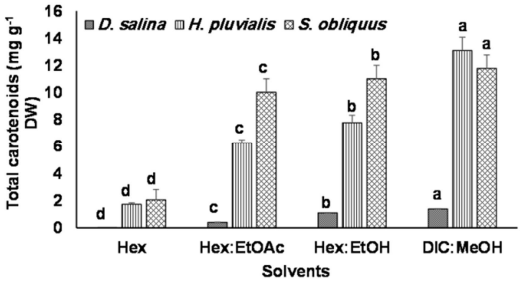

Fig. 2. The effect of different solvents on the total carotenoid content (mg g-1 DW) of *D. salina*, *H. pluvialis*, and *S. obliquus*. Different letters in each series represent significant differences at P≤0.05.

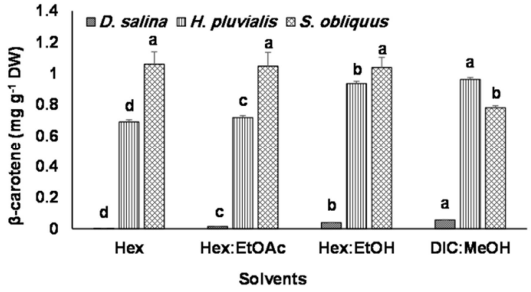

Fig. 3. The effect of different solvents on β-carotene content (mg g-1 DW) of *D. salina*, *H. pluvialis*, and *S. obliquus*. Different letters in each series represent significant differences at P≤0.05.

The content of lutein significantly (P≤0.05) increased with the solvent polarity and depends on microalgae species. The solvent DIC: MeOH extracted the highest amounts of 0.159±0.001 and 0.858±0.055 mg g-1 DW of lutein of *D. salina* and *H. pluvialis*, respectively. While Hex: EtOH extracted the highest amounts of lutein 1.585±0.015 mg g-1 DW of *S. obliquus* (Fig. 4). Microalgae *S. obliquus* yielded the highest amount of lutein followed by *H. pluvialis* then *D. salina*.

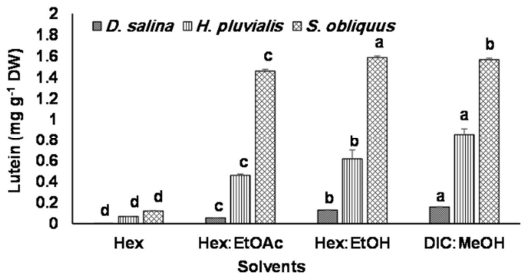

Fig. 4. The effect of different solvents on lutein content (mg g-1 DW) of *D. salina*, *H. pluvialis*, and *S. obliquus*. Different letters in each series represent significant differences at P≤0.05.

The content of zeaxanthin significantly (P≤0.05) increased with the solvent polarity in the case of *D. salina* and *H. pluvialis*, the solvent DIC: MeOH extracted the highest amounts of 0.034±0.001 and 0.270±0.009 mg g-1 DW of zeaxanthin, respectively compared with other solvents. Otherwise, in the case of *S. obliquus* the low polar solvent Hex: EtOAc extracted the highest amount of zeaxanthin 0.121±0.001 mg g-1 DW (Fig. 5). The content of zeaxanthin depends on microalgae species, *H. pluvialis* yielded the highest amount of zeaxanthin followed by *S. obliquus* then *D. salina*.

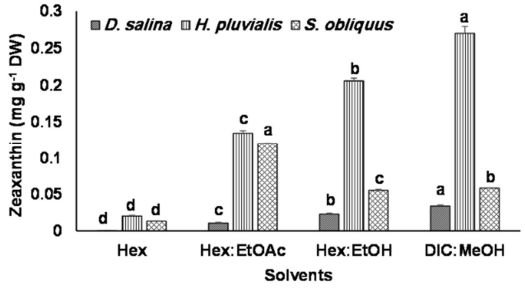

Fig. 5. The effect of different solvents on zeaxanthin content (mg g-1 DW) of *D. salina*, *H. pluvialis*, and *S. obliquus*. Different letters in each series represent significant differences at P≤0.05.

The polarity of solvents positively affected the extraction of astaxanthin, the solvent DIC: MeOH extracted the highest amounts of 0.023±0.001 and 0.077±0.008 mg g-1 DW of astaxanthin from *D. salina* and *S. obliquus*, respectively. The solvent Hex: EtOH extracted the highest amount of 0.059±0.001 mg g-1 DW of astaxanthin from *H. pluvialis* (Fig. 6). The content of astaxanthin depends on microalgae species,

S. obliquus yielded the highest amount of zeaxanthin followed by *H. pluvialis* then *D. salina*.

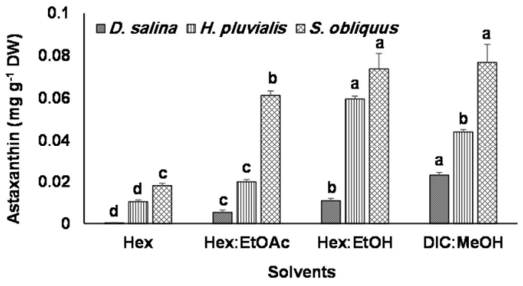

Fig. 6. The effect of different solvents on astaxanthin content (mg g-1 DW) of *D. salina*, *H. pluvialis*, and *S. obliquus*. Different letters in each series represent significant differences at P≤0.05.

3.3. GC-FID Fatty acid profile

The solvent polarity positively affected the content of saturated fatty acids (SFA), especially palmitic acid, the major saturated fatty acid found in *D. salina*. The solvent DIC: MeOH extracted the highest amount (32.58%) of palmitic acid of *D. salina* compared with Hex which extracted the lowest amount (10.46%) as shown in Table 1. The content of omega fatty acids depends on solvent polarity, increasing the solvent polarity increases the sum of omega-3 (ω3), the Sum of ω6, and the Sum of ω7+4, and decreases the sum of ω9. The solvent system Hex: EtOH yielded the highest amount of ω3, especially linolenic acid (22.56%), and ω6, especially linoleic acid (8.95%). While the solvent Hex extracted the highest amount of omega-6 (ω6), palmitoleic acid (23.76%), and oleic acid (23.29%). Overall, the solvent Hex yielded the highest amount of Sum of ω (57.98%) followed by Hex: EtOH (57.6%), Hex: EtOAc (50.53%), then DIC: MeOH (43.02%) of *D. salina* (Table 1).

Data from Table 2 shows that the GC-FID fatty acid profile of *H. pluvialis* varies depending on the extraction solvent. The extraction by Hex: EtOH decreased the content of sum SFA to 22.46% and increased the content of the sum omega fatty acids to 74.94%. The solvents Hex, Hex: EtOAc, and DIC: MeOH mostly show the same extraction efficiency, they extracted sum SFA with amounts of 32.62%, 33.51%, and 34.21% and sum omega fatty acids with amounts of 65.4%, 64.6%, and 63.88%, respectively (Table 2).

Table 1. Fatty acid composition (%) of *D. salina* extracted by different solvents.

Fatty acids	Name	*D. salina* fatty acids (%)			
		Hex	Hex: EtOAc	Hex: EtOH	DIC: MeOH
1- Saturated Fatty Acids (SFA)					
C8:0	Caprylic acid	0.64	5.32	-	-
C11:0	Undecanoic acid	4.17	8.53	2.14	4.24
C12:0	Lauric acid	7.10	-	1.82	3.11
C13:0	Tridecanoic acid	1.04	1.64	-	-
C14:0	Myristic acid	5.30	2.51	1.44	1.81
C15:0	Pentadecanoic acid	3.15	2.84	5.26	3.1
C16:0	Palmitic acid	10.46	17.59	23.05	32.58
C17:0	Heptadecanoic acid	0.73	0.82	3.65	4.88
C18:0	Stearic acid	2.19	4.45	1.70	0.85
C20:0	Arachidic acid	-	-	0.60	1.54
C22:0	Behenic acid	0.25	-	1.21	2.1
Sum of SFA		35.03	43.7	40.87	52.11
2- Omega fatty acids (ω)					
2-1- Omega-3 (ω3)					
C16:4, ω3	Hexadecatetraenoic	-	-	5.71	2.27
C18:3, ω3	Linolenic acid	1.74	12.60	22.56	13.73
C18:4, ω3	Stearidonic acid	0.75	-	-	-
Sum of ω3		2.49	12.60	28.27	16
2-2- Omega-6 (ω6)					
C18:2, ω6	Linoleic acid	7.43	8.07	8.95	4.66
Sum of ω6		7.43	8.07	8.95	4.66
2-3- Omega-9 (ω9)					
C16:1, ω9	Palmitolic acid	23.76	20.44	8.44	9.23
C18:1, ω9	Oleic acid	23.29	6.07	7.27	7.07
C20:1, ω9	Gondoic acid	0.14	-	-	-
Sum of ω9		47.19	26.51	15.71	16.03
2-4- Omega-7+4 (ω7+4)					
C16:1, ω7	Palmitoleic acid	-	0.93	1.03	1.06
C18:1, ω7	Vaccinic acid	0.87	2.42	3.64	4.67
C16:3, ω4	Hexagonic acid	-	-	-	0.6
Sum of ω7+4		0.87	3.35	4.67	6.33
Sum of ω		57.98	50.53	57.6	43.02
Non-Identified Fatty Acid (NIFA)		6.99	5.77	1.53	2.5

Hex: Hexane; Hex:EtOAc: Hexane:Ethyl acetate (1:1, v/v); Hex:EtOH: Hexane:Ethanol (2:1, v/v); DIC:MeOH: Dichloromethane:Methanol (3:1, v/v).

The solvent Hex: EtOH is the best solvent for extracting fatty acids of *H. pluvialis* as it extracted the lowest amount of palmitic acid (11.15%), the main SFA, the highest amount of linolenic acid (30.77%), the main ω3, the lowest amount of linoleic acid (7.80%), the main ω6, and adequate amount of oleic acid (7.11%), the major ω9 (Table 2).

Table 2. Fatty acid composition (%) of *H. pluvialis* extracted by different solvents.

Fatty acids	Name	*H. pluvialis* fatty acids (%)			
		Hex	Hex: EtOAc	Hex: EtOH	DIC: MeOH
1- Saturated Fatty Acids (SFA)					
C8:0	Caprylic acid	0.87	1.60	0.44	-
C11:0	Undecanoic acid	0.71	0.45	-	0.72
C12:0	Lauric acid	2.24	2.55	1.88	4.17
C13:0	Tridecanoic acid	0.29	-	-	-
C14:0	Myristic acid	1.34	1.08	1.08	0.93
C15:0	Pentadecanoic acid	1.65	1.30	1.27	1.32
C16:0	Palmitic acid	14.65	15.83	11.15	16.13
C17:0	Heptadecanoic acid	8.46	8.02	4.06	8.47
C18:0	Stearic acid	1.82	2.09	2.21	2.05
C22:0	Behenic acid	0.59	0.59	0.37	0.42
Sum of SFA		32.62	33.51	22.46	34.21
2- Omega fatty acids (ω)					
2-1- Omega-3 (ω3)					
C16:4, ω3	Hexadecatetraenoic acid	10.02	9.94	13.81	8.43
C18:3, ω3	Linolenic acid	19.27	17.67	30.77	15.71
C18:4, ω3	Stearidonic acid	3.85	3.45	2.13	3.06
Sum of ω3		33.14	31.06	46.71	27.2
2-2- Omega-6 (ω6)					
C18:2, ω6	Linoleic acid	14.46	14.06	7.80	14.94
C18:3, ω6	ɤ- Linoleic acid	0.48	0.42	0.49	-
C20:2, ω6	Eicosadienoic acid	0.17	-	-	1.96
C20:4, ω6	Arachidonic acid	0.10	0.11	1.64	-
Sum of ω6		15.21	14.59	9.93	16.9
2-3- Omega-9 (ω9)					
C16:1, ω9	Palmitolic acid	6.45	6.29	8.74	6.42
C18:1, ω9	Oleic acid	7.35	9.65	7.11	10.97
C20:1, ω9	Gondoic acid	0.26	0.26	0.16	0.19
Sum of ω9		14.06	16.2	16.01	17.58
2-4- Omega-7 (ω7)					
C16:1, ω7	Palmitoleic acid	1.45	1.34	0.79	0.81
C18:1, ω7	Vaccinic acid	1.54	1.41	1.50	1.39
Sum of ω7		2.99	2.75	2.29	2.2
Sum of ω		65.4	64.6	74.94	63.88
Non-Identified Fatty Acid		1.98	1.89	2.60	1.91

Hex: Hexane; Hex:EtOAc: Hexane:Ethyl acetate (1:1, v/v); Hex:EtOH: Hexane:Ethanol (2:1, v/v); DIC:MeOH: Dichloromethane:Methanol (3:1, v/v).

The GC-FID fatty acid profile of *S. obliquus* differs according to the extraction solvent. Overall, the extraction with low polar solvents Hex and Hex: EtOAc resulted in the lowest amounts of sum SFA (24.21% and 23.31%) and the highest amounts of sum omega fatty acids (73.31% and 73.92%), respectively, compared to the high polar solvents Hex: EtOH and DIC: MeOH (Table 3). The solvents Hex and Hex: EtOAc realized the fatty acid high extraction efficiency of *S. obliquus*, they extracted the lowest amount of palmitic acid (11.87% and 11.53%), the main SFA, the highest amount of linolenic acid (29.19% and 29.42%), the main ω3, the lowest amount of linoleic acid (7.12% and 7.38%), the main ω6, and adequate amount of oleic acid (9.23% and 8.19%), the major ω9 (Table 3).

Table 3. Fatty acid composition (%) of *S. obliquus* extracted by different solvents.

Fatty acids	Name	*S. obliquus* fatty acids (%)			
		Hex	Hex: EtOAc	Hex: EtOH	DIC: MeOH
1- Saturated Fatty Acids (SFA)					
C8:0	Caprylic acid	-	-	1.26	0.21
C10:0	Capric acid	-	-	0.27	0.17
C11:0	Undecanoic acid	0.79	0.78	0.30	0.47
C12:0	Lauric acid	2.12	2.03	1.68	1.45
C13:0	Tridecanoic acid	0.37	-	0.29	0.21
C14:0	Myristic acid	1.77	1.62	1.33	1.09
C15:0	Pentadecanoic acid	1.31	1.23	1.36	3.55
C16:0	Palmitic acid	11.87	11.53	14.43	13.35
C17:0	Heptadecanoic acid	3.46	3.40	8.02	3.54
C18:0	Stearic acid	2.28	2.18	2.03	2.39
C20:0	Arachidic acid	-	0.25	-	-
C22:0	Behenic acid	0.24	0.29	0.52	0.34
Sum of SFA		24.21	23.31	31.49	26.77
2- Omega fatty acids (ω)					
2-1- Omega-3 (ω3)					
C16:4, ω3	Hexadecatetraenoic acid	11.55	13.24	10.90	12.91
C18:3, ω3	Linolenic acid	29.19	29.42	20.46	29.10
C18:4, ω3	Stearidonic acid	1.63	1.77	3.11	1.82
Sum of ω3		42.37	44.43	34.47	43.83
2-2- Omega-6 (ω6)					
C18:2, ω6	Linoleic acid	7.12	7.38	13.11	8.35
C18:3, ω6	ɤ- Linoleic acid	0.42	0.43	0.44	0.47
C20:2, ω6	Eicosadienoic acid	-	-	0.59	-
C20:4, ω6	Arachidonic acid	1.97	2.03	0.20	1.54
Sum of ω6		9.51	9.84	14.34	10.36
2-3- Omega-9 (ω9)					
C16:1, ω9	Palmitolic acid	8.91	9.08	6.60	5.42
C18:1, ω9	Oleic acid	9.23	8.19	8.13	10.09
C20:1, ω9	Gondoic acid	0.21	-	0.20	-
Sum of ω9		18.35	17.27	14.93	15.51
2-4- Omega-7+4 (ω7+4)					
C16:1, ω7	Palmitoleic acid	1.13	0.68	1.46	0.85
C18:1, ω7	Vaccinic acid	1.52	1.35	1.44	1.61
C18:3, ω4		0.43	0.35	-	-
Sum of ω7+4		3.08	2.38	2.9	2.46
Sum of ω		73.31	73.92	66.64	72.16
Non-Identified Fatty Acid		2.57	2.77	1.87	0.98

Hex: Hexane; **Hex:EtOAc:** Hexane:Ethyl acetate (1:1, v/v); **Hex:EtOH:** Hexane:Ethanol (2:1, v/v); **DIC:MeOH:** Dichloromethane:Methanol (3:1, v/v).

4. Discussion

The varied use of natural carotenoids in pharmaceutical, food, and cosmetic applications has motivated the development of appropriate extraction methods to recover the highest yield from different food matrices including microalgae. Microalgae, *D. salina*, *H. pluvialis*, and *S. obliquus*, are excellent renewable sources of natural carotenoids worldwide. However, these microalgae form thick, complex, and stiff cell walls, which form the most obstacle to the high extraction efficiency of carotenoids and other bioactive components [7, 9, 26]. Microalgae produce different carotenoids such as β-carotene, lutein, zeaxanthin, and astaxanthin inside lipid globules in the cytoplasm of the cells. Consequently, the cell wall disruption of microalgae is one of the key challenges in carotenoid extraction [27]. Degradation of the microalgae cell wall accelerates the access of solvents inside the cell to dissolve intracellular carotenoids, which increases the extraction yield [28]. In this study, we used the high-speed electrical mill to grind the dry microalgae to a fine powder (≥80 mesh) to lessen the effect of the cell wall barrier on the extraction efficiency. Different investigations used bead milling and grinding of dry microalgae as safe mechanical processes to disrupt the rigid cell wall to maximize the extraction of cell components [7, 29]. Microalgal cell wall disruption using chemical and thermal pre-treatments causes oxidation, degradation, and change in different carotenoids' chemical structures, declining their biological activities [30].

Our results show that the extract yield and the total carotenoids increase significantly ($P \leq 0.05$) depending on the polarity of the solvents and microalgae species. The high polar solvents DIC: MeOH and Hex: EtOH achieved the highest extraction efficiency of the extract yield and the total carotenoids of the studied microalgae, followed by the less polar solvents Hex: EtOAc and Hex. Microalgae *H. pluvialis* and *S. obliquus* yielded the highest extract yield and total carotenoids compared with *D. salina.* The present results are in accord with that of [15], who reported that increasing the polarity of solvents increases the recovery of total carotenoids of microalgae: *Nannochloropsis gaditana*, *Chlorella sp.*, *H. pluvialis*, *Scenedesmus almeriensis*, *Isochrysis galbana*, Tetraselmis suecica, *Protoceratium reticulatum*, and *Karlodinium veneficum*, and the solvent mixture composed of water, ethanol, and hexane achieved the highest recovery. They reported also that the recovery of total carotenoids was species dependent. Consensus to our results, the high polar solvents: methanol, ethanol, and acetone showed higher extraction efficiency of total carotenoids of *H. pluvialis* than ethyl lactate and ethyl acetate [31]. Different authors discussed the varied effects of solvent polarities either single or mixtures on the extraction efficiency of total carotenoids of different microalgae, and they support our results of the positive effect of polar solvents on the extraction efficiency of total carotenoids [32, 33]. The use of solvents with different polarities in this study showed a positive effect on the extraction efficiency of the carotenoid profile: β-carotene, lutein, zeaxanthin, and astaxanthin in the three microalgae depending on the microalgae species. The extraction efficiency of carotenoid individuals strongly depends on the polarity of the solvent and the polarity of the carotenoid compound. As shown in this study, the low polar solvent Hex showed highly efficient in the extraction of β-carotene, low polar carotenoids, in *S. obliquus* microalgae. While the high polar carotenoids: lutein, zeaxanthin, and astaxanthin have been extracted with high efficiency using the high polar solvents: DIC: MeOH, Hex: EtOH, and Hex: EtOAc. The present results are in harmony with earlier studies that stated that the polar solvents (e.g., methanol, ethanol, acetone, ethyl acetate, and dichloromethane) show high extraction efficiency for polar carotenoids such as astaxanthin, zeaxanthin, and lutein, while the low polar solvents (e.g., petroleum ether or hexane) show high extraction efficiency for the nonpolar carotenoids such as β-carotene, α-carotene, or carotenoids in ester forms [18]. The results of [34] showed that hexane and heptane are the most efficient solvents to recover high yields of β-carotene from *Spirulina platensis*, compared with diethyl ether. The acetone and ethanol attained the highest extraction efficiency of the carotenoids profile: violaxanthin, neoxanthin, lutein, α-carotene, and β-carotene of *S. obliquus* and *Gloeothece sp.*. The ethanol, ethyl acetate, and isoamyl acetate achieved high extraction of astaxanthin of *H. pluvialis* [35]. The use of dichloromethane showed high extraction yield, total astaxanthin, and free astaxanthin recovery from *H. pluvialis*, compared with acetone [29], they attributed this due to the higher solubility of astaxanthin in dichloromethane than in acetone. The mixture of dichloromethane with decane showed high extraction efficiency of β-carotene from *D. salina*, compared with pure decane and other solvents [36]. The use of pure EtOH and EtOH: EtOAc mixture (2:1) achieved the highest yields of

astaxanthin and β-carotene from *Phaffia rhodozyma* biomass [37]. Based on the above discussion, the development of a potent method for wide extraction of carotenoids from microalgae is not possible, however, the use of solvents mixtures, polar and non-polar, showed reliable results for the highest extraction of different carotenoids: xanthophylls and carotenes. The mixture of polar and non-polar solvents, used in the present study, can increase the extraction efficiency of carotenoids depending on different factors; the high dissolving efficiency of solvent mixture towards different carotenoids depending on the similar polarity option (the low-polar solvents dissolves the low-polar carotenoids and vice versa), the high-polar solvents have the high ability, compared with low-polar ones, to form a hydrogen bond with carotenoids and extract it from the complexes of the photosynthetic membrane with proteins, and the high ability of the solvent mixture to recover carotenoids from fat globules. This is in accord with former reports [15]. Different studies demonstrated that under certain conditions including nutrient restriction, high salinity, and light stress, *D. salina* can produce large quantities of β-carotene and zeaxanthin, *H. pluvialis* can produced high amounts of astaxanthin, as well as *S. obliques* can produced high amounts of lutein [10, 31, 33, 38, 39]. In the current study, all the algae were grown without any stress conditions and were harvested in the vegetative stage, this might explain the varied concentrations of carotenoids among the investigated algae when compared with previously published results of the same algae.

The GC-FID analysis of fatty acids in this study stated that the three algal strains; *D. salina*, *H. pluvialis*, and *S. obliques* are useful sources of omega fatty acids, especially omega-3, as they contain 52% - 71.51 % sum omega fatty acids out of their total fatty acids. The high content of omega-3 fatty acids, especially α-Linolenic acid, of the three algal strains makes them an outstanding source of nutrition supplements for human health. These results agree with former results of [40], [41], and [42]. Our results emphasize the varied influence of non-polar/polar solvent mixture on the extraction yield and composition of saturated and unsaturated fatty acids from the investigated algal strains and we found that the addition of polar solvent in the extraction mixture markedly improved the extraction yield of omega fatty acids. The solvent mixture of Hex: EtOH recovered the highest yield of omega fatty acids, especially omega-3 from *D. salina* and *H. pluvialis*, and Hex: EtOAc showed the same action with *S. obliques*. In this study, the positive effect of polar solvent in the solvent mixture towards lipids extraction due to increasing the permeability of the solvent mixture through the algal cell wall, consequently, increasing the extraction efficiency of a wide range of lipids from non-polar to polar, depending on the polarity of solvent and lipids. This result is in harmony with former results that concluded that the effective extraction of microalgal lipids is dependent on the polarity of the organic solvent or solvent mixture used, the site of the lipidic compound in the cell, and the cell wall structure [14, 17, 43]. Other authors have reported the positive effect of adding polar solvent in the extraction mixture on the extraction yield and GC-FID profile of the unsaturated fatty acids in microalgae [44–45]. They concluded that polar solvents have higher affinities to polar lipids, which are bound to glycoglycerolipids in the chloroplasts of eukaryotic, so they extract a higher content of unsaturated fatty acids compared to non-polar solvents.

5. Conclusions

The extraction efficiency of carotenoids such as β-carotene, lutein, zeaxanthin, and astaxanthin, and of omega fatty acids, is highly dependent on the polarity of the solvent, the polarity of the extracted component, and the species of microalgae. The high polar carotenoids: lutein, zeaxanthin, and astaxanthin were efficiently extracted by using the high polar solvents: DIC: MeOH, Hex: EtOH, and Hex: EtOAc. The solvent mixture of Hex: EtOH recovered the highest yield of omega fatty acids, especially omega-3 from *D. salina* and *H. pluvialis*, whereas Hex: EtOAc had a similar effect on *S. obliques*. In conclusion, the solvent combinations of Hex: EtOH and Hex: EtOAc are the best for extracting carotenoids and omega fatty acids from the three microalgae investigated.

Conflicts of interest

"There are no conflicts to declare".

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References

[1] El-Baz FK, Hussein RA, Saleh DO, Abdel Jaleel GAR (2019) Zeaxanthin isolated from *Dunaliella*

salina microalgae ameliorates age associated cardiac dysfunction in rats through stimulation of retinoid receptors. Marine drugs 17:290.

[2] El-Baz FK, Saleh DO, Jaleel GAA, et al (2019) *Heamatococcus pluvialis* ameliorates bone loss in experimentally-induced osteoporosis in rats via the regulation of OPG/RANKL pathway. Biomedicine & Pharmacotherapy 116:109017.

[3] El-Baz F K, Aly HF, Salama A, Ali SI, El Askary H I, Khalil A M (2022) Assessment of β-carotenoids extracted from *Dunaliella salina* microalgae: Toxicological aspects. Egyptian Journal of Chemistry1;65(6):727-39.

[4] Novoveská L, Ross ME, Stanley MS, et al (2019) Microalgal carotenoids: A review of production, current markets, regulations, and future direction. Marine drugs 17:640.

[5] El-Baz FK, Salama A, Ali SI, Elgohary R (2021) *Haematococcus pluvialis* carotenoids enrich fractions ameliorate liver fibrosis induced by thioacetamide in rats: modulation of metalloproteinase and its inhibitor. BioMed Research International 2021:6631415.

[6] El-Baz F K, Aly HF, Ali SI, Salama A (2023) Zeaxanthin-enriched fraction of *Dunaliella salina*: Acute and chronic assessments. Egyptian Journal of Chemistry 1;66(6):109-18.

[7] Kim B, Lee SY, Narasimhan AL, et al (2022) Cell disruption and astaxanthin extraction from *Haematococcus pluvialis*: Recent advances. Bioresource Technology 343:126124.

[8] El-Baz FK, Ali SI, Basha M, et al (2020) Design and evaluation of bioenhanced oral tablets of *Dunaliella salina* microalgae for treatment of liver fibrosis. Journal of Drug Delivery Science and Technology 59:101845.

[9] Monte J, Ribeiro C, Parreira C, et al (2020) Biorefinery of *Dunaliella salina*: Sustainable recovery of carotenoids, polar lipids and glycerol. Bioresource technology 297:122509.

[10] Aburai N, Sumida D, Abe K (2015) Effect of light level and salinity on the composition and accumulation of free and ester-type carotenoids in the aerial microalga *Scenedesmus sp.*(Chlorophyceae). Algal Research 8:30–36.

[11] Přibyl P, Pilnỳ J, Cepák V, Kaštánek P (2016) The role of light and nitrogen in growth and carotenoid accumulation in *Scenedesmus sp.* Algal research 16:69–75.

[12] Guedes AC, Amaro HM, Barbosa CR, et al (2011) Fatty acid composition of several wild microalgae and cyanobacteria, with a focus on eicosapentaenoic, docosahexaenoic and α-linolenic acids for eventual dietary uses. Food Research International 44:2721–2729.

[13] Nitsos C, Filali R, Taidi B, Lemaire J (2020) Current and novel approaches to downstream processing of microalgae: A review. Biotechnology Advances 45:107650.

[14] Ryckebosch E, Bruneel C, Termote-Verhalle R, et al (2014) Influence of extraction solvent system on extractability of lipid components from different microalgae species. Algal Research 3:36–43.

[15] Cerón-García MC, González-López CV, Camacho-Rodríguez J, et al (2018) Maximizing carotenoid extraction from microalgae used as food additives and determined by liquid chromatography (HPLC). Food Chemistry 257:316–324.

[16] El-Baz F K, Aly HF, Ali SI, Salama A. (2022) Safety of astaxanthin-rich fraction of *Haematococcus pluvialis* microalgae. Egyptian Journal of Chemistry 1;65(3):479-89.

[17] Mansouri H, Nezhad SE (2020) Improvement in biochemical parameters and changes in lipid profile of *Scenedesmus obliquus* by plant growth regulators under mixotrophic condition. Biomass and Bioenergy 140:105708.

[18] Khanra S, Mondal M, Halder G, et al (2018) Downstream processing of microalgae for pigments, protein and carbohydrate in industrial application: A review. Food and bioproducts processing 110:60–84.

[19] Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriological reviews 35:171–205.

[20] Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Canadian journal of biochemistry and physiology 37:911–917.

[21] Diraman H, Koru E, Dibeklioglu H (2009) Fatty acid profile of *Spirulina platensis* used as a food supplement. The Israeli Journal of Aquaculture – Bamidgeh 61(2):134-142.

[22] Ren L-J, Huang H, Xiao A-H, et al (2009) Enhanced docosahexaenoic acid production by reinforcing acetyl-CoA and NADPH supply in *Schizochytrium sp.* HX-308. Bioprocess and biosystems engineering 32:837–843.

[23] Prum RO, LaFountain AM, Berro J, et al (2012) Molecular diversity, metabolic transformation, and evolution of carotenoid feather pigments in cotingas (Aves: Cotingidae). Journal of Comparative Physiology B 182:1095–1116.

[24] Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J biol Chem 226:497–509.

[25] AOAC (1990) Fatty acids in oils and fats. Preparation of methyl esters. Boron trifluoride method. Official Methods of Analysis of the Association of Official Analytical Chemists.

[26] Tan Y, Ye Z, Wang M, et al (2021) Comparison of different methods for extracting the astaxanthin from *Haematococcus pluvialis*: Chemical composition and biological activity. Molecules 26:3569.

[27] Wang Q, Oshita K, Takaoka M, Shiota K (2021) Influence of water content and cell disruption on lipid extraction using subcritical dimethyl ether in wet microalgae. Bioresource Technology 329:124892.

[28] Uquiche E, Antilaf I, Millao S (2016) Enhancement of pigment extraction from *B. braunii* pretreated using CO_2 rapid depressurization. brazilian journal of microbiology 47:497–505.

[29] Irshad M, Hong ME, Myint AA, et al (2019) Safe and complete extraction of astaxanthin from *Haematococcus pluvialis* by efficient mechanical disruption of cyst cell wall. International Journal of Food Engineering 15(10):20190128.

[30] Kaczor A, Baranska M (2011) Structural changes of carotenoid astaxanthin in a single algal cell monitored in situ by Raman spectroscopy. Analytical chemistry 83:7763–7770.
[31] Castillo A, Pereira S, Otero A, et al (2022) Multicomponent bioactive extract from red stage *Haematococcus pluvialis* wet paste: avoiding the drying step and toxic solvents. Journal of Applied Phycology 34:1537–1553.
[32] Van Leeuwe MA, Villerius LA, Roggeveld J, et al (2006) An optimized method for automated analysis of algal pigments by HPLC. Marine chemistry 102:267–275.
[33] Grujić VJ, Todorović B, Kranvogl R, et al (2022) Diversity and Content of Carotenoids and Other Pigments in the Transition from the Green to the Red Stage of *Haematococcus pluvialis* Microalgae Identified by HPLC-DAD and LC-QTOF-MS. Plants 11:1026.
[34] Dey S, Rathod VK (2013) Ultrasound assisted extraction of β-carotene from *Spirulina platensis*. Ultrasonics sonochemistry 20:271–276.
[35] Myint AA, Hariyanto P, Irshad M, et al (2022) Strategy for high-yield astaxanthin recovery directly from wet *Haematococcus pluvialis* without pretreatment. Bioresource technology 346:126616.
[36] Mojaat M, Foucault A, Pruvost J, Legrand J (2008) Optimal selection of organic solvents for biocompatible extraction of β-carotene from *Dunaliella salina*. Journal of biotechnology 133:433–441.
[37] Mussagy CU, Kurnia KA, Dias AC, et al (2022) An eco-friendly approach for the recovery of astaxanthin and β-carotene from *Phaffia rhodozyma* biomass using bio-based solvents. Bioresource Technology 345:126555.
[38] Rajendran L, Nagarajan NG, Karuppan M (2020) Enhanced biomass and lutein production by mixotrophic cultivation of *Scenedesmus sp.* using crude glycerol in an airlift photobioreactor. Biochemical Engineering Journal 161:107684.
[39] Saha SK, Kazipet N, Murray P (2018) The carotenogenic *Dunaliella salina* CCAP 19/20 produces enhanced levels of carotenoid under specific nutrients limitation. BioMed research international 2018: ID 7532897.
[40] Abd El-Baky HH, El Baz FK, El-Baroty GS (2004) Production of lipids rich in omega 3 fatty acids from the halotolerant alga *Dunaliella salina*. Biotechnology 3:102–108.
[41] Damiani MC, Popovich CA, Constenla D, Leonardi PI (2010) Lipid analysis in *Haematococcus pluvialis* to assess its potential use as a biodiesel feedstock. Bioresource technology 101:3801–3807.
[42] Darki BZ, Seyfabadi J, Fayazi S (2017) Effect of nutrients on total lipid content and fatty acids profile of *Scenedesmus obliquus*. Brazilian Archives of Biology and Technology 60: e17160304.
[43] El-Baz F K, Salah D, Abougharam G, Ali SI, Aly HF (2023) Potential production of omega fatty acids from *Scenedesmus obliquus* microalgae: Evaluation of acute and chronic toxicity. Egyptian Journal of Chemistry 1;66(4):457-65.
[44] Grima EM, González MJI, Giménez AG (2013) Solvent extraction for microalgae lipids. In: Algae for biofuels and energy. Springer, pp 187–205.
[45] Li Y, Ghasemi Naghdi F, Garg S, et al (2014) A comparative study: the impact of different lipid extraction methods on current microalgal lipid research. Microbial cell factories 13:1–9.
[46] Hidalgo P, Ciudad G, Navia R (2016) Evaluation of different solvent mixtures in esterifiable lipids extraction from microalgae *Botryococcus braunii* for biodiesel production. Bioresource Technology 201:360–364.