PRESSURIZED LIQUIDS AS AN ALTERNATIVE PROCESS TO ANTIOXIDANT CAROTENOIDS EXTRACTION FROM HAEMATOCOCCUS *PLUVIALIS* MICROALGAE

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ABSTRACT

In this work, extraction of antioxidant carotenoids from *Haematococcus pluvialis* microalga, has been studied combining pressurized liquid extraction (PLE), using hexane and ethanol as extracting solvents, and analytical techniques such as TLC and HPLC with DAD. The effect of the extraction temperature (50, 100, 150 and 200ºC) and the polarity of the solvent has been studied in terms of *in vitro* antioxidant activity and chemical composition considering two different morphological cells (green vegetative cells and red cysts). Results demonstrate that the extraction temperature had a positive influence in the extraction yield while its effect in the antioxidant activity was negative, lowering the activity of the extracts with an increase of the extraction temperature. The best yields were obtained with ethanol at the higher extraction temperature while the best antioxidant activity was also achieved using ethanol but at lower temperatures. Chemical composition was determined by TLC and HPLC with DAD. Several compounds were identified in the samples and concentration of astaxanthin was obtained. Results pointed out that the extracts contained different carotenoids in both, the green and the red phase, and that depending on its contribution a stronger antioxidant activity would be expected.

1 Introduction

Haematococcus pluvialis is a green motile unicellular algae. When subjected to favourable conditions, it grows as motile biflagellated green cells but under stress conditions, such as, nutrient deficiency, high light intensity or salt stress, the cells lose their motility, increase in size and form red cysts or aplanospores with thick cell walls. In this form, the alga can survive for long periods, even under harsh conditions (Hagen, Siegmund & Braune, 2002).

In green cells, chlorophylls along with the carotenoids lutein and β-carotene dominate the total pigment content; thus cells appear green (Lu, Vonshak, Gabbay, Hirschberg & Boussiba, 1995**)**. As encystment is induced, photosynthetic activity decreases and the amount of astaxanthin increases dramatically from a few picograms per cell to a few hundreds at the end of the process. At this stage, astaxanthin esters (mono and di) constitute up to 98% of the total carotenoids profile and reach up to 4% of total cellular dry weight (Boussiba, Bing, Zarka, Yuan, & Chen, 1999).

Haematococcus pluvialis has received considerable scientific and biotechnological attention in recent years as a natural source of the carotenoid astaxanthin, since it is used as food coloring agent in aquaculture and poultry (Lorenz & Cysewski, 2000.) and it possesses a potent antioxidant capacity (Miki, 1991; Kobayashi & Sakamoto, 1999; Naguib, 2000). Moreover, many biological activities including anti-inflammatory, anti-cancer, anti-aging and age-related diseases, promotion of immune response are believed to be related to its antioxidant activity (Guerin, Huntley & Olaizola, 2003).

Different techniques have been widely applied to extract carotenoids from *H. pluvialis,* mainly organic solvent extraction after cell wall disruption (Mendes-Pinto, Raposo, Bowen, Young & Morais, 2001; Sarada, Vidhyavathi, Usha, & Ravishankar, 2006). However, these traditional methods are difficult to apply on a large scale, because they

require large amount of solvents and multiple extraction steps (Lim, Lee, Lee, Haam, Kim, 2002). Opposite to that, supercritical fluid extraction and pressurized liquid extraction have been proposed as useful green extraction techniques.

Supercritical carbon dioxide (SC-CO_2), in particular, has been widely used for industrial applications due to its many processing advantages, which play key roles in enabling the solvent to readily penetrate the solid biomass matrix as well as in extraction solutes (Youn, Roh, Weber, Wilkinson & Chung, 2007). Furthermore, low critical temperature of carbon dioxide means that SC-CO_2 system could be operated at moderate temperature, preventing the degradation of the substances due heat induction (Machmudah, Shotipruk, Goto, Sasaki & Hirose, 2006).

Nevertheless, although astaxanthin molecule is considered containing no strong polar moieties, its large molecule inhibited its solubility in pure SC-CO_2 due to its low volatility (Dandge, Heller & Wilson, 1985), being necessary the use of co-solvents such as ethanol or vegetable oils to increase the yield extraction (Machmudah et al, 2006; Krichnavaruk, Shotipruk, Goto & Pavasant, 2008).

PLE is a more recent extraction technique proposed to obtain bioactive compounds. It uses less solvent, in a shorter period of time, is automated, and involves retaining the sample in an oxygen and light-free environment than traditional organic solvent extraction. PLE is based on the use of conventional solvents at controlled temperatures and pressures and has been well established for extraction of valuable compounds from natural sources (Benthin, Danz & Hamburger, 1999).

Moreover, in previous studies PLE turned out a faster and easier to use extraction technique with higher yield extraction than SC-CO_2. Nevertheless, the extract obtained from pure SC-CO_2 contains fewer polar impurities than PLE (Herrero, Jaime, Martín-Álvarez, Cifuentes, Ibáñez, 2006; Jaime et al, 2007). Therefore, although PLE has been

proposed as an efficient extraction technique of carotenoids from *H. pluvialis* (Denery, Dragull, Tang & Li, 2004), no studies have focused on simultaneous optimization of PLE procedure and functional-chemical characterization of the extracts.

Therefore, the aim of this study was to evaluate the carotenoid composition and antioxidant capacity of different pressurized fluid extracts from green and red phase cells of *Haematococcus pluvialis*.

2 Materials and methods

2.1 Samples and chemicals

Haematoccoccus pluvialis (BNA 10/024, National Bank of Algae, Canary Islands, Spain), were grown in modified Bold´s Basal Medium (Nichols & Bold, 1965) enriched with $NaNO_3$ (0.75 g/l). Cells (green phase) were cultured photoautotrophycally in 20 L Carboys bubbled with air, at 25°C, in light:dark cycles (16:8) with white fluorescent lamps providing 80 µmol m^{-2} s^{-1}. To induce astaxanthin biosynthesis (red phase) exponentially grown cultures were transferred to nitrogen starvation medium and continuously illuminated with 200 µmol m^{-2} s^{-1} during 6 days. Cells were collected by centrifugation, freeze dried and stored at inert atmosphere before extraction.

Ethanol was obtained from Scharlau Chemie S.A. (Barcelona, Spain). Hexane was purchased to Panreac Quimica S.A. (Barcelona, Spain). Water was purified using a Milli-Q system (Millipore Corporation, Billerica, MA, USA). The sea sand used was supplied by Panreac Quimica (Barcelona, Spain). β-carotene standard, 2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and potassium persulfate were obtained from Sigma (Madrid, Spain), whereas Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid) standard were purchased to Fluka Chemie AG (Buchs, Switzerland). Authentic astaxanthin, lutein and neoxanthin standard

isolated from phytoplankton were purchased to DHI Water & Environment (Hørsholm, Denmark).

2.2 Pressurized fluid extraction

Dry cells were pretreated by freezing and mashing the microalgae with liquid nitrogen in a ceramic mortar. The process was repeated three times in order to induce cell-wall lysis.

Extractions of *Haematococcus pluvialis* were performed using an Accelerated Solvent Extractor (ASE 200, Dionex Corporation, Sunnyvale, CA, USA) equipped with a solvent controller unit. Table 1 shows the experimental conditions used; two different solvents (i.e. hexane and ethanol) were employed to achieve extracts with different composition. Extractions were carried out in duplicate. Moreover, extractions were performed at four different extraction temperatures (50, 100, 150 and 200ºC) and 20 minutes as extraction time. Previously, an extraction cell heat-up was used for a given time, that changed according to extraction temperature (the heat-up time is automatically fixed by the equipment, i.e., 5 min when the extraction temperature was 50 and 100ºC, 7 min at 150 ºC and 9 min at 200 ºC). All extractions were performed in 11 mL extraction cells, containing 2.0 g of sample.

The extraction procedure was as follows: (i) sample is loaded in the cell; (ii) cell is filled with solvent up to a pressure of 10.34 MPa, (iii) initial heat-up time is applied; (iv) a static extraction with all system valves closed is performed; (v) the cell is rinsed (with 60% cell volume using extraction solvent); (vi) solvent is purged from the cell with N_2 gas and (vii) depressurization takes place. Between extractions, a rinse of the complete system was made in order to overcome any extract carry over. To minimize the loss of volatiles and to avoid sample degradation, the extracts were quickly cooled

down to freezing temperatures by placing the vials in a water-ice bath. This was done for all the extracts at the different temperatures tested. Once cold, the extracts were dried using a Rotavapor R-200 (from Büchi Labortechnik AG, Flawil, Switzerland).

2.3 TLC analysis

300 μg of PLE extracts were spotted on a 20 × 20 cm silica gel 60 F_{254} TLC plate (Merck, Germany). Plates were put in a closed chamber and eluted by 100 mL of petroleum ether: acetone mixture in the proportion 75:25 (v/v) as mobile phase. Lutein, β-carotene and astaxanthin were used as standard.

2.4 HPLC analysis.

Analysis were performed with an HPLC Agilent HP 1100 Series (Agilent, Palo Alto, CA, USA) equipped with a diode array detector and an automatic Agilent 1200 Series injector.

Carotenoid compounds were analysed with a YMC C30 (YMC, Schermbeck, Germany) C30 analytical column (5 μm, 250 x 4.6 mm I.D.). The mobile phase was a mixture of acetone and water as an isocratic mixture of 84% acetone and 16% water for the first 21 min, followed by a 4 min linear gradient to 97% acetone and 3% water for the reminder of the 50 min run. The flow rate was kept at 1 mL/min. Detection was accomplished by using a diode array system at a wavelength of 480 nm (scan from 190 to 700 nm) (Denery et al, 2004).

The major carotenoids were identified by comparison of retention times and spectra against authentic standard (astaxanthin, lutein, neoxanthin, β-carotene). Astaxanthin esters were identified considering their spectra and their different retention time and also through comparison with data appearing in the literature (Denery et al, 2004).

Concentrations of astaxanthin and astaxanthin esters were obtained from a standard calibration curve prepared from authentic astaxanthin and considering for all of them the same extinction coefficient.

2.5 Antioxidant Activity Determination. TEAC Assay.

The TEAC (Trolox Equivalent Antioxidant Capacity) assay described by Re, Pellegrini, Proteggente, Pannala, Yang and Rice-Evans (1999) was used to measure the antioxidant activity of the PLE extracts from *Haematococcus pluvialis*. Briefly, $ABTS^{\bullet+}$ radical cation was generated by reacting 7 mmol/L ABTS with 2.45 mmol/L potassium persulfate after incubation at room temperature for 16 h in the dark. The $ABTS^{\bullet+}$ radical solution was diluted with ethanol to an absorbance of 0.70 ± 0.20 at 734 nm. 10 μL of *H. pluvialis* at five different concentrations extract was added to 0.990 μL of diluted $ABTS^{\bullet+}$ radical solution. The reaction was measured until the absorbance reached a plateau. Trolox was used as reference standard, and results were expressed as TEAC values (mmol Trolox/ g extract). All analyses were done at least in triplicate.

2.6 Astaxanthin hydrolysis

Hydrolysis of astaxanthin esters in crude extract was carried out with cholesterol esterase according to Jacobs, LeBoeuf, McCommas & Tauber (1982) procedure. Total hydrolysis of astaxanthin esters was verified by TLC analysis.

3 Results and discussion

3.1 PLE from *H. pluvialis*.

Several authors reported the need for *H. pluvialis* pre-treatment (in the red phase) previous to extraction as the way to optimise carotenoids extraction. For *H. pluvialis* in

the red phase, this pretreatment implies cell wall cysts destruction. Several disruption processes, comprising treatments with extracellular enzymes, DMSO, HCl, NaOH, autoclave, sonication and mechanical disruption (Mendes-Pinto et al., 2001; Sarada et al., 2006; Yuan and Chen, 1998; Bubrick, 1991), have been described, followed by solvent extraction procedures. Among all of them, mechanical disruption (homogenization) at cryogenic temperature has been proposed as one of the best treatment to minimize the loss of carotenoids (Bubrick, 1991). Even though the only reference of *H. pluvialis* extraction with PLE (Denery et al., 2004) did not use any processing of cyst cells previous to extraction, based on the above mentioned references and on initial observations done in our laboratory (data not show), mechanical disruption at freezing temperatures was selected previous to PLE extraction since it also provided higher extraction yields of biological active compounds from the disrupted cells.

To know the influence of temperature and solvent polarity in the extraction of antioxidant compounds from *H. pluvialis* using PLE, two different extracting solvents (hexane and ethanol) and four different conditions were tested (50, 100, 150 and 200 ºC), also considering both, green and red phases. Table 1 shows the results in terms of extraction yields. As can be observed, the extraction yield increased with the temperature. This effect could be due to an increase of the mass transfer from the sample to the pressurized solvent with the temperature. Moreover, the extraction yield enhanced as polarity of the solvent increased (ethanol).

Opposite to the expected behaviour the extraction yields obtained from the red phase of the microalgae were generally higher than those obtained form the green one. Nevertheless, as all the cells were pretreated to induce cell lysis, it seems reasonable to consider that both types of cells have the same broken structure when pressurized

solvent extraction is applied. Thus, higher extraction yields of red cells than green cells can be explained by the higher solubility of cellular material in the solvents employed, at the conditions used in the extraction.

3.2 Analysis of PLE extracts

Analytical TLC of green cells (Figure 1) showed chlorophylls as the main components, especially in ethanol extracts. Moreover, it is well known extraction at high temperatures with organic solvents causes the appearance of pheophytins, pyropheophytins, chlorophyllides, etc., as degradation products of chlorophylls (Jaime et al., 2005). Grey-greenish and maroon spots in the middle of the analytical TLC correspond to pheophytine-like compounds, whereas blue-greenish spots of ethanol extracts at R_f 0.2-0.25 might be due to the presence of chlorophilide-like compounds (Mínguez-Mosquera, 1997). As can be seen in Figure 1, hexane caused a higher formation of chlorophyll derivatives than ethanol. Similar results were achieved in the pigment composition of PLE extracts from *Spirulina platensis* (Jaime et al., 2005). Chlorophyll a and b, lutein and β-carotene have been reported as the main pigments in green cells, together with little amounts of violaxanthin and neoxanthin (Lu et al., 1995; Orosa, Franqueira, Cid & Abalde, 2005). The presence of β-carotene on the top of the plate and lutein (R_f = 0.17) in green cells extracts were confirmed with standard. Chlorophyll a (R_f = 0.1-0.16) and b (R_f = 0.03-0.08) were tentatively identify as green-bluish and green-yellowish spots, whereas violaxanthin and neoxanthin might fit with yellow spots at Rf = 0.09 and Rf = 0.03, respectively, according to Cerón et al., (2006) and Minguez-Mosquera (1997). This preliminary identification was further confirmed by HPLC with DAD detection, as discussed later.

Encystment of *Haematococcus pluvialis* cells by nitrogen starvation and high light intensity exposure cause important changes in their pigment composition. Under these stress environmental growing conditions, reactive oxygen species, especially singlet oxygen, are produced and cell induces ketocarotenoid astaxanthin accumulation to protect itself against oxidative damage. The accumulation of astaxanthin is associated with the decline in photosynthetic activity in red cysts, so initial green cells turn red (Lu, Vonshak, Zarka & Boussiba, 1998).

In *H. pluvialis*, astaxanthin is synthesized from β-carotene by the addition of two keto groups to carbons C4 and C4' by β-carotene ketolase (Huang, Chen, Sandmann, 2006) followed by addition of two hydroxyl groups to C3 and C3' by β-carotene hydroxylase (Linden, 1999). Therefore, the pathway for astaxanthin biosynthesis in *H. pluvialis* can be summarized as follows: β-carotene $\rightarrow$ echinenone $\rightarrow$ canthaxanthin $\rightarrow$ adonirubin $\rightarrow$ astaxanthin (Boussiba, 2000). The identification of small amounts of echinenone, canthaxanthin and adonirubin in encystment process of *H. pluvialis* is in agreement with the proposed pathway (Renstrøm, Borch, Skulberg & Liaaen-Jensen, 1981).

Analytical TLC of cyst cells extracts (Figure 2) showed important differences in pigment composition compared to vegetative cells. As expected, small amounts of chlorophylls can be observed in red cells, while some degradation products can be seen in PLE extracts obtained at high temperatures. Astaxanthin monoesters (Rf = 0.37-0.50) and diesters (Rf = 0.79-0.91) seem to be the main components of these extracts. β-carotene (Rf = 1.0), lutein (Rf =0.17) and free astaxanthin (Rf = 0.20) presence was confirmed with standard. According to Cerón et al., (2006), bands at Rf = 0.64 and Rf = 0.30 might suggest the existence of canthaxanthin and echinenone in the extracts.

A general behaviour with the extraction temperature can be observed for both, green and red phases and considering the two solvents. By observing Figures 1 and 2, it can

be seen that pigment extraction slightly decreases as temperature increase from 50 to 150°C, being more pronounced at 200°C.To obtain further information of the extracts and to be able to compare among them, extracts obtained at 100ºC using ethanol and hexane with red and green cells were analysed by HPLC, as described above; results are shown in Tables 1 and 2 for, respectively, green and red cells and in Figures 3 and 4, where a comparison between green and red cells extracted using PLE with ethanol at 100°C is given.

Regarding to green phase cells (Table 2, Figure 3), lutein was shown as their main pigment. Other identified carotenoids were neoxanthin and β-carotene. These results are in agreement with Orosa et al., (2005) and Lu et al., (1995) reports and with our previous analysis by TLC. The existence of violaxanthin in green cells has been reported for some authors (Linden, 1999; Renstrøm et al., 1981), nevertheless its presence in these samples could not be confirmed by HPLC comparing with an authentic standard.

Other compounds found in large amount in the green cells of *H. pluvialis* were chlorophylls; as can be seen in Table 2, several chromatographic peaks were identified as chlorophyll or chlorophyllic-like compounds.

In general, ethanol extracts showed a higher lutein and a lower β-carotene content while chlorophyllic-like compounds and other minor carotenoids can be found in both, hexane and ethanol extracts, in similar amounts.

During encystment, an increase in lipid content which correlates with astaxanthin accumulation was observed (Boussiba, 2000). *Haematococcus* primarily contains monoesters of astaxanthin linked to 16:0, 18:1 and 18:2 fatty acids. Fatty acids are esterified onto the 3, 3’ hydroxyl group(s) of astaxanthin after biosynthesis of the

carotenoids, thereby increasing its solubility and stability in the cytoplasm (Lorenz & Cysewski, 2000).

As expected, astaxanthin-derived compounds constituted up to 90% of total pigments in encysted cells. Astaxanthin esters (mono and di) were the main components of red cells extracts (> 80% of normalized area) (Table 3, Figure 4) in both ethanol and hexane extracts whereas free astaxanthin constituted up to 3% of normalized area. Meanwhile, lutein was shown as the main free carotenoid in these extracts. Hexane extract showed higher astaxanthin content (35.1 mg/g dry weight) and a lower percentage of lutein than ethanol extract (20.7 mg/g dry weight). The presence of canthaxanthin and echinenone could not be confirmed by HPLC using pure standard.

3.3 Antioxidant activity of the PLE extracts.

TEAC values (mmol trolox/g extract) of PLE extracts from *Haematococcus pluvialis* are shown in Table 4. In general, ethanol extracts presented better antioxidant activity than hexane extracts at all temperatures tested. The behaviour of the antioxidant activity as a function of the extraction temperature is also clear since it can be inferred from the data that the higher extraction temperature, the lower the TEAC value of the extracts and therefore, the lower the antioxidant activity. This effect was more pronounced at 200ºC. This decrease on the antioxidant capacity seems to be related with the lower carotenoid content of the extracts, as shown in TLCs (Figures 1 and 2).

Surprisingly, green phase extracts showed more antioxidant activity than the red ones. Many studies have reported the high antioxidant capacity of astaxanthin, compared to other carotenoids, due to the presence of keto groups at the 4 and 4’ position and hydroxyl groups at the 3 and 3’ position in the β-ionone ring (Naguib, 2000). Moreover, astaxanthin seems to be a safer antioxidant even at high oxygen

concentrations, opposite to β-carotene behaviour that acts as pro-oxidant under conditions of high pO_2 (Hix, Lockwood & Bertram, 2004).

However, even if our results seemed to be contradictory to the previously reported, it has to be considered that the most part of the studies performed with astaxanthin have been done employing pure synthetic astaxanthin, and only few of them used natural astaxanthin, like the one obtained from *H. pluvialis*. Synthetic astaxanthin is available as the free form of astaxanthin whereas *H. pluvialis* astaxanthin is accumulated in the form of di- or monoesters of astaxanthin. Of course, free or esterified carotenoids should have a different antioxidant activity. For example, Miki (1991) found a strong activity of free natural astaxanthin as an inhibitor of lipid peroxidation, better than ohers carotenoids, whereas astaxanthin diester showed a very low activity. Moreover, Kobayashi and Sakamoto (1999) reported a higher $^1O_2$ quenching activity of free astaxanthin, followed by astaxanthin monoester and astaxanthin diester in an ethanol medium when IC_{50} was expressed as μg of carotenoids/mL. On the other hand, Cerón et al., (2006) concluded that astaxanthin diesteres exhibits the highest antioxidant capacity against $DPPH^{\bullet}$ free radical, followed by astaxanthin monoesters and free astaxanthin. Moreover, they reported that fatty acids could contribute, in addition to astaxanthin, to the antioxidant activity exhibited by the extracts. However, $DPPH^{\bullet}$ show a maximum of absorption at 482 nm and 517 nm, respectively, and therefore a clear interference can be expected when measuring the antioxidant activities using this methodology; this could explain the results obtained by Cerón et al., (2006) which are clearly in disagreement with the previously reported and with those shown in the present study.

In order to prove if fatty acids change the antioxidant activity of astaxanthin, a basic hydrolysis of the extract obtained from red cysts at 150ºC with ethanol as extraction solvent was carried out. The TEAC value of the hydrolyzed extract increased from

0.168 up to 0.434 mmol trolox/g extract. Therefore, with this assay we demonstrate that di and monoesters of astaxanthin had a lower antioxidant activity than free astaxanthin. Moreover, synthetic astaxanthin showed, as expected, the highest TEAC value, 2.433 ± 0.015 mmol trolox/g.

Although some authors have suggested that hydroxyl groups of zeaxanthin does not participate in antioxidant mechanisms (Woodall, Lee, Weesie, Jackson & Britton, 1997), the antioxidant activity of the extract that undergone saponification from *Haematococcus pluvialis* was higher than the original extract, suggesting a high contribution of hydroxyl groups to delocalization of electron in the astaxanthin molecule. This different behaviour could be due to the contribution of ketone groups of astaxanthin, and thus, astaxanthin exists in an equilibrium, with the enol form of the ketone, thus the resulting dihydroxy conjugated polyene system possesses a hydrogen atom capable of breaking the free radical reaction in a similar way to that of α-tocopherol (Naguib, 2000).

Therefore, antioxidant activity of *H. pluvialis* extracts seemed to be related to their free carotenoids content, mainly lutein, that exists in more concentration in green cells showing higher TEAC values than red cells.

Nevertheless, a further step is needed when considering the use of natural extracts as a source of functional ingredients. Related to the *in vivo* antioxidant activity of free and/or esterified carotenoids, Khachik, Beecher, Goli, Lusby & Smith (1992) demonstrated that only free xanthophylls were detected in plasma and peripheral tissues, thus suggesting a hydrolytic step before absorption. Moreover, Wingerath, Stahl & Sies (1995) concluded that di-esterification seems to be a common phenomenon in xanthophylls during gastrointestinal digestion, as they are not detected in chylomicrons or serum.

With this information it can be concluded that extracts from *H. pluvialis* red cells would have a higher antioxidant activity *in-vivo* than that of the green ones because of their high astaxanthin content that, even in form of di- and monoesters, can be hydrolysed to free astaxanthin in the human body. Furthemore, *H. pluvialis* is a good source of astaxanthin as synthetic astaxanthin stability and activity are lower than those of natural product (Lim et al., 2002).

4 Concluding remarks

In the present work we have demonstrated the usefulness of PLE as a clean and fast extraction method to obtain antioxidant extracts from *Haematococcus pluvialis* in both, green and red growing phases. Extracts obtained using ethanol showed the highest yield and antioxidant activity being this activity associated to the presence of free carotenoids in the sample. Results have also demonstrated that the antioxidant activity of red cells extracts could be greatly improved after hydrolysis of the mono- and diesters of astaxanthin, reaction that seems to occur after ingestion of these products. Thus, the results presented in this study showed the interest of the combined use of PLE and analytical techniques to optimise extraction processes focused to the development of functional ingredients from natural sources.

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5 REFERENCES

Benthin, B., Danz, H., & Hamburger, M. (1999). Pressurized liquid extraction of medicinal plants. *Journal of Chromatography A, 837,* 211-219.

Boussiba, S. (2000). Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. *Physiologia Plantarum., 108,* 111-117.

Boussiba, S., Bing, W., Zarka, A., Yuan, J.P., & Chen, F. (1999). Changes in pigment profiles of *Haematococcus pluvialis* during exposure to environmental stresses. *Biotechnology Letters, 21*, 601-604.

Bubrick, P. (1991). Production of astaxanthin from *Haematococcus. Bioresource Technology, 28*, 237-239.

Cerón, M.C., García-Malea, M.C., Rivas, J., Acien, F.G., Fernández, J.M., Del Río, E., Guerrero, M.G., & Molina, E. (2006) Antioxidant activity of *Haematococcus pluviales* cells grown in continuous culture as a function of their carotenoid and fatty acid content. *Applied Microbiology and Biotechnology, 74(5),* 1112-1119.

Dange, D.K., Heller, J.P., Wilson, K.V. (1985). Structure solubility correlations: organic compounds and dense carbon dioxide binary systems. *Industrial & Engineering Chemical Product Research Development, 24*, 162-166.

Denery, J.R., Dragull, K., Tang, C.S., & Li, Q.X. (2004). Pressurized fluid extraction of carotenoids from *Haematococcus pluvialis* and *Dunaliella salina* and kavalactones from *Piper methysticum*. *Analytica Chimica Acta, 501*, 175-181.

Guerin, M., Huntley, M.E., & Olaizola, M. (2003). *Haematococcus* astaxanthin: applications for human health and nutrition. *Trends in Biotechnoogy., 21(5)*, 210-216.

Hagen, C., Siegmund, S., & Braune, W. (2002). Ultrastructural and chemical changes in the cell wall of *Haematococcus pluvialis* (Volvocales, Chlorophyta) during aplanospore formation. *European Journal of Phycology, 37*, 217-226.

Herrero, M., Jaime, L., Martín-Álvarez, P.J., Cifuentes, A., & Ibáñez, E. (2006). Optimization of the Extraction of Antioxidants from Dunaliella salina Microalga by Pressurized Liquids. *Journal of Agricultural & Food Chemistry, 54*, 5597-5603

Hix, L.M., Lockwood, S.F., & Bertram, J.S. (2004). Bioactive carotenoids: potent antioxidants and regulators of gene expression. *Redox Report, 9(4),* 181-191.

Huang, J.C., Chen, F., & Sandmann, G. (2006). Stress-related differential expression of multiple β-carotene ketolase genes in the unicellular green alga *Haematococcus pluvialis*. *Journal of Biotechnology, 122*, 176-185.

Jacobs, P.B., LeBoeuf, R.D., McCommas, S.A., & Tauber, J.D. (1982). The cleavage of carotenoid esters by cholesterol esterase. *Comparative Biochemistry and Physiology B, 72*, 157-160.

Jaime, L., Mendiola, J., Herrero, M., Soler-Rivas C., Santoyo, S., Señorans, F.J., Cifuentes, A., & Ibañez, E. (2005). Separation and characterization of antioxidant compounds from *Spirulina platensis* microalga combining pressurized liquid extraction, TLC and HPLC-DAD. *Journal of Separation. Science, 28(16),* 2111-2119.

Jaime, L., Mendiola, J.A., Ibáñez, E., Martin-Álvarez, P.J., Cifuentes, A., Reglero, G., & Señoráns, F.J. (2007). β-carotene isomers composition of sub- and supercritical carbon dioxide extracts. Antioxidant activity measurement. *Journal of Agricultural & Food Chemistry, 55* (26), 10585-10590.

Khachik, F., Beecher, G.R., Goli, M.B., Lusby, W.R., & Smith, J.C. (1992). Separation and identification of carotenoids and their oxidation products in extracts of human plasma. *Analytical Chemistry, 64*, 2111-2122.

Kobayashi, M., & Sakamoto, Y. (1999). Singlet oxygen quenching ability of astaxanthin esters from the green alga *Haematococcus pluvialis*. *Biotechnology. Letters, 21*, 265-269.

Krichnavaruk, S., Shotipruk, A., Goto, M., & Pavasant, P. (2008). Supercritical carbon dioxide extraction of astaxanthin from *Haematococcus pluvialis* with vegetable oils as co-solvent. *Bioresource Technology, 99*, 5556-5560.

Lim, G.B., Lee, S.Y., Lee, E.K., Haam, S.J., & Kim, W.S. (2002). Separation of astaxanthin from red yeast *Phaffia rhodozyma* by supercritical carbon dioxide extraction. *Biochemical Engineering. Journal, 11*, 181-187.

Linden, H. (1999). Carotenoid hydroxylase from *Haematococcus pluvialis*: cDNA sequence, regulation and functional complementation. *Biochimica et Biophysica Acta, 1446*, 203-212.

Lorenz, T., & Cysewski, G.R. (2000). Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Trends in Biotechnology, 18(4)*, 160-167.

Lu, F., Vonshak, A., Gabbay, R., Hirschberg, J., & Boussiba, S. (1995). The biosynthetic pathway of astaxanthin in a green alga *Haematococcus pluvialis* as indicated by inhibition with diphenylamine. *Plant cell physiology, 36*, 1519-1524.

Lu, F., Vonshak, A., Zarka, A., & Boussiba, S. (1998). Does astaxanthin protect *Haematococcus pluvialis* against Light damage? *Zeitschrift fur naturforschung C, 53c*: 93-100.

Machmudah, S., Shotipruk, A., Goto, M., Sasaki, M., & Hirose, T. (2006). Extraction of astaxanthin from *Haematococcus pluvialis* using supercritical CO2 and ethanol as entrainer. *Industrial Engineering Chemical Research, 45*, 3652-3657.

Mendes-Pinto, M.M., Raposo, M.F.J., Bowen, J., Young, A.J., & Morais, R. (2001). Evaluation of different cell disruption processes on encysted cells of *Haematococcus pluvialis*: effects on astaxanthin recovery and implications for bio-availability. *Journal of Applied Phycology, 13*, 19-24.

Miki, W. (1991). Biological functions and activities of animal carotenoids. *Pure and Applied Chemistry, 63(1),* 141-146.

Mínguez-Mosquera, M.I. (1997) In: Clorofilas y carotenoides en tecnología de alimentos. Secretariado de publicaciones. Universidad de Sevilla, pp 111-154.

Naguib, Y.M.A. (2000). Antioxidant activities of astaxanthin and related carotenoids. *Journal of Agricultural & Food Chemistry, 48*, 1150-1154.

Nichols, H.W., & Bold, H.C. (1965) *Trichsarcina polymorpha* gen. et sp. Nov, *Journal of Phycology, 1(1),* 34-38.

Orosa, M., Franqueira, D., Cid, A., & Abalde, J. (2005). Analysis and enhancement of astaxanthin accumulation in *Haematococcus pluvialis*. *Bioresource Technology, 96,* 373-378.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical. Biology & Medicine., 26,* 1231-1237.

Renstrøm, B., Borch, G., Skulberg, O.M., & Liaaen-Jensen, S. (1981). Optical purity of (3S, 3S')-astaxanthin form *Haematococcus pluvialis*. *Phytochemistry., 20,* 2561.

Sarada, R., Vidhyavathi, R., Usha, D., & Ravishankar, G.A. (2006). An efficient method for extraction of astaxanthin from green alga *Haematococcus pluvialis*. *Journal of Agricultural & Food Chemistry, 54,* 7585-7588.

Wingerath, T., Stahl, W., & Sies, H. 1995. β-cryptoxanthin selectively increases in human chylomicrons upon ingestion of tangerine concentrate rich in β-cryptoxanthin esters. *Archives of Biochemistry and Biophysics, 324,* 385-390.

Woodall, A.A., Lee, S.W.M., Weesie, R.J., Jackson, M.J., & Britton, G. (1997). Oxidation of carotenoids by free radicals: relationship between structure and reactivity. *Biochimica et Biophysica Acta, 1336,* 33-42.

Youn, H.-S., Roh, M.-K., Weber, A., Wilkinson, G.T., & Chun, B.-S. (2007). Solubility of astaxanthin in supercritical carbon dioxide. *Korean Journal of Chemical Engineering*, *24* (5), 831-834.

Yuan, J.-P., & Chen, F. (1998). Chromatographic separation and purification of trans-astaxanthin from the extracts of *Haematococcus pluvialis*. *Journal of Agricultural & Food Chemistry*, *46*, 3371-3375.