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Characterization of Extracts from *Haematococcus pluvialis* Red Phase by using Accelerated Solvent Extraction

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The request for natural products such as antioxidant pigments derived from microalgae, i.e. ß-carotene, lutein and astaxanthin, is growing. In this context, astaxanthin, a powerful antioxidant produced by *Haematococcus pluvialis*, used as an additive in animal feed and as a food supplement, has been extracted by accelerated solvent extraction using acetone and ethanol as green and safe solvents, and hexane and chloroform:methanol (1:1) performing the best operating conditions. The obtained extracts showed not only the recovery of mainly astaxanthin but also other carotenoids, such as lutein and in lesser part of ß-carotene. In addition, the composition of the extracts was analyzed by highlighting the content of other valuable bio-products such as proteins, carbohydrates, lipids and Total Dietary Fibers. The best extraction performance was found using acetone and ethanol as solvent.

1. Introduction

The microalgae *Haematococcus pluvialis*, is a promising source of astaxanthin, a colored and antioxidant carotenoid that accumulates during the red growth phase. At this phase, stress factors as high light intensity or nitrogen deprivation triggered the transformation of ovoid green cells of *H. pluvialis* into round, red, and resistant biggest cysts, rich in astaxanthin which can make up 1-3% of the dry weight (Cerón et al. 2007) and more than 80% of the total carotenoids (Shah et al. 2016).
Astaxanthin ($C_{40}H_{52}O_4$, 3,3′-dihydroxy-β,β-carotene-4,4′-dione) as synthetic and natural form are currently mainly used as feed additive for salmonids, fish and ornamental birds and in nutraceutical sector as an antioxidant supplement. Astaxanthin market in 2018 was worth 550 million U.S. dollars with a forecast that could reach about 720 million U.S. dollars in the next 10 years considering a CAGR equal to 4.8% (Global Market Insights, 2018).. However, the synthetic form of astaxanthin still dominates most of the market since natural forms are not yet competitive due to the cost of microalgae cultivation and extraction process (Ruiz et al. 2016). Astaxanthin extraction processes from *H. pluvialis* have been investigated in several scientific papers using different technologies such as solid-liquid extraction by means of Sohxlet apparatus (Ruen-ngam et al. 2010), pressurized liquid extraction using Accelerated Solvent Extractor (ASE 200) (Denery et al. 2004, Molino et al., 2018a,b), supercritical fluid extraction, CO_2-SFE (Pan et al. 2012, Molino et al. 2018c, Di Sanzo et al. 2018) with the main objective to implement the recovery of astaxanthin. However, it would also be interesting to consider not only the extraction of astaxanthin from *H. pluvialis* but also of other products such as lipids, proteins, carbohydrates that can have an important value in different industrial applications. Since their content in H. pluvialis is around 30% dry weight for lipids, between 15-25% dry weight for proteins, and between 36-40% dry weight for carbohydrates (Shah et al. 2016).. The objective of this work is to extract astaxanthin from *Haematococcus pluvialis* using Accelerated Solvent Extraction testing the best operating conditions at 40 °C and 100 bar using acetone as solvent, at 67 °C and 100 bar using ethanol and chloroform:methanol (1:1 v/v), at 20 °C and 100 bar with hexane. Composition of the extracts was

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characterized in terms of total carotenoids (astaxanthin, lutein, beta-carotene), proteins, carbohydrates, lipids, and Total Dietary Fibers (TDFs).

2. Materials and methods

Haematococcus pluvialis lyophilized microalgae, purchased by MICOPERI BLUE GROWTH®, (Rimini, Italy) was mixed with inert material (Diatomaceous Earth, 0.8 grams) and mechanically pretreated by Retsch PM200 planetary ball mill at 400 rpm for 5 min. The obtained pretreated biomass was extracted by ASE 200, Accelerated Solvent Extractor (Dionex, Salt Lake City, UT, USA), following the procedure reported by Molino et al. 2018a. The best operating conditions were performed for the extraction of astaxanthin from *H. pluvialis* as reported in Molino et al. 2018a (Table 1). Every extraction cycles (n°4) were carried out for 20 min for a total time of 80 min till to obtain the biomass discoloring.

Table 1: Operating condition for astaxanthin extraction from H. pluvialis

Solvent	Temperature(°C)	Pressure (bar)	Extraction cycle (n°)	Extraction time (min)
chloroform:methanol (1:1 v/v)	67	100	4	20
ethanol	67	100	4	20
hexane	20	100	4	20
Acetone	40	100	4	20

The obtained extracts were quantified gravimetrically after a drying process under nitrogen flow by the TurboVAP Zymark® and their composition has been characterized.
Haematococcus pluvialis biomass and each extracts were analyzed in terms of moisture and ash according to the official methods EN ISO 712 and EN ISO 2171. Proteins and carbohydrates were quantified respectively according to the kjeldahl method (UNI EN ISO 20483) and HPLC-ELSD analysis (UNI EN 15086). Fatty acids was analyzed by GC-FID (Agilent 7820A) (UNI EN ISO 12966), while Total Dietary Fibers(TDFs) was quantified according AOAC 985.29. Total carotenoids were extracted from *H. pluvialis*, as reported by Li et al. 2012 and analyzed by uHPLC-DAD (Agilent 1290 Infinity II). uHPLC analysis was carried out to quantify astaxanthin, lutein (Ruegnam et al. 2010) and ß-carotene (UNI EN 12823-2).

3. Results and discussion

Haematococcus pluvialis red phase (HPR) microalgae was firstly characterized before the extraction of astaxanthin by ASE 200 as reported in Table 2. Moisture and ash content is equal to 70.0 mg/g and 60.15 mg/g dry weight basis resulting in line with others works, as well as the amount of protein (Kim et al. 2015, Shah et al. 2016). Lipids and carbohydrates have been found in lower amounts than in previous work, where their content is about 10 times higher. The unexpected low amount of carbohydrates is however balanced by the amount of TDFs. In fact, the content of TDFs, which are also the most abundant compounds also includes the polysaccharide fraction that gives strength and solidity to the cell wall of the red cysts of H. pluvialis. In addition, astaxanthin is the most widely produced carotenoid, followed by lutein and ß-carotene in smaller quantities as reported by Shah et al., 2016.

Table 2: Haematococcus pluvialis cellular composition characterization

Compounds	Protein	Carbohydrates	Lipids	Total carotenoids	Astaxanthin	Lutein	β - carotene	TDFs
(mg/g dry weight)	256.94	63.0	26.02	28.70	20.01	7.70	0.99	585.17

At the end of accelerated solvent extraction, liquid extracts and exhausted biomass had been analyzed. Liquid extracts were dried for the gravimetric quantification, whom quantity are reported in Table 3 and the exhausted biomass was used for the determination of ashes (Table 4).

Table 3: Extracts quantification expressed as mg/g dry weight (nd: not detectable)

Extracts (mg/g)	Ethanol 67 °C	Acetone 40 °C	Hexane 20 °C	Chloroform:methanol 1:1 (v/v) 67 °C
1st	328.22	326.20	185.16	286.42
2nd	21.01	67.88	15.13	34.70
3rd	12.81	19.00	20.16	14.70
4th	3.55	nd	19.52	1.11
Total	365.58	413.08	239.96	336.93

Although chloroform:methanol has widely been used to extract principally lipids from microalgae for their better extraction yields due to the different polarities of the solvent mixture, or hexane for the extraction of non-polar molecules (Mercer and Armenta 2011), in this work the largest quantities of extract were obtained using GRAS (Generally Recognized as Safe) solvents such as acetone at 40 °C and 100 bar and ethanol at 67 °C 100 bar after 80 minutes of extraction. Total extracts was equal to 413.08 mg/g dry weight using acetone and equal to 365.58 mg/gdry weight using ethanol. In all cases, the great quantity of extracts was obtained after the first 20 minutes of extraction.

Table 4: Ash content in exhausted biomass at different operating conditions

Ash (mg/g)	HPR	Ethanol 67 °C	Acetone 40 °C	Hexane 20 °C	C/M 67 °C
	60.15	52.83	50.29	58.20	55.61

Ash content in exhausted biomass resulted lower than initial HPR biomass (~ 60.15 mg/g dry weight) under all operating conditions . The biomass subjected to hexane extraction at 20 °C, after 80 min showed the higher value of ash and the lower extraction yield (239.96 mg/g dry weight). So, if the extraction yield decreased according to the following order of solvents acetone>ethanol>C/M>hexane, in the same order the quantity of ash increased.

Each obtained extracts was characterized in terms of total carotenoids, distinguishing astaxanthin, lutein and beta-carotene content as reported in Table 5.

The largest amount of carotenoids, equal to 22.06 mg/g dry weight, was extracted using acetone at 40 °C after 80 min of extraction, recoveringup to 76.9 % of the carotenoids in the initial biomass. The best recovery of astaxanthin using acetone was also demonstrated by Ruen-ngam et al. 2010 using different extraction process as Sohxlet, ultrasound and microwaves extractions. The largest quantity of astaxanthin and lutein was extracted already after 20 min of extraction, obtaining 99.0%and 96.7% of astaxanthin and lutein respectivelyExtracts obtained at 20 °C, 100 bar, after 80 min, using hexane as solvent showed the lowest carotenoid recoveryequal to 11.26 mg/g dry weight. Despite the lower extraction yieldin this condition, the best recovery of beta-carotene was obtained equal to 75.7%.

The composition of obtained extracts have been characterized by analyzing proteins, carbohydrates, lipids and TDFs expressed as mg/g dry weight as shown in Table 6 and Figure 1 Protein and TDFs are the most abundant compounds found in the extracts but in this case it is important to specify that TDFs have been obtained for difference by others compounds.

The acetone extract at 40 °C contains the highest amount of protein (206.91 mg/g dry weight and TDF 165.39 mg/g dry weight). Carbohydrates are more abundant in the extract in hexane at 20 °C and in ethanol at 67 °C and after 40 minutes of extraction is equal to 6.56 mg/g dry weight and 6.08 mg/g dry weight respectively.

The extracts that presented the highest amount of lipids are those in hexane at 20 °C and C/M at 67 °C that after 80 minutes of extraction, presented a content of 25.08 mg/g dry weight at 21.88 mg/g dry weight respectively. Under both conditions of extraction, the lipids contained in the extracts constituted 96.4% and 84.1% of the total lipid content compared to the initial biomass. Moreover, it was observed that in C/M, after the first extraction cycle of 20 min, almost all the lipids equal to 21.00 mg/g dry weight were extracted, while in hexane, after the first extraction cycle, only 57.52% of the total content were extracted. So it is very clear in this case, how the use of different organic solvents can affect the extraction process of bio-products.

Table 5: Carotenoids composition expressed as mg/g dry weight of Haematococcus pluvialis extracts (nd: not detectable; dL: Detection Limit)

	Astaxanthin	Lutein	ß-carotene	Total carotenoids
Ethanol 67 °C 1st	13.46	5.56	nd	19.01
Ethanol 67 °C 2nd	1.01	0.20	nd	1.21
Ethanol 67 °C 3rd	0.12	0.04	nd	0.16
Ethanol 67 °C 4th	0.05	0.02	nd	0.07
Total	14.64	5.82	nd	20.45
Acetone 40 °C 1st	17.28	4.47	nd	21.75
Acetone 40 °C 2nd	0.15	0.12	nd	0.28
Acetone 40 °C 3rd	0.02	0.02	nd	0.04
Acetone 40 °C 4th	<dl	<dl	nd	0.00
Total	17.45	4.62	nd	22.06
Hexane 20 °C 1st	4.78	0.96	0.70	6.45
Hexane 20 °C 2nd	1.57	0.34	0.05	1.97
Hexane 20 °C 3rd	1.25	0.32	<dl	1.57
Hexane 20 °C 4th	0.98	0.30	<dl	1.27
Total	8.58	1.93	0.75	11.26
C/M 67 °C 1st	8.41	4.40	nd	12.81
C/M 67 °C 2nd	3.27	0.60	nd	3.87
C/M 67 °C 3rd	1.06	0.81	nd	1.87
C/M 67 °C 4th	0.06	0.08	nd	0.14
Total	12.79	5.89	nd	18.68

Table 6: Extracts characterization of main cellular compounds expressed as mg/g dry weight (nd: not detectable)

	Proteins	Carbohydrates	Lipids	TDFs
Ethanol 67 °C 1st Ethanol 67 °C 2nd Ethanol 67 °C 3rd	179.36	4.33	26.02	110.38
Ethanol 67 °C 4th	14.67	1.75	1.67	1.72
Total	nd	nd	0.33	12.32
	nd	nd	0.21	3.28
	194.03	6.08	17.34	127.69
Acetone 40 °C 1st Acetone 40 °C 2nd Acetone 40 °C 3rd	173.51	1.00	16.65	113.29
Acetone 40 °C 4th	33.40	0.36	0.47	33.38
Total	nd	nd	0.24	18.72
	nd	nd	0.21	nd
	206.91	1.36	17.56	165.39
Hexane 20 °C 1st Hexane 20 °C 2nd Hexane 20 °C 3rd	140.18	4.72	14.36	20.16
Hexane 20 °C 4th	5.39	1.84	5.81	0.17
Total	nd	nd	3.13	15.46
	nd	nd	1.78	16.46
	145.57	6.56	25.08	52.25
C/M 67 °C 1st	164.40	0.52	21.00	87.69
C/M 67 °C 2nd	29.31	0.06	0.48	0.99
C/M 67 °C 3rd	nd	nd	0.22	12.61
C/M 67 °C 4th	nd	nd	0.19	0.78
Total	193.71	0.58	21.88	102.07

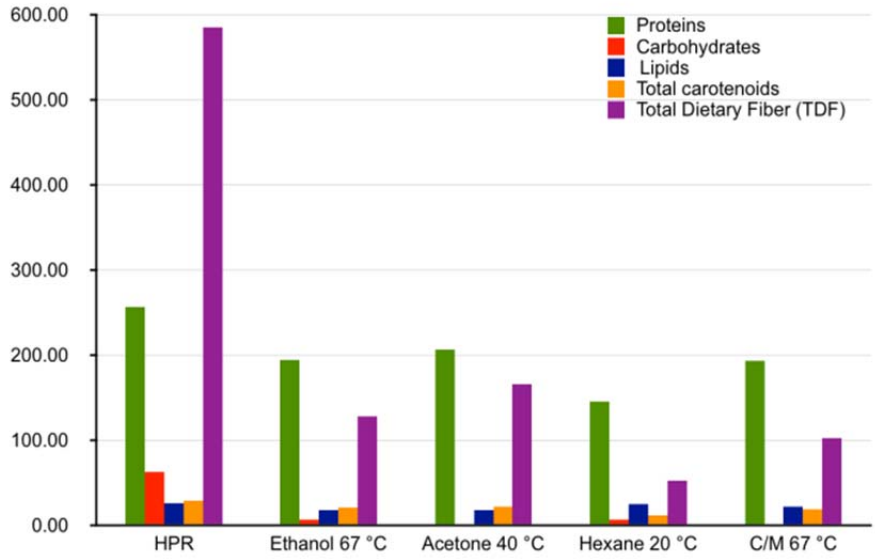

Figure 1: Characterization of H. pluvialis extracts expressed as mg/g dry weight

4. Conclusions

Extracts obtained from H. pluvialis using Accelerated Solvent Extractor have shown that their content does not only consist of astaxanthin, lutein and beta-carotene, but also of other compounds. The compounds most commonly found in extracts were proteins, TDFs, and lipids. The best extraction yields in terms of total carotenoids, protein, TDFs and lipid content were obtained using a GRAS solvent such as acetone at 40°C and 100 bar.

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