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Optimization of Mild Extraction Methods for the Efficient Recovery of Astaxanthin, a Strong Food Antioxidant Carotenoid from Microalgae

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Microalgae represent an important source of bioactive compounds for food applications. In this study, multifunctional extracts from *Haematococcus pluvialis* (*H. Pluvialis*) microalga have been developed in order to be used as food additives. Total extracts were recovered by ultrasound assisted extraction using eco-friendly and food grade solvents. Five different ratios ranging from 1/100 to 1/5 g biomass/ mL solvent were studied in different ultrasound intensities in order to optimise the extraction of carotenoids, especially of astaxanthin. The produced extracts were analysed using High Pressure Liquid Chromatography (HPLC-DAD) and UV-Vis spectroscopy techniques. Acetone was the most efficient solvent for the extraction of carotenoids in all tested ratios. However, D-limonene and medium chain triglycerides extracts also gave a satisfying recovery of carotenoids. D-limonene was the preferred solvent since extracts with high amounts of carotenoids were recovered, permitting their direct use in food products and avoiding further condensation steps.

1. Introduction

Antioxidants are compounds able to face oxidative stress and improve significantly human health, gaining enormous attention by the biomedical research community (Carlsen et al., 2010). Carotenoids represent an important group of antioxidants, which is widely used as additive in food products the last decade not only because of the proved health benefits, but also because of the positive effect on the color, microstructure and rheological properties of food products (Kwang et al.,2008; Sun et al.,2009). Nowadays, the increasing demands for high quality food products together with the use of naturally derived ingredients lead to the replacement of synthetic antioxidants with natural carotenoids, mainly in products intended for human consumption (S. Papadaki et al., 2016).

Astaxanthin (3,30-dihydroxy-b-b-carotene-440-dione), is a xanthophyll carotenoid with superior antioxidant capacity, which is 38-fold higher than that of b-carotene and 500-fold higher than vitamin E (Shimidzu et al., 1996; Zhang et al., 2016). Astaxanthin has gained popularity due to its antioxidant activity, antitumor effects, antidiabetic and anti-inflammatory properties (Ambati et al., 2014). It cannot be synthesized by humans, but it can be extracted from various natural sources and living organisms that biosynthesise it, such as shrimps, salmons and algae (Ambati et al., 2014). However, astaxanthin intake is low and almost limited to fish species, posing seaweed, and mainly microalgae, as its most important sources. On a commercial basis, astaxanthin is derived chiefly from *Haematococcus pluvialis (H. pluvialis)* microalga. *H.pluvialis* 's form of astaxanthin has been utilized in human nutritional supplements and constitutes the form on which all human clinical trials have been conducted (Industry Experts, 2015). *H.pluvialis* contains high amounts of astaxanthin that can reach more than 4% of dry weight (Cuellar-Bermudez et al., 2015).

The green alga *H. pluvialis* is a freshwater species of *Chlorophyta* that is naturally found in temperate zones around the world (Panis and Carreon, 2016; Zhang et al., 2016).). According to its physiology, *H.pluvialis,* during unfavorable growth conditions, such as nutrient deficiency and excessive light, initiates

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carotenogenesis and lipid accumulation, while undergoing morphological transformation from green vegetative cells to deep red forming deep red cysts (Wan et al., 2014; Gao et al., 2015). Apart from the high carotenoid content, *H.pluvialis* contains fatty acids, proteins, carbohydrates, and minerals (Borowitzka, 2013). Scientists are trying to optimise its cultivation system in order to achieve the highest concentration of astaxanthin within *H.pluvialis* red cysts (Chekanov et al., 2014).

In order to exploit the functional properties of microalgae, their bioactive compounds must be recovered using efficient methods. The recovery of bioactive compounds is mainly based on conventional extraction techniques, such as mechanical agitation, pressurized liquid technique and Soxhlet extraction, due to the low processing cost and ease of operation. Despite their advantages, these methods use toxic solvents and need large amounts of solvent and extended time. Moreover, the possibility of thermal degradation of bioactive compounds cannot be ignored, due to the high temperatures applied during the long times of extraction (Ruen-ngam et al., 2011). Currently, efficient, eco-friendly, clean, scalable and low cost extraction techniques are applied, such as Ultrasound-Assisted Extraction (UAE) that can reach high extraction yields within short time (Zou et al., 2013). The principle of ultrasound assisted extraction is based on the increase of mass transfer between the solvent and the biomass by improving the diffusion through the effective collapse of the cell membrane (J. Mason et al., 2011). UAE is an easy and comfortable process to scale-up (Deenu et al., 2013). In industrial scale, UAE is a suitable method for carotenoid extraction from microalgae in terms of both environmental (S. G. Papadaki et al., 2016) and economic (Deenu et al., 2013) aspects.

In addition, the increasing legislative restrictions on the presence of organic solvents in food products coupled to their negative effects on the nutritional and functional properties of compounds, have driven the search for “greener” alternatives than organic solvents, which are commonly used to extract valuable lipophilic compounds from microalgae (European Parliament Council, 2008; The European Parliament And The Council Of The European Union, 2009). Among them, D-limonene and medium chain triglycerides (MCT) oil can be used to replace organic solvents. D-limonene is a dietary supplement containing a natural cyclic monoterpene and is a major component of the oil extracted from citrus peels with potential chemo-preventive and antitumor activities (Sun, 2007; Golmakani et al., 2014). MCT oil is a unique form of dietary fat with excellent properties (St-Onge et al., 2008). Another advantage is the fact that D-limonene and MCT extracts can be directly used in foods, without removing the solvent (Raposo et al., 2012; Raposo et al., 2015).

Mild and eco-friendly extraction techniques in combination with the replacement of organic solvents with natural alternatives represent an important sector in food processing. The aim of this study is the optimization of ultrasound assisted extraction method for the recovery of total carotenoids and astaxanthin from *H. Pluvialis*, using organic solvent (acetone) and green and food grade solvents like Medium Chain Triglycerides (MCT) oil and D-limonene. Acetone extracts were used as control samples in order to evaluate the performance of the other solvent systems, since acetone is a high yielding solvent regarding the recovery of carotenoids from microalgae (Casella et al., 2019). Different extraction conditions (ultrasound power and solid to solvent ratio) were applied and total carotenoid and astaxanthin content were determined. All extracts were analysed using HPLC-DAD analysis in order to evaluate and quantify astaxanthin content.

2. Materials and Methods

All reagents and solvents used in the extractions were of analytical grade, while solvents used in analysis were of HPLC grade. Acetone, petroleum ether, methanol, t-butyl-methyl-ether, water, sodium sulphate anhydrous and phosphoric acid were purchased from Fisher scientific (UK). Cholesterol esterase, trans-beta-apo-8’-carotenal and astaxanthin were purchased from Sigma–Aldrich (USA). MCT oil and orange oil (D-limonene) were received by the company ASPIS S.A. (Argos, Greece). *H. pluvialis* cracked and dried biomass was donated by Algatechnologies Ltd.

2.1 Extraction experiments

Dry *H. pluvialis* samples were extracted using UAE. The solvents used were acetone, MCT and D-limonene. UAE was carried out in the Ultrasonic Microwave Reaction System XO-SM50 (Nanjing Xianou Instruments Manufacture co., Ltd., Nanjing City, China), as it is also described in our previous study (Stramarkou et al., 2017) The parameters that were tested included the quantity of the sample and the ultrasound intensity. Specifically, samples of *H.pluvialis* were placed in a 100 mL beaker with the selected solvent at biomass: solvent ratios: 1:100, 1:50, 1:20, 1:10, 1:5 g/ mL solvent. The extraction probe was inserted at 2 cm above the beaker bottom and the system operated at 25 kHz frequency, at 450 and 648 Watt and temperature 50 ^{o}C for a total duration of 20 min. The specific ultrasound powers were chosen to be studied with the intention of comparing the effect of a medium power, which is used as reference in our research (Stramarkou et al., 2017), with a higher one. A full design experiment was set up in order to estimate how the ratio solid to solvent, ultrasound intensity and solvent could affect the extraction’s yield and the final concentration of each extract.

The extracts were filtered with Whatman No.1 filter paper and the filtrates were centrifuged (NF400, Nuve, ANKARA / TURKEY) at 3500 rpm for 20 min. The supernatants were stored at 0 ^{o}C prior to further analysis. All the experiments were conducted in triplicate

2.2 Characterization of the extracts

2.2.1. Total carotenoid content

Quantitative determination of total carotenoids was accomplished with UV-Vis spectrometry. Final concentration of total carotenoids was identified according to Parsons' method (1984) (Timothy Parsons, 1984), using the absorbance at 480 (A480) and 510 (A510) nm, through the equation:

$$C = (7.6 * A_{480} - 1.49 * A_{510}) * D \quad (1)$$

Where C is the content of total carotenoids, A is the absorbance and D is equal to: extracted volume (mL)/ dry weight of sample used for extraction (mg). The carotenoid content was expressed as g of carotenoids per g of dry biomass (d.b.). The measurements were conducted in duplicates.

2.2.2. Hydrolysis of astaxanthin

Astaxanthin is encysted in *H.pluvialis* cells consisting of approximately 70% monoesters, 25% diesters and only 5% are met in free form (Kang and Sim, 2008). Therefore, enzymatic hydrolysis should be performed in order to quantify the astaxanthin content. Hydrolysis of astaxanthin esters in crude extract was carried out with cholesterol esterase according to protocol: 1.5 mL of extract, 1.5mL of cholesterol esterase (4 units/ mL) and 0.5 mL of internal standard. The mix is incubated at 37 ^{o}C for 2h. After enzymatic reaction 1 mL petroleum ether and 0,5g $NaSO_4$ were added in the mix and agitated for 30s in vortex. Then petroleum ether layer was separated and kept in a clean tube. This procedure is revised 3 times in order to recover all the amount of hydrolysed astaxanthin.

2.2.3. HPLC analysis of astaxanthin

HPLC analysis was performed with an HPLC Shimandzu HP 1100 Series (USA) equipped with a diode array detector and an automatic Agilent 1200 Series injector. Carotenoid compounds were analysed with an YMC C30 (Germany) analytical column (5 m, 250 x 4.6 mm I.D.). The solvents consisted of methanol, t-Butylmethylether and 1% Phosphoric acid aqueous solution and the flow rate was 1 mL/min. The linear gradient applied for t-Butylmethylether was: 0 min, 15%; 15 min, 30%; 23 min, 80%; 27 min, 80%; 27.1 min, 15%; 35 min, 15%, while concentration of 1% Phosphoric acid solution was constant and equal to 4%. Detection of astaxanthin was accomplished by using a diode array system at a wavelength of 474 nm. Astaxanthin was identified by comparison to external standard (Astaxanthin standard) and was quantified by use of internal standard (trans-β-Apo-8'-carotenal) and standard curve.

2.3 Statistical analysis

Two-way analysis of variance (ANOVA) and Tukey's multiple range test were applied to detect differences among the different extraction conditions in terms of carotenoid and astaxanthin content. Statistical analyses were performed with the STATISTICA software (Statistica Release 7, Statsoft Inc, Tulsa, OK, USA). The differences were considered to be significant at $p<0.05$.

3. Results and Discussion

The carotenoid recovery from *H. pluvialis* biomass is strongly dependent on the nature of solvent and the extraction conditions. The total carotenoid content per g dry biomass in different extraction ratios (biomass/solvent) (1:100, 1:50, 1:20, 1:10, 1:5) using acetone, MCT and D-limonene as solvents and ultrasonic power at 648 is presented at Fig. 1a and at 450 W is presented at Fig. 1b, whereas Table 1 presents the statistical analysis letters of values in Fig.1.

The total carotenoid content is strongly related to both sonication power and solid to solvent ratio for acetone and D-limonene extracts, while MCT extracts show no important statistical difference. At 648 W, the reduction of the solvent (increase of the ratio) caused decrease of extraction yield. The fact that extraction yield at 648W and 1g/50mL solid to solvent ratio (average value :0.026 g carotenoids/ g d.b.) is lower than the yield at 648W and 1g/20mL solid to solvent (average value: 0.034 g carotenoids/ g d.b.) is explained through reverse osmosis phenomenon as it is described from Papadaki et al. 2014 (Papadaki et al., 2014). Acetone was more efficient than D-limonene and MCT oil, especially in lower biomass to solvent ratios (1/100, 1/ 50 and 1/20), where acetone extracts showed significant differences with D-limonene and MCT oil extracts. Between the two green solvents, D-limonene was more effective since it recovered an average carotenoid content equal to

0.026 g/ g d.b, whereas the respective content recovered by MCT oil was 0.019 g/ g d.b, and was preferred over MCT oil because of its odour that makes it compatible with foods and beverages.
On the other hand, extractions that took place at 450 W had lower carotenoid content equal to 0.019 g/ g d.b. and the total carotenoid yield was not affected significantly by the solid to solvent ratio as statistical values in Table 2 show. This behaviour is based on slight sonication and partial cell disruption that eliminates solvent's penetration (Rostagno et al., 2003). Cell disruption is an important parameter for the effective extraction due to the fact that when cell collapse is extended the solvent is better mixed with the bioactive compounds (Desai and Parikh, 2015).

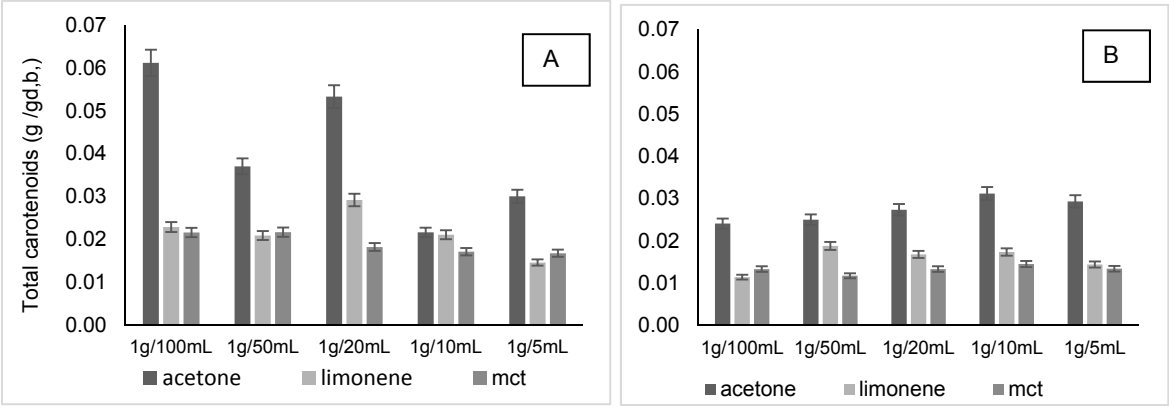

Figure 1. Total carotenoid content of extracts in terms of g total carotenoid/g dry biomass (d.b.) at A. 648 W and B. 450 W.

Table 1. Statistical analysis letters of total carotenoid content values presented in Fig 1. Values not sharing the same superscript (separately for each Figure) are significantly different (p<0.05).

Solid/ solvent ratio (g/ mL)	Solvent system	Statistical analysis letters at 648 W	Statistical analysis letters at 450 W
1/100	acetone	a	a
	limonene	d,b,h	e
	MCT	g,c,j	j,e,g
1/ 50	acetone	b,l	a
	limonene	e,f,c	f
	MCT	g,c,j	j,e
1/ 20	acetone	a,h	a,b
	limonene	e,b,c	f,h,g
	MCT	g,n,f	j,g,e
1/ 10	acetone	c,n	a
	limonene	e,f,m,j	f,h,g
	MCT	g,n,f	j,e,g,k
1/ 5	acetone	c,j,l	c,d
	limonene	f,n	e,h
	MCT	g,n,f	j,e,g,k

Regarding astaxanthin, Tables 2 and 3 present astaxanthin content of *H.pluvialis* extracted by UAE using acetone, MCT oil and D-limonene as solvents and different solid to solvent ratios at 648 and 450 W.
Astaxanthin is located along in cell membrane, so ultrasound power has to disrupt the cell's fragments to smaller fragments for the better isolation. This fact indicates the relation between ultrasound power and duration and as a result all parameters have to be taken into account in order to obtain the optimum extract.
Astaxanthin content showed the same trend as total carotenoid content. Specifically, astaxanthin content and astaxanthin percentage in total carotenoids were higher in smaller ratios with the extraction ratio 1:20 being more efficient in most cases for both powers with an average value of 19.65 mg astaxanthin/ g d.b. As far as sonication power was concerned, it was observed that at 648 W larger amounts of the valuable carotenoid were recovered compared to 450 W (1.78 folds higher), especially at 1g/100mL, 1g/50mL and 1g/20mL ratios. Acetone was again the best performing solvent in both ultrasound powers with an average astaxanthin content equal to 23.73 mg/ g d.b. and MCT oil recovered the lowest quantities of astaxanthin with an average content of 10.81 mg/ g d.b.

Finally, the results of this study showed that ultrasound exerted a mechanical effect, allowing great penetration of solvent into the cells, increasing the contact surface area between the solid and liquid phase. As a consequence, astaxanthin quickly diffused from the solid phase to the solvent (Rostagno et al., 2003).

Table 1. Astaxanthin content of extracts in terms *of mg/g dry biomass (d.b.) and percentage of astaxanthin in total carotenoids (%) at 648 W.*

Solvent system	Solid/ solvent ratio (g/ mL)	Astaxanthin content (mg/ g d.b.)	Percentage of astaxanthin in total carotenoids (%)
acetone	1/100	42.45 ^{a} ± 0.07	69.59 ^{a} ± 0.11
	1/50	26.43 ^{b} ± 0.06	71.44 ^{b} ± 0.17
	1/20	40.79 ^{c} ± 0.03	76.97 ^{c} ± 0.07
	1/10	16.77 ^{d} ± 0.03	79.87 ^{d} ± 0.13
	1/5	24.37 ^{e} ± 0.04	81.23 ^{e} ± 0.14
limonene	1/100	16.92 ^{d} ± 0.03	61.60 ^{f} ± 0.10
	1/50	17.62 ^{f} ± 0.04	66.55 ^{g} ± 0.16
	1/20	25.97 ^{g} ± 0.06	74.46 ^{h} ± 0.24
	1/10	19.25 ^{h} ± 0.03	77.56 ^{c} ± 0.13
	1/5	15.91 ^{i} ± 0.05	78.90 ^{i} ± 0.26
MCT oil	1/100	12.94 ^{J} ± 0.02	76.91 ^{c} ± 0.13
	1/50	13.98 ^{k} ± 0.03	83.9 ^{j} ± 0.21
	1/20	14.15 ^{l} ± 0.05	89.55 ^{k} ± 0.29
	1/10	13.96 ^{k} ± 0.03	91.65 ^{l} ± 0.22
	1/5	13.4 ^{m} ± 0.02	93.56 ^{m} ± 0.15

Table 2. Astaxanthin content of extracts in terms *of mg/g dry biomass (d.b.) and percentage of astaxanthin in total carotenoids (%) at 450 W.*

Solvent system	Solid/ solvent ratio (g/ mL)	Astaxanthin content (mg/ g d.b.)	Percentage of astaxanthin in total carotenoids (%)
acetone	1/100	14.28 ^{a} ± 0.02	62.08 ^{a,b} ± 0.10
	1/50	14.96 ^{b} ± 0.04	62.35 ^{a} ± 0.15
	1/20	17.45 ^{c} ± 0.01	64.63 ^{c} ± 0.21
	1/10	20.39 ^{d} ± 0.03	65.76 ^{d} ± 0.16
	1/5	19.39 ^{e} ± 0.03	66.87 ^{e} ± 0.11
limonene	1/100	6.00 ^{f} ± 0.01	44.41 ^{f} ± 0.07
	1/50	11.74 ^{g} ± 0.03	65.30 ^{d} ± 0.16
	1/20	10.96 ^{h} ± 0.04	65.83 ^{d} ± 0.21
	1/10	11.80 ^{g} ± 0.02	66.85 ^{e} ± 0.11
	1/5	10.00 ^{i} ± 0.03	67.86 ^{g} ± 0.22
MCT oil	1/100	5.77 ^{J} ± 0.01	54.50 ^{h} ± 0.09
	1/50	7.18 ^{k} ± 0.02	61.80 ^{b} ± 0.15
	1/20	8.56 ^{l} ± 0.03	64.49 ^{c} ± 0.05
	1/10	10.03 ^{i} ± 0.02	65.58 ^{d} ± 0.11
	1/5	8.14 ^{m} ± 0.01	66.69 ^{e} ± 0.11

Number of replicates: 2, +/- shows the standard deviation between replicates. Values not sharing the same superscript (separately for each Table) are significantly different.

4. Conclusions

In this study, the major parameters, i.e. solvent system, ultrasound intensity and solid to solvent ratio, that affect extraction's performance were evaluated. The aim of this study was to design a process that produces food grade extracts with high concentration of astaxanthin. Therefore, D-limonene and MCT oil were evaluated towards their ability to recover carotenoids from *H. pluvialis* biomass. D-limonene and MCT oil showed lowest yielding compared to acetone extracts, thus sufficient to be used as food ingredients. Moreover, limonene and MCT oil showed similar performance, with D-limonene to perform better at 1g/20mL solid to solvent ratio and higher ultrasound power. D-limonene is proposed as the preferred solvent system towards acetone and MCT oil for both sufficient carotenoids recovery and mostly for its ability to extract carotenoids without any undesirable odour that permits the direct use of the developed extracts in final food products. Overall, the highest yield is reached at 648 Watt for all solvents systems and ratios, indicating the importance of ultrasound power. In conclusion, the presented results indicate the potential of industrial application of this study, thus the design of a downstream processing that critical points of the extraction has to be optimized towards maximum extraction yield and lower cost is needed.

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