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Purification of Astaxanthin from Microalgae by using Commercial Activated Carbon

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Microalgae are among the most interesting eukaryotic photosynthethic microorganisms able to use solar energy, nutrients and carbon dioxide to convert them into proteins, carbohydrates, lipids and other valuable organic compounds including carotenoids. Astaxanthin is one of the most interesting antioxidant molecules which has attracted crescent interest due to its positive effects on health and the numerous applications in different sectors, from nutraceutical to cosmetic and aquaculture. Even though the astaxanthin properties are well-known, its price still remains high if associated to the algal form, exceeding ~6000 Eur/Kg. This can be explained by considering the process expenses related to the extraction and purification steps of microalga intracellular metabolites. In fact, the downstream stage of this biotechnological process often accounts for more than 60–70% of total production costs. Optimized extraction and purification operations might contribute to microalgae market with the advantage to commercialize a natural existing astaxanthin form.
The aim of this paper is the evaluation of the use of commercial activated carbon Darco™ G-60 for the purification of astaxanthin from an extraction broth. Astaxanthin was firstly extracted from *Haematococcus pluvialis* red phase supplied by Micoperi Blue Growth, an Italian Company that is working for a long time and it is specialized in the microalgae growth. Extraction was performed by Accelerated Solvent Extractor (ASE®200 DIONEX) at 100 bar and 67°C by using ethanol as green solvent with the main advantage to separate all the unipolar fractions as well as insoluble fractions from astaxanthin extracts (fibers, carbohydrate, ashes).
In the second step, astaxanthin was purified with a column filled with activated carbon. Experimental tests by changing the mass of activated carbon were carried out (50mg, 100mg and 200mg)and with a flow rate in the range 0.9-1.0ml/min. All the experimental tests were carried out at room temperature (20°C). Results showed that by using activated carbon, it is possible to obtain an adsorption capacity of DARCO G60 in the range 21,9-23,9 mg/g.

1. Introduction

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a potent red xanthoplyll belonging to carotenoids class. Its content is available in natural forms in microalgae, yeast, fishes (salmonids), and shrimps. Astaxanthin is very popular in nutraceuticals sector for its antioxidant properties and its use is also widespread in aquaculture sector as feed additives for salmonids. Astaxanthin is naturally present in salmonids and shrimps but is not produced by these organisms.It accumulates as a result of the feeding of microorganisms and microalgae that contain it. Now consumer awareness is changing the astaxanthin market by requiring bio-based products that are supplied in the form of oleoresin, meal, extracts and encapsulated form as soft-gel (Lim et al., 2018). Notwithstanding the market of astaxanthin is still dominated by the synthetic form which has a cost 10 times lower than the natural one of about 800 euros per kg (Liu et al., 2018, Molino et al., 2019a) but despite the economic disadvantages, naturally produced astaxanthin has many advantages such as a greater antioxidant function, its consumption is healthier and more environmentally friendly (Khoo et al., 2019). The main natural

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sources of astaxanthin are the yeast *Phaffia rhodozyma* and the microalgae *Haematococcus pluvialis*. Nowadays, microalgae are among the most interesting eukaryotic photosynthethic microorganisms able to use solar energy, nutrients and carbon dioxide to convert them into proteins, carbohydrates, lipids and other valuable organic compounds including carotenoids (Marino et al., 2019, Molino et al., 2019b). *Haematococcus pluvialis* is considered a promising source of astaxanthin as it can produce up to 5% astaxanthin by dry weight during a particular stage of the life cycle when the cells become red cysts rich in astaxanthin (Haque et al., 2016). During this stage, called red phase, astaxanthin constituted the highest percentage of carotenoids around 80-90%. The production process of astaxanthin derived from *Haemotococcus pluvialis* can be summarized in some essential steps, such as the cultivation of the microalgae, biomass harvesting that contribute for 20-30% to the total production cost, collected biomass pre-treatment and astaxanthin extraction(Panis et al., 2016). Extraction processes has been abundantly studied for astaxanthin from *Haematococcus pluvialis* by testing different technologies such as extraction by using organic solvent as ethanol, acetone, hexane and chloroform and methanol mixture (1:1 v/v)(Molino et al., 2018a) or by new unexplored solvent as butanol, cyclohexane, ethyl acetate (Samori et al., 2019), extraction by supercritical fluids by using CO_2 (Sanzo et al., 2018), acid pre-treatment and homogenization (Kang et al., 2008) with the aim of improving extraction astaxanthin yield.
Recent insight about the improving of carotenoids quality extracts is the purification by using adsorbent such as decolorized activated carbon or bentonite to remove and recovery beta-carotene from crude palm oil or to study the desorption behavior of bentonite respect to betacarotene (Novaković et al., 2015, Ulfah et al., 2017).
Adsorption technique was recently applied as a procedure to recovery compounds as carboxylic acids from fermentative broth by using commercial activated carbon (Yousuf et al., 2016) or to study the adsorption of caffeine, ibuprofen and triclosan, pharmaceutical and personal care products from water by using Darco™ G-60 (Kaur et al., 2018).According to our knowledge, commercial activated carbon as Darco™ G-60 has not so far been tested as a means of absorbing and desorption of astaxanthin from extracts obtained from *Haematococcus pluvialis* to investigate active carbon behavior and to improve astaxanthin recovery.
The objective is to test the adsorption capacity of active carbon Darco™ G-60 against an astaxanthin solution extracted from the microalgae *Haematococcus pluvialis* by Accelerated Solvent Extractor (ASE®200 DIONEX). Each test were performed at three different concentrations of bed material and adsorbed and desorbed astaxanthin content was evaluated during the time.

2. Materials and Methods

Haematococcus pluvialis red phase (HPR) lyophilized microalgae was supplied by MICOPERI BLUE GROWTH®, (Rimini, Italy). Microalgae biomass was characterized in terms of moisture content (70.0 mg/g on fresh weight basis) and ash (60.15 mg/g) on a dry weight basis and principal compounds as proteins, carbohydrates, lipids and carotenoids as reported in table 1. Astaxanthin content was equal to 20.01 mg/g dryweight while, lutein and beta-carotene was also detected with a content of 7.70 mg/g dry weight and 0.99 mg/g dry weight, respectively. Protein and total dietary fibers are the most abundant compounds in HPR biomass.

Table1: Haematococcus pluvialis lyophilized microalgae characterization

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

Characterization was performed principally by standard methods as reported by Molino et al., 2018b. Before astaxanthin extraction, HPR microalgae was mechanically pre-treated by Retsch PM200 planetary ball mill at 400 rpm for 5 minutes to enhance astaxanthin extraction yield.Diatomaceous Earth for ASE(0.8 grams) was used as inert material both for pre-treatment and extraction. Astaxanthin extracts were obtained by Accelerator solvent extraction by ASE 200 Dionex (Salt Lake City, UT, USA) (Figure 1a). Extractions were performed at 100 bar, 67 °C by using ethanol as GRAS solvent as reported in previous works (Molino et al., 2018 c; Casella et al., 2019). Astaxanthin extracts were purified by using an experimental adsorption system apparatus (Figure 1b) by using a chromatographic column packed by activated carbon, Darco™ G-60 (Fisher

Chemical FLD127500, CAS Number 7440-44-0). Characteristics of activated carbon are reported in the table 2. with a density of 1.8g/mol and a molecular weight equal to 12.011 g/mol.

Table 2: Darco™ G-60 activated carbon characteristics

Activated carbon	Particle size (mesh)	Surface area (m^2/g, approx.)	Pore volume (ml/g, dry basis)	pH	Moisture (%)	Ash (%)	Water solubles (% max.)	Tamped bulk density (lb/cu ft)
Darco™ G-60	100-325	600	0.95	6-8	12	3.5	0.5	25

a) b)

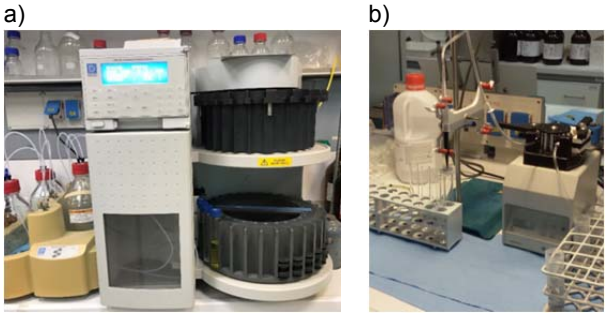

Figure 1: a) ASE® 200 Accelerated Solvent Extractor, b) adsorption system apparatus

Before and after adsorption trials, astaxanthin was analysed by uHPLC-DAD (Agilent 1290 Infinity II)by using a reversed phase column, Zorbax C18 column, an isocratic mobile phaseas methanol/water (95:5, v/v) as a mobile phase solvent by Ruegnam et al. 2010. Astaxanthin and isomers were detected at 478 nm by using a Diode array detector (DAD). For the analysis of astaxanthin, saponification was necessary to enhance free form by using methanolic-KOH 0.05M (Yuang and Chen 1999). Analytical astaxanthin standard was used for identification and quantification of total isomers.

3. Results and Discussion

A solution containing astaxanthin at concentration of about 120mg/l was feed in the adsorption column till to the complete saturation of the activated carbons. The feed flow rate was in the range 0.9-1.0ml/min. Three solids weight were used: 50mg, 100mg and 200mg. All experimental tests were carried out at room temperature (20°C). Results are showed in Figure 2.

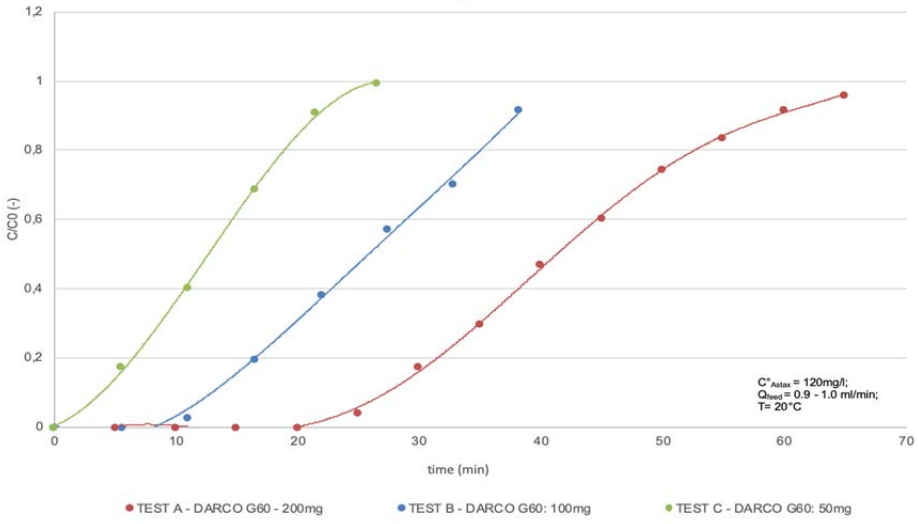

Figure 2: Adsorption test at different quantity of DARCO G60 quantity.

During the adsorption tests, it was possible to observe that during the first few minutes, the activated carbon was able to adsorb all the pigments of the solution producing a discolored solution, while after the breakthrough of the bed material the solution color come back as in the sample. An example of this is showed in the picture below (Figure 3).

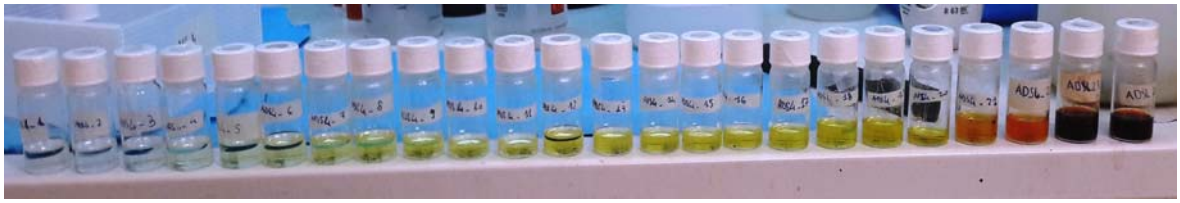

Figure 3: Adsorbing Test: Discolouring effect of the pigment across the activated carbon

As shown in Figure 3, the effect of the solid weight was very noticeable. In fact, by increasing it, all the figures are shifted in the right direction but their maintained the same slope due to the amplitude related to the area of matter transfer that depended exclusively on the matter transport phenomenon on the solid, being therefore independent by the quantity of introduced solid.
The maximum quantity of adsorbed astaxanthin was evaluated through a differential balance of matter in the adsorbent bed according to Eq(1):

$$Q * C_0 * dt - Q * C * dt = m * d\varpi \quad (1)$$

in which $Q$was the feed flow rate, C and C_0were the concentrations of astaxanthin during the time and at t=0min, $d\varpi$ was the adsorbed quantity of astaxanthin in the infinitesimal time dt. The integration of this differential equation from zero to the complete saturation of the bed material in which $\omega = \omega^*(C_0)$ allowed to evaluate the maximum adsorbed quantity by means of Eq(2):

$$\omega^* = \frac{Q C_0 * \int_0^\infty \left(1 - \frac{C}{C_0}\right) * dt}{m} \quad (2)$$

In the present work, by applying Eq(1) and Eq(2) (as finite form) in all the three tests it was possible to evaluate the adsorption capacity of the bed material as shown in the Table 3.

Table 3: astaxanthin feed and adsorption capacity

	Time	Astaxanthin feed	Astaxanthin Adsorbed	Adsorption capacity
	min	mg	mg	mg/g
TEST A	65,0	7,8	4,8	23,9
TEST B	38,0	3,9	2,2	21,9
TEST C	26,5	3,0	1,1	22,1

On the basis of the results of table 3, it was possible to confirm that the absorbing capacity of DARCO G60 in these operative conditions was 22.6±1.1mg/g.

4. Conclusions

Astaxanthin is undoubtedly one of the most beneficial nutraceutical carotenoids which is attracting more and more interest from the scientific community. The majority of astaxanthin available on the market today is made artificially. However, there are many challenges for natural based astaxanthin to compete with the synthetic form. *Haematococcus pluvialis* microalgae represents the prominent source of astaxanthin. The production process of the algal carotenoidincludes: 1) cultivation of the microalgae strain;2) biomass harvesting; 3) biomass pre-treatment and 4) astaxanthin extraction. The use of activated carbon as downstream technology for astaxanthin purification could be considered a valid strategy. In this paper, from solid to liquid phase of organic compounds has been done by using accelerated solvent extraction. In this stage, all the pigments including astaxanthin have been transferred into liquid phase. In fact, this stage was carried out till to the complete discolouring of the microalgae biomass (*Haematococcus pluvialis* red phase). Liquid phase was used as feed for an adsorption bed material (DARCO G60) by changing the weight of the adsorption bed at

the end to evaluate the adsorption capacity of DARGO G60 on astaxanthin. Preliminary results show that in the adopted operative conditions, the astaxanthin adsorption capacity onto DARCO G60 was 22.6 mg/g. In this contest, a possible strategy for astaxanthin purification can be the selective desorption of astaxanthin by modulating the operational parameters and using a solvent with a greater polar affinity with the main focus to obtain astaxanthin at high purity.

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