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PRODUCTION AND RECOVERY OF ASTAXANTHIN FROM THE MICROALGA *HAEMATOCOCCUS PLUVIALIS*

Tesi di Laurea in Biocarburanti e Bioraffinerie

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ABSTRACT

Nowadays, a great interest in natural astaxanthin derived from *Haematococcus pluvialis* microalga is spreading internationally for nutraceutical and cosmetic use. Astaxanthin is a secondary keto-carotenoid that has many health-promoting effects, including a very strong antioxidant activity. Astaxanthin is naturally accumulated by *H. pluvialis* cells when subjected to stress conditions. The present thesis focuses on the production and extraction of natural astaxanthin through innovative sustainable methods. Specifically, different stress factors (acidic pH, salinity and ascorbic acid) were tested in combination to high light and nutrient deprivation to improve astaxanthin accumulation in *H. pluvialis* cells. Ascorbic acid was found to be the best factor for promoting astaxanthin production. Almond oil and Hydrophobic Natural Deep Eutectic Solvents (NaDESs) based on terpenes were selected as candidates to obtain formulates enriched in astaxanthin directly available for human consumption or for the cosmetics industry; all solvents were applied in a milking extraction mode, dedicated to the maintenance of algal viability. Menthol: oleic acid was the best NaDES in terms of cell viability, followed by α-bisabolol, menthol: lauric acid and thymol: oleic acid, but astaxanthin recovery was very low. On the other hand, almond oil allowed to reach a good compromise between cell viability and astaxanthin recovery. Both NaDES and almond oil allowed to re-cultivate *H. pluvialis* after a first milking extraction, without affecting the turning of algal cells into vegetative and stressed phases. This phenomenon makes the extraction process more sustainable by reducing energy and economic costs associated to the algal biomass loss. In the second part of the thesis natural and synthetic astaxanthin effects on cancer cells Hep G2 were investigated. Although both natural and synthetic astaxanthin were confirmed to be potent antioxidant, only the last one seemed to influence lipid metabolism and slightly mitochondrial respiration.

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1 INTRODUCTION

1.1 MICROALGAE AS RENEWABLE RESOURCE

Microalgae are an extremely various group of organisms accounting for approximately 50% of photosynthesis on this planet.[1] Microalgae ensure certain exceptional advantages over terrestrial plants: they are photoautotrophs and therefore do not necessary require organic substances as energy. Solar radiation, water, CO_2, and inorganic nutrients are the best requirement for algal growth.[2] Consequently, their large-scale cultivation in open ponds or photobioreactors is theoretically simple and cheaper. Recent U.S. studies *(Olaizola, 2003)* have focused on the coupling of photobioreactors with CO_2 emissions from industrial plants in order to obtain mutual benefits in terms of production/waste costs and sustainability.[3]

Algal biomass has been used for centuries as food and medicine. The health-promoting effects of microalgae were discovered as early as 1500 BC.[4] However, microalgae biomass gained interest as a source of chemicals and pharmaceuticals only recently.
Figure 1 shows the main algae industrial applications.[5]

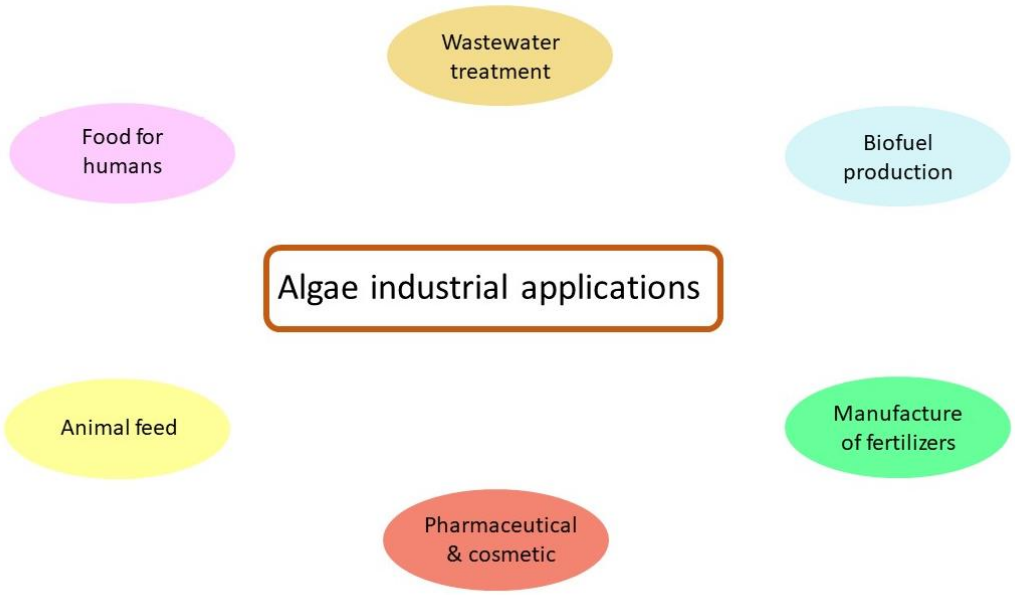

Figure 1 The main industrial application for algae.

Nowadays, microalgae are considered extremely important producers of high-value nutraceutical compounds in order to justify high cost of microalgae processing technologies (cell cultivation, harvesting, dewatering, extraction of metabolites and their purification).[6] Examples of high-value compounds are vitamins and antioxidants, that have many health-promoting effects.[2] Recent research has been suggested microalgae as a therapeutic solution for a number of serious conditions, including diabetes, heart disease, autoimmune diseases, cancer, and cognitive decline, in the form of dementia and Alzheimer disease.[7,8] Moreover, nutritional content of microalgae is rapidly gaining importance as alternative and renewable source to substitute the conventional ingredients in the human diet or animal feed,[9] such as the use of the carotenoid astaxanthin for feeding salmonid and crustaceans and providing the pink colour characteristic of that species, or the use of algae proteins as substituents for animal proteins in vegan or vegetarian diets/supplements.[10]

1.1.1 ALGAL PIGMENTS: CAROTENOIDS

Microalgae absorb visible light through a lot of compounds known as pigments, such as chlorophylls and carotenoids. Another function of pigments is to protect microalgae against various stress factors like high irradiance by quenching reactive oxygen species (ROS). Antioxidant activity of algal pigments depends on their structural features like extended system of conjugated double bonds. More than 700 different carotenoid molecules[2] are known in nature, but very few are used commercially, for example β-carotene, astaxanthin, zeaxanthin, lutein, and lycopene (see Figure 2).[11]

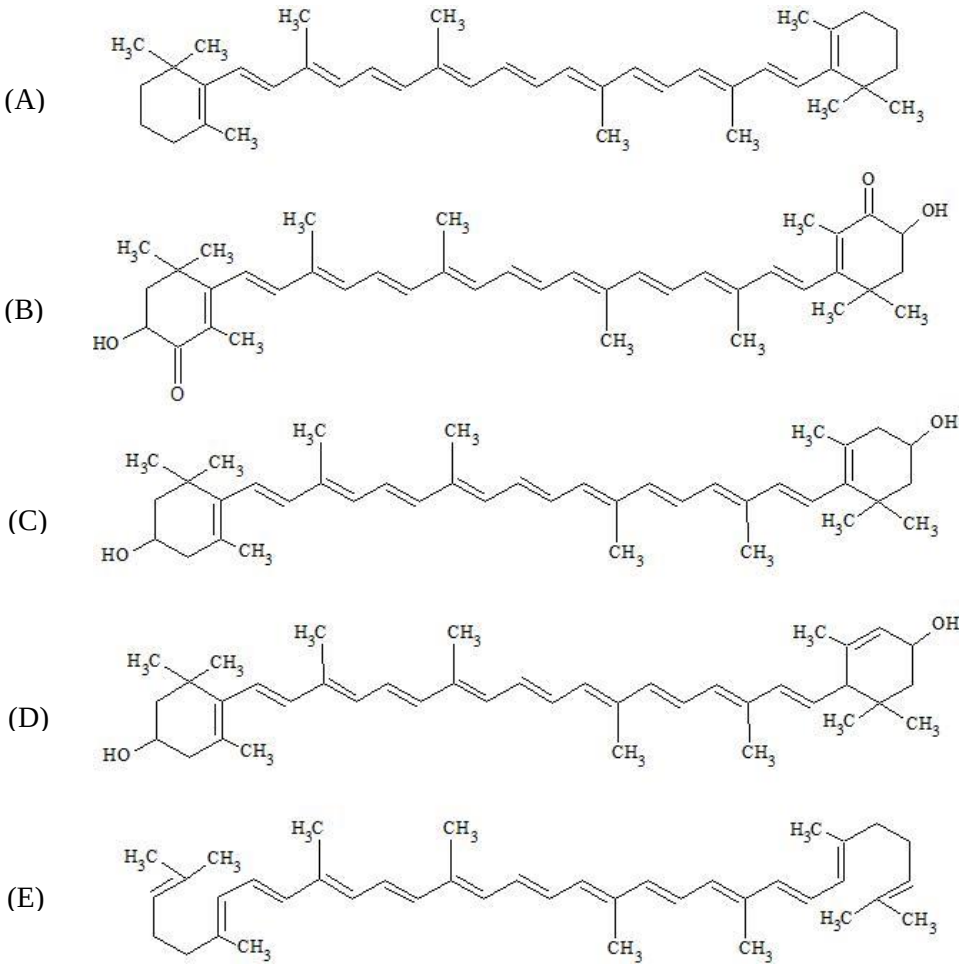

Figure 2 Chemical structure of (A) β-carotene; (B) astaxanthin; (C) zeaxanthin; (D) lutein; (E) lycopene.

Due to their brilliant colours, these pigments are also considered as an eco-friendly alternative to the synthetic food colorants.[2] Due to their health-promoting properties, algal pigments have found many commercial applications in food, feed, and pharmaceutical and cosmetic markets. In fact, many algal metabolites and pigments have antiaging, antioxidant, anticancer, antiobesity and neuroprotective properties making them suitable for use in nutraceutical, pharma and cosmetics.[2]

1.2 *HAEMATOCOCCUS PLUVIALIS* AND ASTAXANTHIN PRODUCTION

The main natural source of astaxanthin, object of the present thesis, is a freshwater unicellular biflagellate green microalga called *Haematococcus pluvialis*. This alga belongs to the class Chlorophyceae and it is diffused worldwide, but it is most common in temperate regions. It can be found in brackish water and in temporary puddles. Normally, *H. pluvialis* utilizes the available phosphate, nitrate and other nutrients to grow and reproduce. Under stress conditions like high light

or nutrient deprivation, *H. pluvialis* accumulates a large amount of astaxanthin as protective barrier against harsh ultraviolet rays.[I]

H. pluvialis cultures are very sensitive to contamination by other microorganisms as well as to extreme environmental conditions (high and low temperature or light). Closed photobioreactors thus represent the most successful system to industrially grow this species.[12] Photobioreactors consist of a set of plastic or glass tubes with solar collectors generally tilted to maximize the capture of light radiation. In photobioreactors algal biomass is mixed with airlift pumps, and CO_2 is introduced against the current. In general, the process of *H. pluvialis* cultivation in photobioreactors consists of two-steps: the "green phase" and the "red phase". In the "green phase" algal culture grows under optimal conditions, with regular cycles of alternating light and dark 12:12 or 16:8 h.[13,14] The typical irradiation for *H. pluvialis* cultivation is about 80 $\mu E/m^2/s$, [13] and optimal pH is between 7.00–7.85.[15,16] When cell density becomes high, algal biomass is subjected to stress conditions to induce a transition phase that leads to the formation of red cysts. The origins of this stress can be caused by a combination of multiple factors. The combination of abiotic stress factors has recently been reported to be a very effective and economical approach to improve astaxanthin accumulation in microalgae.[17] High irradiance and nitrogen starvation, are commonly used to induce astaxanthin accumulation in *H. pluvialis*.[17] In fact, nitrogen limitation can lead to approximately twice the rate of astaxanthin production than the limitation of phosphorus.[6] To decrease nitrogen concentration, the alteration of the C/N ratio must be induced by aerating *H. pluvialis* culture with CO_2, together with continuous high light.[18] Some studies affirm that astaxanthin production can be also increased by adding NaCl (0.25–0.5% w/v) to the media, together with the addition of sodium acetate.[19] When *H. pluvialis* is exposed to both high light and NaCl, genes involved in the biosynthesis increase to a high level by accumulating astaxanthin in algal cells.[20] Although the increase in astaxanthin due to NaCl is confirmed in literature, the expression of these genes behaves differently amongst strains. The reason is not known because currently the regulation and expression of carotenogenesis genes leading to astaxanthin formation is still not well understood in *H. pluvialis*.[21] Another stress factor is high temperature: at 30°C the transition from green phase to red phase occurs rapidly.[6]

Few studies have reported the antioxidants addition to algal culture to promote astaxanthin accumulation. As antioxidant, butylated hydroxytoluene (BHT) has been employed to promote astaxanthin and lipid accumulation in *H. pluvialis*.[17,22] The results of *Zhao et al.* (2018) study

[I] Astaxanthin accumulation in *H. pluvialis* was discovered by the English biochemist Tisher in 1944.[24]

suggest that the addition of 2 mg/L of BHT treatment is the optimal concentration for promoting astaxanthin and lipid production in *H. pluvialis* under high-light and nitrogen-deficient conditions.[17]

1.2.1 *HAEMATOCOCCUS PLUVIALIS* LIFE CYCLE

H. pluvialis life cycle consists of two phases based on cell morphology and physiology:

- "green vegetative phase",[6] a motile phase;
- "red encysted phase",[6] a non-motile phase.

Zoospores, motile cells, non-motile cells, and aplanospores are four cellular forms with different characteristics (Figure 3).

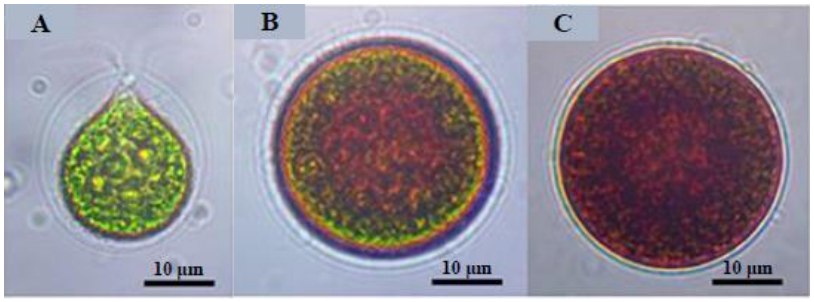

Figure 3 Light microscopic images of *H. pluvialis* life cycle. (A) Motile cell; (B) Non-motile cell; (C) Aplanospore. Source: image adapted from the Journal *Astaxanthin-producing green microalga Haematococcus pluvialis: From single cell to high value commercial products, M. Shah et al., 2016*.

Motile cells are spherical biflagellated cells 8-20 μm long.[6] They have two flagella of equal length emerging from anterior end, and a cup-shaped chloroplast with numerous pyrenoids. A lot of contractile vacuoles are irregularly distributed near the protoplast surface of the cell.[23] The carotenoid fraction of motile cells consists mainly of lutein (75-80%) and β-carotene (10-20%).[24]
Under unfavourable environmental conditions, motile cells start losing flagella, and expand their cell size, by developing a non-motile form. Non-motile cells are formed by a thick cell wall and a green periphery, due to the presence of chloroplasts. Few astaxanthin granules start to accumulate in the mid-region of the cell, around the cell nucleus, by forming a red centre. Non-motile cells appear to be in a resting stage however they still have proliferative ability. As a matter of fact, if environmental stress continues, non-motile cells will be able to produce aplanospores, to resist against extreme environmental conditions.
Aplanospores can be defined as daughter cells originated by asexual reproduction of a non-motile cell.[25] These cells are defined as "cysts" and they contain two distinct structures, a thick and rigid trilaminar sheath, and secondary cell wall. Mature aplanospores accumulate secondary carotenoids

including astaxanthin, which gathers in lipid droplets (triglycerides) deposited in the cytoplasm. This phenomenon is the responsible of characteristic red colour of these cells. Astaxanthin content in *H. pluvialis* is reported to be about 1.5-5% of its dry weight.[26]

The main cellular proliferation of *H. pluvialis* is asexual reproduction. Under favourable conditions, both motile cells and non-motile cells are able to release respectively zoospores and aplanospores, in order to initiate a new vegetative growth cycle. Motile cells usually produces 2, sometimes 4, and exceptionally 8 zoospores by mitosis, while non-motile cells are able to produce 2, 4 or 8, and occasionally 20–32, aplanospores.[25] Zoospores are released during asexual reproduction process (Figure 4): a mother cell develops into a sporangium that swells and the wall breaks to release some daughter cells. Zoospores are flagellated cells smaller than 10 μm in size and with a high-speed motility. Over time a zoospore transforms into a motile cell, under favourable environmental conditions. However, if a transition from stress conditions to favourable conditions occur, a non-motile cell is also able to produce zoospores.

Another kind of cells reproduction is the vegetative one, which consists of a direct cell division in the motile phase and a cell budding in the non-motile phase.

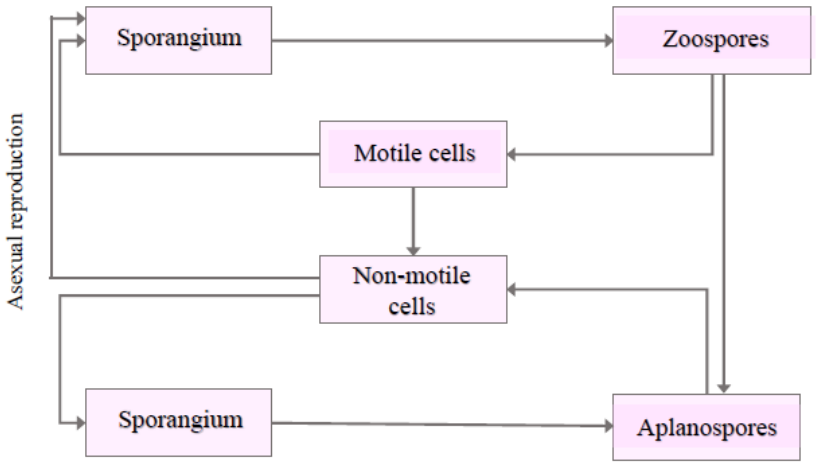

Figure 4 Life cycle and cellular forms of *H. pluvialis*.

M. Wayama et al. (2013) study focuses on the morphological changes of *H. pluvialis* cells during life cycle phases. Figure 5 shows a motile cell and gives a clear visualization of membrane structures and thylakoids (Figure 5 A, B). Conspicuous pyrenoids with electron-dense matrix are observed in the stroma (Figure 5 A, B). Few astaxanthin granules are located around the nucleus, and highly developed chloroplasts are located at the periphery.

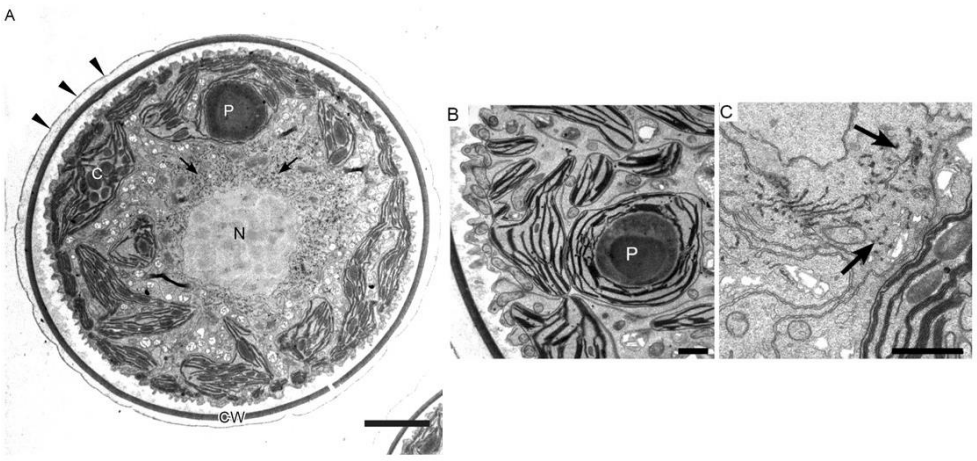

Figure 5 Transmission electron micrographs of *H. pluvialis* motile cells: (A) general ultrastructure. The cell wall is surrounded by extracellular matrix (arrowheads). Arrows indicate astaxanthin granules. (B) Chloroplast and pyrenoid. (C) High-magnification view of astaxanthin granules (arrows). C, chloroplast; CW, cell wall; N, nucleus; P, pyrenoid. Scale bars in A and B, C: 5 µm and 1 µm respectively. Source: picture adapted from *Three-Dimensional Ultrastructural Study of Oil and Astaxanthin Accumulation during Encystment in the Green Alga Haematococcus pluvialis, M. Wayama, 2013.*[27]

In *H. pluvialis* non-motile cells (intermediate cells) conspicuous pyrenoids with electron-dense matrix are located in the stroma and are surrounded by thick starch capsules. Oil droplets containing astaxanthin are electron-dense and located around the nucleus (Figure 6 A, B). These droplets were rounded and of various sizes, probably depending on cells age. Thylakoids are partially degraded.

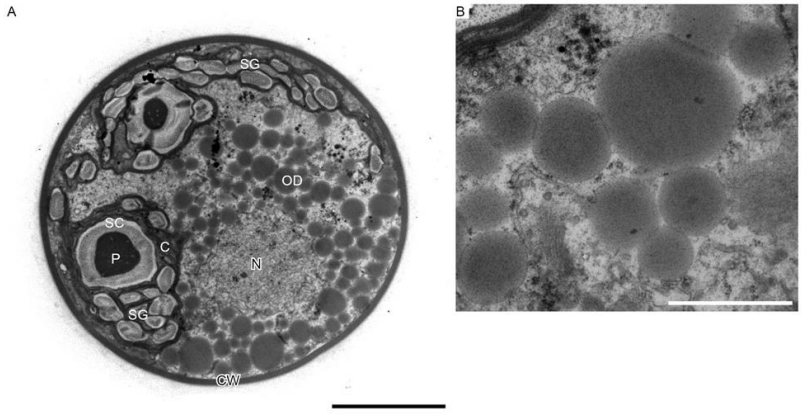

Figure 6 Transmission electron micrographs of intermediate *H. pluvialis* cells. (A) General ultrastructure. (B) High-magnification view of astaxanthin oil droplets. C, chloroplast; CW, cell wall; N, nucleus; OD; oil droplet; P, pyrenoid; SC, starch capsule; SG, starch grain. Scale bars in A and B: 5 µm and 1 µm, respectively. Source: picture adapted from *Three-Dimensional Ultrastructural Study of Oil and Astaxanthin Accumulation during Encystment in the Green Alga Haematococcus pluvialis, M. Wayama, 2013.*[27]

In *H. pluvialis* cells oil droplets containing astaxanthin are different among cyst cells. They differ in size, electron-density, and some droplets are occasionally fused (Figure 7 D). Chloroplasts are highly degenerated and localized in the interspace between oil droplets (Figure 7 C).

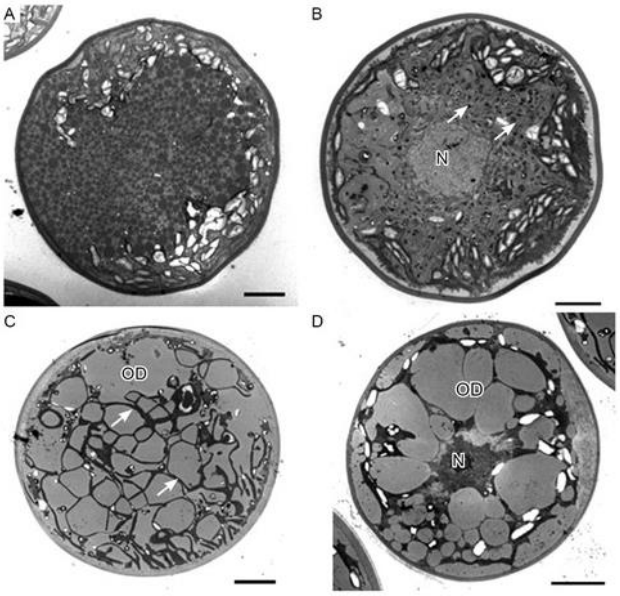

Figure 7 Transmission electron micrographs of *H. pluvialis* cyst cells. (A) General ultrastructure of cyst cells, showing small granules that contain astaxanthin. (B) General ultrastructure of a cyst cell, showing astaxanthin accumulation in oil droplets. (C) General ultrastructure of a cyst cell, showing large oil droplets. Chloroplasts localize in the interspace between oil droplets (arrows). (D) Some oil droplets are fused. E. C, chloroplast; N, nucleus; OD, oil droplet. Scale bars in A–D: 5 µm. Source: picture adapted from *Three-Dimensional Ultrastructural Study of Oil and Astaxanthin Accumulation during Encystment in the Green Alga Haematococcus pluvialis, M. Wayama, 2013*.[27]

1.2.2 *HAEMATOCOCCUS PLUVIALIS* CHARACTERIZATION

H. pluvialis composition varies greatly between the green and the red phase of its life cycle. Under optimal conditions (green phase) protein content is higher than in the stress phase (29-45% versus 17-25%), while for the lipid and carbohydrate content is the opposite (15.17% in green phase and 36-40% in red phase, Table 1).[28] Proteins content in the red stage includes mainly aspartic acid, glutamic acid, alanine, and leucine, while the carbohydrates content in the red phase is mostly starch, an energy reserve to withstand a long stress period. In the green phase, total lipid content varies from 20 to 25%, with approximately 10% lipids composed predominantly of short (C16, C18) polyunsaturated fatty acids deposited in the chloroplasts. In both phases neutral lipids are predominant. Stress conditions induce the synthesis of triacylglycerols (TAG) that accumulate as

cytoplasmic lipid droplets. Lipid composition (especially palmitic (16:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids) varies greatly among different strains, because it depends on several factors such as culture environment, stress conditions, culture parameters, strain origin. Carotenoid levels are higher in the red phase. In the green phase primary carotenoids (such as β-carotene, zeaxanthin, lutein) and chlorophylls are predominant. In the red phase primary carotenoids are transformed in secondary carotenoids, mainly astaxanthin (80-90% of total carotenoids).[29,30] During the transition phase astaxanthin starts to accumulate as fatty acid mono- or diesters (usually esters of palmitic, oleic, or linoleic acids) in cytoplasmic lipid droplets.[31] *H. pluvialis* fatty acid composition is reported in Table 2. *Zhekisheva et al. (2002)* demonstrated that under stress phase fatty acid content increased and that the predominant fatty acids were palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3).[32]

Table 1 *H. pluvialis* composition in green and red phases. n.d, no data. Image adapted from *The carotenoid astaxanthin from Haematococcus pluvialis, Grewe e Griehl, 2012.*[33]

Composition content (% DW)	Green phase	Red phase
Proteins	29-45	17-25
Carbohydrates	15-17	36-40
Lipids (% of total)	20-25	32-37
Carotenoids (% of total)	0.5	2-5
Neoxanthin	8.3	n.d
Violaxanthin	12.5	n.d
β-carotene	16.7	1.0
Lutein	56.3	0.5
Zeaxanthin	6.3	n.d
Astaxanthin	n.d	81.2
Adonixanthin	n.d	0.4
Adonirubin	n.d	0.6
Canthaxanthin	n.d	5.1
Echinenone	n.d	0.2
Chlorophylls	1.5-2	0

Table 2 Fatty acids composition in *H. pluvialis*. Source: *A Technical Review of Haematococcus Algae, R. T. Lorenz, 1999.*[34]

Fatty acids	% of total fatty acids
C12:0	0.1
C14:0	0.5
C16:0	29.0
C16:1	0.6
C17:0	0.2
C17:1	1.3
C18:0	2.1
C18:1	25.9
C18:2	20.8
C18:3	12.8
C18:4	1.4
C20:0	0.6
C20:1	0.3
C20:2	1.2
C20:3	0.5
C20:4	1.4
C20:5	0.6
C22:0	0.4
C24:0	0.2
C24:1	0.1

1.3 ASTAXATNHIN

1.3.1 ASTAXANTHIN BIOCHEMISTRY

Biosynthesis of astaxanthin in *H. pluvialis* is a complex process which is induced by stress factors. The process of astaxanthin biosynthesis in depicted in Figure 8.

The first intermediate of carotenoids biosynthesis is isopentenyl pyrophosphate (IPP) together with its isomer dimethylallyl diphosphate (DMAPP). IPP is synthesized from pyruvate and glyceraldehyde-3-phosphate (two photosynthesis products), through 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway. DMAPP derives from IPP isomerization, carried out by 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) enzyme.

Elongation of the isoprenoid chain starts with a molecule of DMAPP and a subsequent linear addition of three molecules of IPP, that is catalysed by geranylgeranyl pyrophosphate synthase (GGPS) enzyme. This process ends with the formation of geranylgeranyl pyrophosphate (GGPP), a C_{20} compound. Another enzyme, phytoene synthase (PSY), condensates two GGPPs in order to obtain phytoene, a C_{40} compound. Phytoene is the precursor of astaxanthin and other carotenoids.

The following four desaturation steps permit the increase of the number of conjugated carbon-carbon, by forming lycopene. These steps are catalysed by three enzymes: phytoene desaturases (PDS), ζ-carotene desaturase (ZDS), and plastid terminal oxidase (PTOX 1). The last one acts as co-factor for electron transfer. Lycopene undergoes cyclization catalysed by lycopene cyclases, LCY-e and LCY-b. LCY-e is responsible for producing α-carotene, while LCY-b catalyses β-carotene. β-carotene forms a precursor of astaxanthin, and the process of astaxanthin formation is catalysed by the enzymatic activity of β-carotene ketolase (BKT) and hydroxylase (CrtR-b), through the formation of some intermediates.

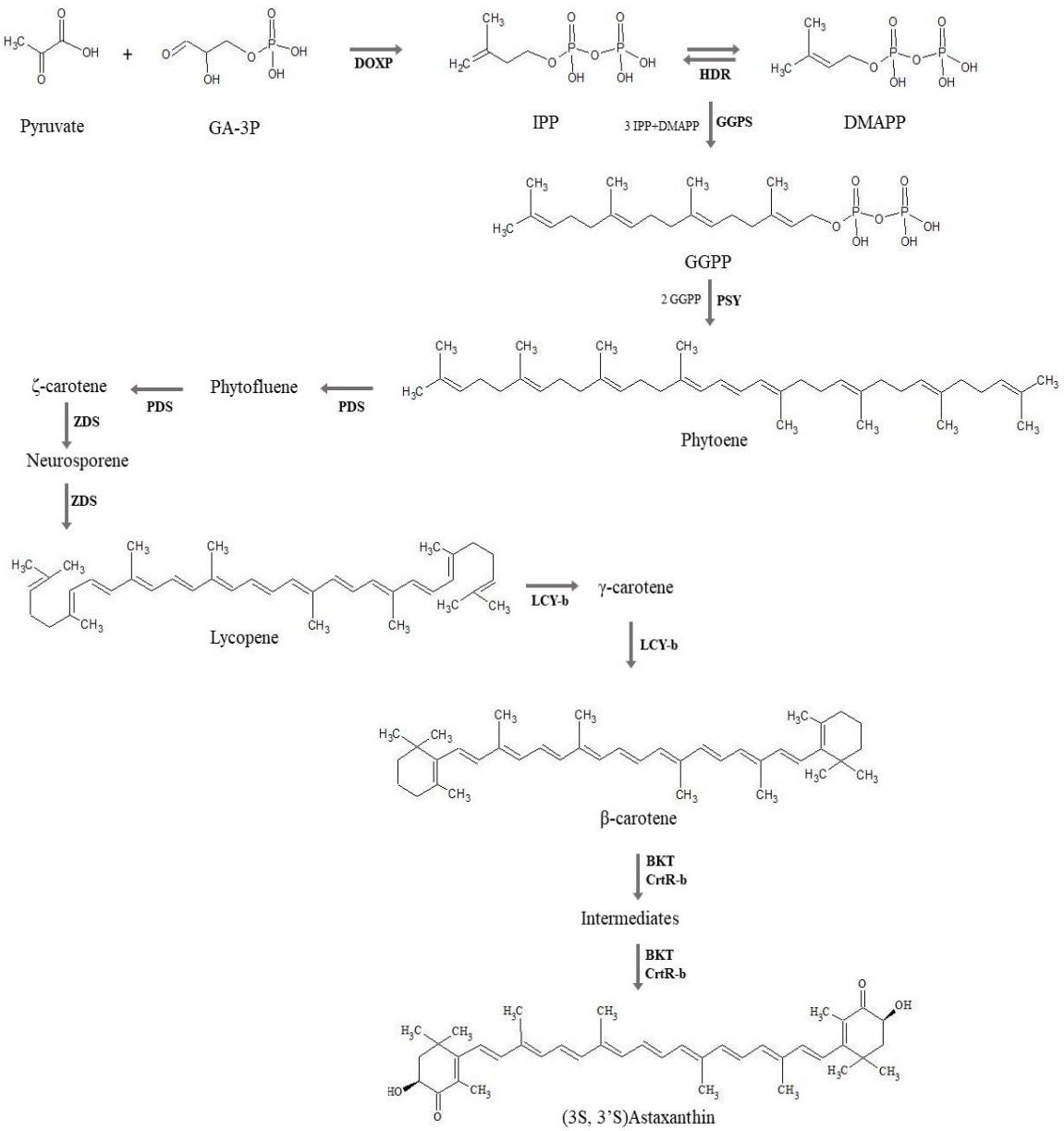

Figure 8 Pathway of (3S-3'S)-astaxanthin biosynthesis in *H. pluvialis*.

1.3.2 ASTAXANTHIN CHEMISTRY

Carotenoids are red, orange or yellow coloured lipophilic polyene compounds ending with "ionone" rings.[2] Two main groups of carotenoids can be identified:

- carotenes, hydrocarbon-based oxygen-free carotenoids;
- xanthophylls, alcohol-based carotenoids.

Astaxanthin (3,30-dihydroxy-b,b-carotene-4,40-dione, $C_{40}H_{52}O_4$) is a keto-carotenoid[35] which belongs to xanthophylls group, and contains both hydroxyl groups and ketones. The presence of double bonds in the aliphatic chain of 40 carbon atoms confers absorption of light at wavelengths between 400 and 500 nm, which gives the characteristic red colour.[36] Astaxanthin has lipophilic and hydrophilic properties and has both polar (at the ends of the molecule) and apolar (in the middle of the chain) functional groups. Astaxanthin molecular weight is 596,84 g/mol.

Astaxanthin exists in stereoisomers, geometric isomers, free and esterified forms,[10] and all of these forms are found in natural sources.[37] Since astaxanthin has two identical chiral centres at the positions of 3 and 3′ it can be found in three different stereoisomers, the meso-form (3R, 3′S); (3R, 3′R); (3S, 3′S),[6] depending on the spatial orientation of the hydroxyl groups (OH) in chiral carbon (Figure 9). (3S, 3'S) and (3R, 3'R) are the most abundant in nature.[37] The predominant form in *H. pluvialis* is (3S, 3'S),[6] and it is in mono (the most abundant form)[38] and di-ester form, where astaxanthin hydroxyl group reacts with one or two fatty acids (Figure 10),[34] such as palmitic, oleic, linoleic acid (Table 4). Esterification gives astaxanthin greater lipophilicity and stability in the cytoplasm.[24]

(A

(B)

(C)

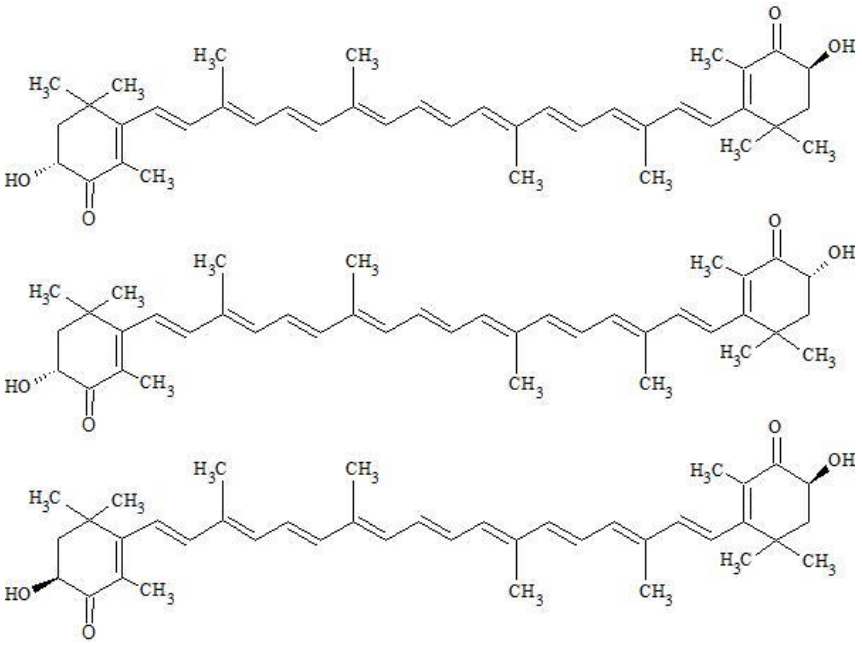

Figure 9 Chemical structure of astaxanthin. (A) 3R, 3'S; (B) 3R, 3'R; (C) 3S; 3'S.

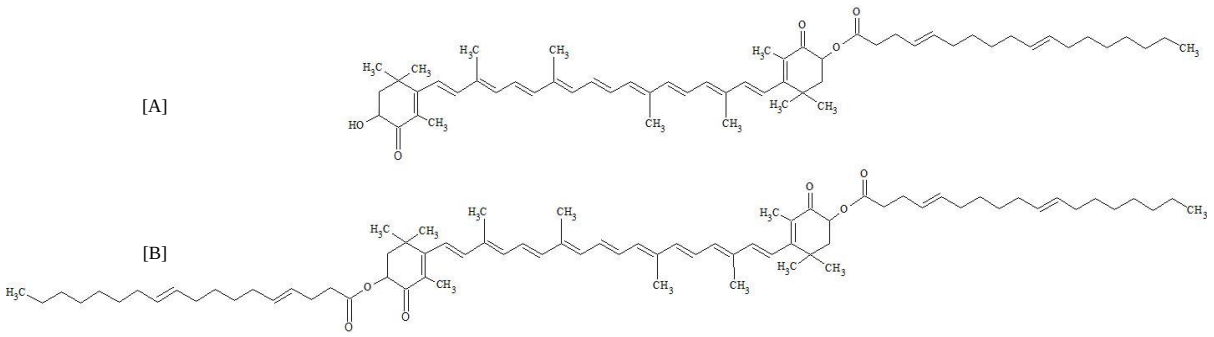

Figure 10 (A) Mono-esterified and (B) di-esterified astaxanthin.

Synthetic astaxanthin is a racemic mixture of (3R,3'S), (3R,3'R), (3S,3'S) isomers in the ratio of 2:1:1, that give greater stability to the substance.[10] Free astaxanthin is less stable than the esterified one: the first easily loses its anti-oxidative activity when exposed to heat and light.[39] Each double bond may exist in two different configurations of geometric isomers: *cis* or *trans*. *Cis* isomers are thermodynamically less stable than *trans* isomers. In nature *trans* isomer is the most diffused form of carotenoids.[40] As a matter of fact, *H. pluvialis* includes almost entirely (88%) all-*trans* astaxanthin (Table 3). However, *trans* natural astaxanthin is readily isomerized to *cis-trans*, especially *9-cis* and *13-cis*, for stearic reasons.[41] *X. Liu and T. Osawa (2007)* study compares antioxidant activity of *trans* astaxanthin with that of *9-cis* and *13-cis* (Figure 11). The study states that the last ones, especially *9-cis* astaxanthin, have a higher antioxidant activity if compared with all-*trans* astaxanthin.[41]

Table 3 Isomers in natural and synthetic astaxanthin. Table taken from *Astaxanthin in cardiovascular health and disease: mechanisms of action, therapeutic merits, and knowledge gaps, F. Visioli and C. Artaria, 2017.*[42]

	Natural astaxanthin from *H. pluvialis*			**Synthetic astaxanthin**		
	3S, 3S'	3R, 3R'	3R, 3S'	3S, 3S'	3R, 3R'	3R, 3S'
Trans form	88%	-	-	25%	25%	50%
Cis form	8% (9-*cis*) 4% (12-*cis*)	-	-	-	0.8%	-

Table 4 Forms of natural and synthetic astaxanthin. Table taken from *Astaxanthin in cardiovascular health and disease: mechanisms of action, therapeutic merits, and knowledge gaps, F. Visioli and C. Artaria, 2017.*[42]

		Natural astaxanthin from *H. pluvialis*	**Synthetic astaxanthin**
Esterified astaxanthin	Mono-	90%	-
	Di-	8%	-
Free astaxanthin		2%	100%

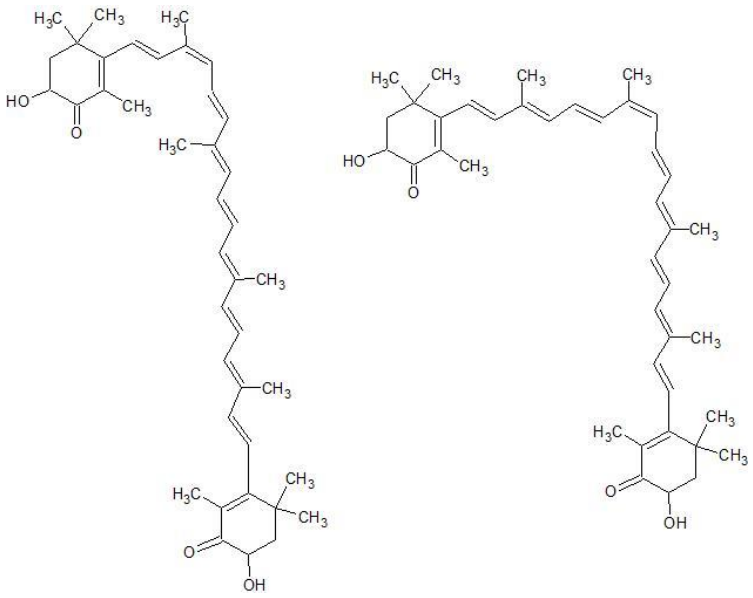

Figure 11 From left to right: *9-cis* and *13-cis* astaxanthin.

1.3.3 ASTAXANTHIN PROPERTIES AND MARKET

Astaxanthin is a high-value compound which is very attractive for important industrial markets thanks to its interesting properties, such as food grade, colouring and antioxidant agent.

Astaxanthin was originally isolated from a lobster by Kuhn and Sorensen[43] and it was first commercially used for pigmentation in the aquaculture industry. In nature, it is mainly synthesized by aquatic and non-aquatic microorganisms such as bacteria, fungi, yeasts and microalgae, and it accumulates in marine fauna (zooplankton, crustaceans and fish). Source of natural astaxanthin are the waste processing of crustaceans, krill, and the *Phaffia rhodozyma* yeast, but astaxanthin concentration is extremely low (0.05% - 0.40%) if compared with *H. pluvialis* (1.5 - 5%).

The most important commercial application is the use of astaxanthin in animal feed as pigmenting agent, in particular for aquaculture and poultry.[24] Food and Drugs Administration in 2009 officially approved astaxanthin as additive in feed for colouring body tissues (for example trout, salmon and crustaceans) or animal products (like eggs). European Food Safety Authority (EFSA) as well considers animal feed colouring astaxanthin for human consumption.[24] Astaxanthin products are used for food and nutraceutical industry, found in the form of tablets, capsules, soft gels, energy drink, syrups, oils, creams, and granulated powders.[37] Some astaxanthin products are a combination of other carotenoids, multivitamins, herbal extracts and omega-3, 6 fatty acids.[37] The current market is dominated by synthetic astaxanthin (95%)[6], with a total market value above $240 million per year.[44,45] The market price of synthetic astaxanthin is about 2000 $/kg, while the price of the natural astaxanthin derived from *H. pluvialis* is strongly fluctuating (it depends on a lot of factors, such as

H. pluvialis origin, location of the production and astaxanthin purity) and varies between 2500-7000 $/kg.[46] However, the interest in natural astaxanthin is currently increasing for human consumption, and this phenomenon has promoted large-scale cultivation of microalgae. Synthetic astaxanthin cost of production is about 1000 $/kg, while the natural one is estimated to be above 3000 $/kg.[12,45] The latter is more expensive because of technological problems associated with large-scale microalgae cultivation. As a matter of fact, there are some problems related to the production of astaxanthin from *H. pluvialis*: difficulties in avoiding contamination by micro-organisms on a commercial scale, slow growth of biomass, inadequate and economically unviable cultivation and expensive extraction technologies for astaxanthin.[6]

However, astaxanthin derived from *Haematococcus pluvialis* showed improvements in skin wrinkle and in skin texture; for this reason, in cosmetics industry there are several astaxanthin-based skin-care products. For example, it has been reported that in 2009 Fujifilm Corporation launched an astaxanthin-based product, "Astalift Whitening Essence" to combat aging-related skin spots.[2] Also the Japanese cosmetic giant Kose has marketed astaxanthin-based product "AstaBlanc" for combating wrinkles and spots.[2] Again, AstaReal AB (Sweden) has marketed "AstaReal Natural Astaxanthin", which is claimed to revitalize photodamaged skin, remove wrinkle, and enhance the elasticity of the skin.[2]

Because of its chemical structure, astaxanthin has a higher antioxidant activity than other carotenoids[10] and it has a lot of health-promoting properties:

- antioxidant activity;
- anticancer activity;
- immune boosting activity;
- anti-inflammatory activity;
- neuroprotective activity;
- derma protective activity;
- photoprotective activity.

Astaxanthin is a potent biological antioxidant. It can absorb the excited energy of singlet oxygen onto its chain by preventing other molecules or tissue from damage.[2] In vitro and in vivo assays have shown enhancement by astaxanthin of antibody production by mouse spleen cells[47] and of partially restoration of decreased humoral immune responses in old mice.[48] Studies on human blood cells, have also demonstrated enhancement by astaxanthin of immunoglobulin production.[49] Furthermore, astaxanthin has an anti-inflammatory activity: studies have shown the efficiency of astaxanthin in reducing the induced swelling of rat paw[50] and in decreasing symptoms of gastric inflammation.[51] Moreover some researches have demonstrated astaxanthin ability to downregulate

increased nuclear factor kappa B activity,[52] which is the responsible for cell proliferation and cancer. Finally, it has been reported that astaxanthin helped to protect the retinal photoreceptors in rats exposed to acute UV-light injury.[53]Astaxanthin does not show any significant side effects. However, recommended daily intake of astaxanthin varies within a small range:

- 6 mg/day in Japan;
- 5 mg/day in United States;
- 4 mg/day in Europe.[24]

Some studies demonstrate that astaxanthin long-term repeated dose exposure (1,5 and 3 months) does not lead to bioaccumulation in humans. Several human clinical studies confirm the safety of astaxanthin, even at doses well above regulatory levels. Recent studies state that doses of astaxanthin up to 40 mg/day for 8 weeks or 30 mg/day for 1 year are tolerable to the human body.[24]

1.3.4 COMPARISON BETWEEN NATURAL ASTAXANTHIN AND OTHER CAROTENOIDS

Carotenoids are synthesized by plants and microorganisms but not by animals. Fruits and vegetables constitute the major sources of carotenoids in human diet: yellow-orange vegetables contain β-carotene and α-carotene, dark green vegetables contain lutein, and tomatoes products contain lycopene (Table 5).[54]

Table 5 Food sources of the main carotenoids in North American diet. Image adapted from: *Carotenoids and human health, A.V. Rao and L.G. Rao, 2007.*

Carotenoid	Food source	Amount
β-Carotene	Apricot, dried	17600
	Carrots, cooked	9771
	Spinach, cooked	5300
	Green Collard	5400
	Canteloupe	3000
	Beet Green	2560
	Broccoli, cooked	1300
	Tomato, raw	520
α-Carotene	Carrots, cooked	3723
Lycopene	Tomatoes, raw	3100
	Tomato juice	10000
	Tomato paste	36500
	Tomato ketchup	12390
	Tomato sauce	13060
Lutein	Spinach, cooked	12475
	Green collard	16300
	Beet, green	7700
	Broccoli, cooked	1839
	Green peas, cooked	1690

Unlike the carotenoids mentioned above, natural astaxanthin derives from marine sources, such as fishing wastes (Figure 12) and microalgae, in particular *Haematococcus pluvialis*.

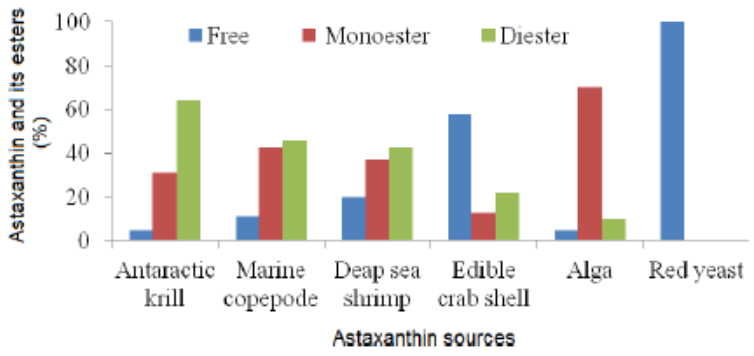

Figure 12 Astaxanthin marine sources.

These sources are more sustainable than terrestrial plants cultivation. Microalgae cultivation advantages are:

- lack of competition with food sources;
- avoidance of "land use";
- possibility of cultivation in marginal lands and extreme areas;
- avoidance of seasonality and extreme climate events;
- higher photosynthetic efficiency and, consequently, faster growth;
- easier manipulation to modify microalgal chemical composition;
- possibility of coupling with other industrial activity (wastewater, CO_2 capture).

Natural astaxanthin is considered the strongest antioxidant among carotenoids. Astaxanthin advantages compared to the other carotenoids are:

- crossing the blood-brain barrier by allowing antioxidant and anti-inflammatory protection to the brain and to the central nervous system;
- crossing the blood-retinal barrier by allowing antioxidant and anti-inflammatory protection to the eyes;
- reaching all the organs, skin and muscles (most carotenoids accumulate mainly in the liver);
- working as a super-powerful antioxidant and quickly eliminating free radicals.[55]

Astaxanthin and β-carotene chemical structures are very similar. β-carotene is the main precursor of vitamin A and it is an important compound for human health. However, β-carotene antioxidant activity is less strong than astaxanthin. Lutein seems to enhance eyes health.

As mentioned above (section 1.3.3), the main property of astaxanthin is to be a powerful antioxidant that has positive effects on cancer, diabetes, the immune system, and ocular health.[56] As a matter of fact, its chemical structure allows to quench free radicals (ROS) thanks to its numerous conjugated double bonds. Antioxidant treatment can alleviate some of the detrimental effects of oxidative stress.[56] *A. M. Wolf et al. (2010)* study shows that astaxanthin is able to protect rat adrenal

pheochromocytoma (PC12) cells against oxidative stress, and it stimulates respiration by maintaining a higher membrane potential.[56]

Environmental conditions of extreme heat induce mitochondrial fragmentation and ROS production.[57] Excessive amount of ROS contributes to heat-related skeletal muscle injury. Astaxanthin preserves mitochondrial function by accumulating into the mitochondrial membrane and it donates electrons to free radicals to reduce them into more stable forms.[37]

Another particularity of astaxanthin is given by its linear, polar-nonpolar-polar molecular structure, which allows it to insert into the membrane and to span the entire membrane (Figure 13). In this position, astaxanthin can intercept reactive molecular species within the membrane's hydrophobic interior and along its hydrophilic boundaries.[58]

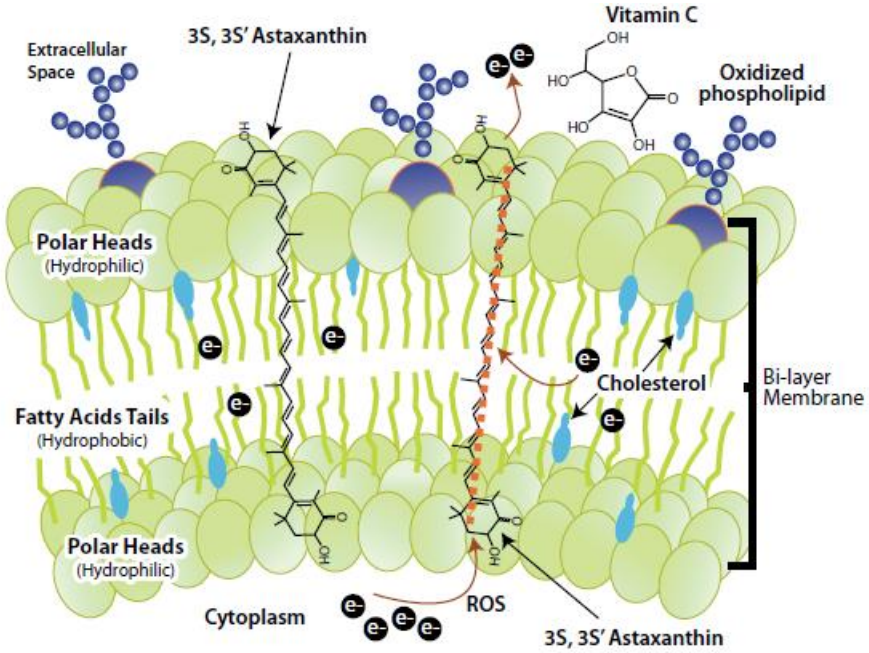

Figure 13 Astaxanthin localization into the membrane. Source: *Astaxanthin, Cell Membrane Nutrient with Diverse Clinical Benefits and Anti-Aging Potential, P. Kidd, 2011.*

W. Aoi et al. (2017) study states that esterified astaxanthin antioxidant activity is higher than that of the non-esterified form, since esterified astaxanthin is easier assimilable. It has been reported that its antioxidant activity is 10 times higher if compared with other carotenoids, such as β-carotene, canthaxantin, zeaxanthin and lutein;[10] 65 times higher than vitamin C; 100 times higher than α-tocopherol.[6] The *Creighton University* study shows that natural astaxanthin is the best free radical scavenger and that it is 20.9 times stronger than synthetic astaxanthin. Astaxanthin ability to cross the blood-brain barrier allows to prevent eyes injuries (macular degeneration, retinal arterial and venous occlusion, glaucoma) better than lutein.

Whereas some carotenoids, such as β-carotene, lycopene, zeaxanthin, can become pro-oxidants under certain conditions by causing cell disfunction, astaxanthin never becomes pro-oxidant.[59]

Related to its antioxidant properties, astaxanthin protects the skin from sunlight by preventing its photoaging: wrinkles, freckles, age spots and skin staining (it was found to decrease melanin production by 40%).[55] Some studies state that astaxanthin protection against UVA light-induced oxidative stress is superior if compared to lutein and β-carotene.[55] During physical activity, astaxanthin was demonstrated to increase strength and endurance. It is in fact able to scavenge free radicals that form in muscles. Another effect observed *in vivo* demonstrates that astaxanthin supplementation significantly reduces fat accumulation and lactic acid levels. Furthermore, during mice physical activity, astaxanthin promotes AMPK gene activation that allows mitochondrial biogenesis and inhibits fatty acids synthesis by reducing ATP consumption.[60]

Astaxanthin is also a very effective anti-inflammatory because it works to suppress different inflammatory mediators. Related to its anti-inflammatory properties, natural astaxanthin is considered excellent against tendons inflammation, rheumatoid arthritis, ocular inflammation, muscular inflammation during physical activity.

Moreover, natural astaxanthin helps to prevent heart disease, such as heart attack or stroke. This happens because astaxanthin improves blood flow capacity by decreasing low density lipoprotein (LDL, bad cholesterol) and triglycerides, and by increasing high density lipoprotein (HDL, good cholesterol). A human clinical trial was done on men with high cholesterol: subjects were supplemented with 4 mg of astaxanthin for thirty days. At the end of the study, subjects taking astaxanthin showed an average decrease in total cholesterol and of LDL of 17%, and an average decrease of triglycerides of 24%.[55]

1.3.5 REACTIVE OXYGEN SPECIES AND MITOCHONDRIAL RESPIRATION

Reactive oxygen species (ROS) production depends on normal metabolic activity, lifestyle factors (smoking, exercise, diet) and environmental pollution (chemicals, car exhaust, smoke, sunlight exposure).

The mitochondrial electron transport chain is one of the major cellular generators of ROS due to electrons leak in the electron transport chain, especially in complex I and III.[61] ROS include superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl free radical ($\cdot OH$).[62–64] ROS are highly reactive, and their presence is believed to be important in the aging process: in the pathogenesis of neurodegenerative diseases,[65–67] in other pathologies such as cancer, diabetes, atherosclerosis, stroke, arthrosis, and also skin aging (wrinkles, dry skin, skin cancer).[68] However, low levels of ROS are essential for normal cell physiological functions (proliferation, differentiation, migration and cell death). Normally, ROS production is in equilibrium with

antioxidant defences and this contributes to keep in check the ROS levels [61] Oxidative stress is defined as "a serious imbalance between the generation of ROS and antioxidant defences in favour of ROS, causing excessive oxidative damage".[69] Excess cellular levels of ROS cause damage to cellular biomolecules (proteins, nucleic acids, lipids, membranes and organelles), which can lead to activation of cell death processes such as apoptosis.[68]

Mitochondria play important roles in cellular energy metabolism. Moreover, they are implicated in cell death and in several human diseases. Mitochondria are described as the "powerhouses" of the cells because of their crucial role in the conversion process of the energy released from nutrients oxidation into the biological energy "currencies" of the cells: the ATP molecule. Mitochondria achieves this goal transferring electrons from reduced coenzymes (NADH and $FADH_2$) to molecular oxygen through the electron transport chain located in the inner mitochondrial membrane. Part of the free energy released from the electrons transfer through the complexes of the electron transport chain is used to create a transmembrane proton flow from the mitochondrial matrix to the intermembrane space. In particular, three of the four enzyme complexes involved in the electron transfer are proton pumps and they are complexes: I (or NADH-coenzyme Q oxidoreductase), III (ubiquinol-Cyt c oxidoreductase or bc1 complex) and IV (Cyt c oxidase). Protons flow create a transmembrane electrochemical gradient that constitutes what Mitchell called the 'proton-motive force. The force that drives the ATP synthesis through ATP synthase. Protons passage activates the turning of parts of this molecular machine allowing the addition of a phosphate to ADP.[70] Mitochondrial oxidative phosphorylation process is depicted in Figure 14.

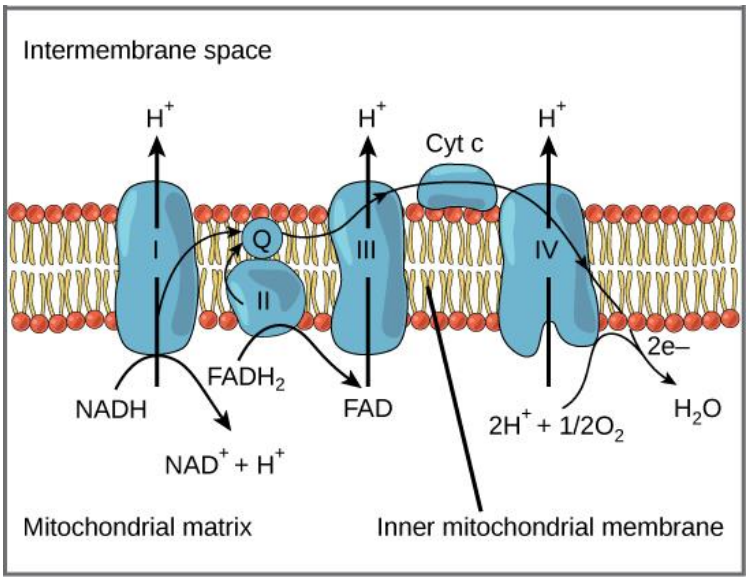

Figure 14 Electron transport chain. Source: *Biology (Chapter 7,4), 2015.*

1.4 ASTAXANTHIN EXTRACTION

Due to its lipophilic nature, astaxanthin can be recovered with apolar solvents. Several pre-treatments are necessary to extract astaxanthin from *H. pluvialis*, because of the thick cell wall of encysted cells. Since thick cell wall is characterized by an extraordinary resistance against mechanical and chemical attack, astaxanthin extraction process requires expensive techniques.[23] Generally pre-treatments are: centrifugation to concentrate algal biomass, washing with water and cells rupture.

The main treatments used in industry are listed below:

- Microwaves (75 °C for 5 minutes) and subsequent extraction with acetone.[71,72] This is a fast and effective extraction technique (75% extraction efficiency). Disadvantages are the possible degradation of macromolecules and the death of algal culture.
- Autoclave (30 min, 121°C, 1 atm), or mechanical cells disruption (with a cell homogeniser) followed by centrifugation, addition of ethanol, diethyl ether and n-hexane, drying with nitrogen, and extraction with acetone. These techniques are efficient with rates higher than 85%.[73] The main disadvantages are a large utilization of toxic solvents and the death of algal cells.
- Acid treatment with HCl (0.1 M), followed by centrifugation, addition of toxic solvents and extraction with acetone. The main disadvantage of HCl treatment is the significant loss of some unstable carotenoids (including astaxanthin) due to 3-hydroxy, 4-keto end-group.[73] However, other researches show that astaxanthin was recovered with hydrochloric acid and acetone respectively at the 80% and 70%.[37]
- Vegetable oils (soybean oil, corn oil, olive oil and grapeseed oil) with vigorous stirring of the culture. Olive oil is considered the best oil for its ability to extract 93% of astaxanthin.[11] This process is sustainable thanks to process simplicity and to the use of bio-solvents, by excluding cell harvest procedure and the gravitational sedimentary separation of astaxanthin extract.
- Pressurized fluid extraction (PFE) utilizes conventional solvents at controlled temperatures and pressures. The main difference from other techniques is that PFE uses less amount of solvent in a shorter period. Moreover, it allows the sample conservation by avoiding contact with light and oxygen. *Denery et al. (2003)* study[II] states that the best solvent for total astaxanthin extraction with PFE method (1500 psi, 40°C, 2–5 min) is a mixture of methylene chloride and methanol in a 1:3 molar ratio (1.09 % of astaxanthin recovery).[38]

[II] *Denery et al. (2003)* study focused on the extraction of carotenoids from *H. pluvialis*, *Dunaliella salina*, and *Piper methysticum.*

Carotenoids recovery rate is comparable to traditional extraction methods, but with lower environmental impact.

- Supercritical CO_2 ($scCO_2$) is considered the most efficient and widely used method, because it requires a low extraction time, is relatively cheap, and non-toxic.[6] This process has a low environmental impact, because it entails no residue of harmful solvents, and have easy separation from extracts.[74] Since supercritical CO_2 has special physicochemical properties between those of a liquid and a gas (high compressibility, low viscosity, and low surface tension), it is able to diffuse easily through the natural solid matrix by increasing carotenoids extraction, if compared to the conventional liquid solvents. Carotenoids thermal degradation is avoided because CO_2 has a low critical temperature and pressure (31.1°C and 1084.86 psi). To enhance the extraction *H. pluvialis* culture must be pre-treated, such as mechanical disruption, acid treatment, or enzymatic treatment.[75] By coupling $scCO_2$ extraction with vegetable oils, extraction efficiency increases. In particular olive oil extraction power is comparable to the co-solvent ethanol.[76] The main disadvantages of $scCO_2$ extraction is algal cells death.
- Enzymatic treatment consists of a mixture of 0.1% protease K and 0.5% driselase in a phosphate buffer that was added to the sample for 1 hour (pH 5.8, 30°C).

Traditional solvent extraction techniques are widely applied in the industry because they are fast and efficient. However, they utilize large quantities of organic solvents, are labor-intensive, and can expose the extracts to excessive heat, light and oxygen, promoting the potential isomerization and oxidation of labile compounds.

1.4.1 INNOVATIVE EXTRACTION: THE MILKING TEST

The main problem of cultivation of some algal species is their low productivity. As a matter of fact, secondary metabolites are produced when algal growth is limited. For this reason, a critical issue in developing algal biotechnology is the optimization of culture parameters.[77–79] Traditional industrial treatments (downstream process) generally involve dewatering, drying, grinding, extraction, and purification steps.[79] Drying and grinding require energy inputs and kill the microalgal biomass that then needs to be repeatedly regrown. Moreover, extraction and purification processes generate organic wastes by increasing production costs. It is estimated that downstream operation costs usually represent 50%-80% of the total processing expense, besides being unsustainable for the environment.[5]

Cultures in which cell mass is reused for continuous production can be a solution to overcome these problems. The innovative milking test strength is keeping algal cells alive during carotenoids extraction process; microalgae can thus be re-cultivated and more extractions can take place (Figure 15).

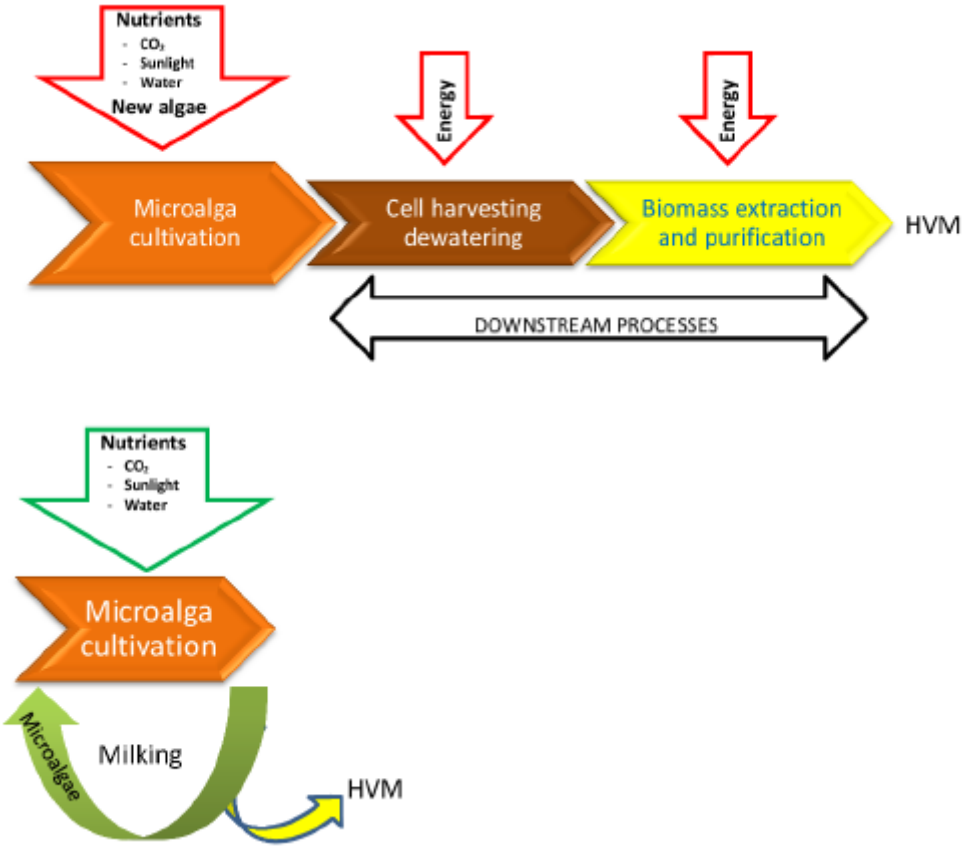

Figure 15 Comparison between traditional (downstream process) and innovative (milking) processes for producing high-value molecules (HVM). Downstream procedure is a linear process which require starting new microalgal cultures after each extraction; while milking test is a circular process in which the same microalgal culture is re-cultivated after extraction. Source: *Diatom Milking: A Review and New Approaches, V. Vinayak et al., 2015.*

The milking test idea can be explained by the image of milking cows, where cows are continuously "reused" for milk production.[80] This principle can be applied to various high-value compounds, such as secondary metabolites derived from microalgae (e.g. β-carotene from *Dunaliella salina*, hydrocarbons from *Botryococcus braunii*), which are not necessary for algal survival.

The milking test is based on Liquid-Liquid Extraction (LLE) with hydrophobic solvents. LLE principle is the formation of a multiphase system of two non-miscible liquid phases (Figure 16): hydrophobic solvent enriched with high-value compounds, and culture medium.

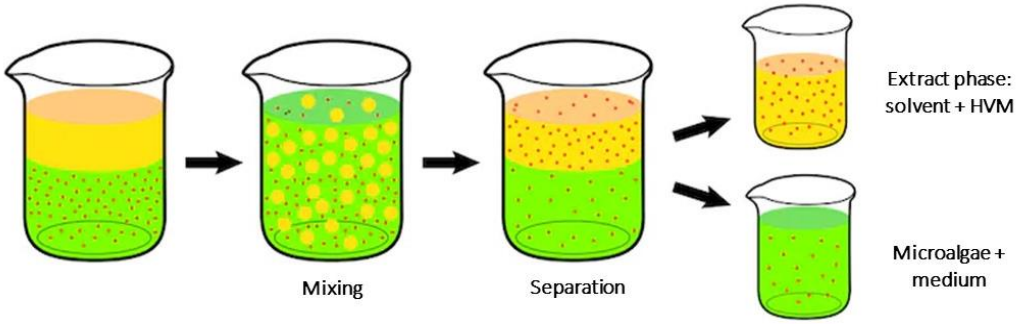

Figure 16 Liquid-Liquid Extraction diagram: solvent addition to algal culture, mixing, separation through centrifugation. An extract phase consisting of solvent and high-value compounds (HVM), and a raffinate phase with algal culture and medium are obtained. Source: image adapted from *https://www.shutterstock.com/it/image-illustration/schema-liquidliquid-extraction-beaker-510367069*.

LLE is considered an economical and environmentally friendly technique.[81] As a matter of fact the use of LLE reduces energy consumption and avoids volatile solvent discharge to the environment.

Good solvent candidates for astaxanthin extraction are lipophilic solvents with chemical properties compatible with secondary metabolites and with the morphological cell structure, in order to preserve metabolites integrity and productivity. Moreover, the milking test avoids pre-treatment processes, such as the harvesting and dewatering steps, that have a large impact on energy consumption and economics of the process.[82]

There are few examples of milking tests reported in literature. The concept of milking was introduced by Frenz and collaborators in 1989 to describe the extraction of hydrocarbons from the green algae *Botryococcus braunii*, through a short time (10 minutes) recovery with non-polar solvents like hexane.[83] The second extraction on the same culture was repeated after 10 days, and it led to increase in both biomass density and hydrocarbon contents. However, the recovery yield depends on the algal culture physiological state, and on cell density. As a matter of fact, if cell density is high the recovery yield decreases because cells tend to aggregate, by impeding the contact with hexane. Another study concerns the extraction of β-carotene from *Dunaliella salina*: a biocompatible organic phase circulated in a two-phase (an aqueous and an organic phase) bioreactor at different circulation rate (150 and 200 mL/min). β-carotene extraction rate increased significantly at the higher recirculation rate, without decreasing cell viability.[84] *Racheva R. et al. (2018)* study tests the use of a biocompatible nonionic surfactants, Triton X-114, to extract lipids from the green microalgae *Acutodesmus obliquus*.[85] The optimal extraction yield was 39,3% with a cell viability yield of 80%. Cell viability did not change significantly over the extraction time. Pulsed electric field (PEF) is another technology useful for milking test. PEF advantages are high extraction yield, low energy consumption, large-scale process. It can be applied to yeast, microalgae and

cyanobacteria. In this method cell membranes are punctured by electric pulses, allowing the cell components to ooze out.[5] The study of *Praveenkumar et al. (2015)* is one of the few researches about milking of astaxanthin from *H. pluvialis*.[86] It consists in an advanced single crystalline gold nanoscalpel (Au-NS, with a thickness under 300 nm and a width of 1-3 µm) to incise *H. pluvialis* cells allowing astaxanthin coming out, maintaining the integrity of the membrane. After the first extraction, astaxanthin accumulation in incised cells was twice that of the untreated cells. *Samorì et al. (2019)* research aims to explore biocompatible hydrophobic solvents as alternative to traditional hydrocarbons for astaxanthin extraction from *H. pluvialis* through milking test.[82] Hydrophobic solvents tested without any pre-treatment were butanol, cyclohexane, ethyl acetate (EtOAc), dimethyl carbonate (DMC) and diethyl carbonate (DEC), methyl isobutyl ketone (MIBK), 2-methyltetrahydrofuran (2-MeTHF), 3-methylbutyl acetate (isoamyl acetate) and almond oil. The different solvents and algal culture were tested in a 1:3 volume ratio for 5, 10, 30 minutes of contact and 48 hours for almond oil. The most efficient extraction time was of 30 minutes, and the best solvent was 2-MeTHF (>80%), but cell viability was null. Almond oil, after 48 hours, was able to recover only 10% of total astaxanthin. However, almond oil is totally algae-compatible because cell viability after 48 hours of extraction was 85%. After the extraction with almond oil, *H. pluvialis* was re-cultivated with fresh nutrients and optimal environmental conditions and, after 26 days, a second extraction was done. Although astaxanthin total content was not changed, recovery was 2-3 times higher. This was probably due to cell wall modifications caused by the first extraction. Substantially, to obtain higher yields, a complete transition cycle of vegetative-red phase should be induced before the extraction, by obtaining young red cysts. In addition to almond oil, other vegetable oils (corn, soybean, olive, karate, sunflower and soybean mixed, exhausted oil) were tested in *M. Semeraro's* thesis.[87] After 30 minutes of extraction cell viability was high but astaxanthin extraction was about 1%. The results are similar to astaxanthin recovery with almond oil after the same extraction time.

1.5 DEEP EUTECTIC SOLVENTS

Developing new green solvents is one of the key subjects in Green Chemistry[88] that aims at reducing the use of hazardous media by offering new environmentally acceptable techniques. [89]

Deep eutectic solvents (DESs) are among the most promising solvents of the last decades.

DES are generally composed by two or three cheap and safe components which are capable of combining each other, through hydrogen bond interactions, to form an eutectic mixture.[90] The combination of hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD) mixed at a

specific molar ratio, results in a DES characterized by a melting point lower than that of each individual component, mainly due to the generation of intermolecular hydrogen bonds.[91] This is the reason why some solid chemicals can become liquid by mixing in certain ratios.[88]
The creating DES was demonstrated for mixtures of quaternary ammonium salts[92] with a range of amides and carboxylic acids,[93] and later extended to choline chloride with alcohols,[94] and urea with sugars or organic acids.[95,96] DESs are easier to prepare with high purity at low cost:[88] in fact their preparation is based on just mixing and, if necessary, heating; the absence of purification steps is a real advantage from a synthetic and also an economic point of view.[97]

The application of DES has been so far limited to organic reactions,[95,96,98] organic extractions,[99] electrochemistry,[100–102] and enzyme reactions.[94] In order to increase the number of candidates for DESs and extend their applications, attention has been directed towards natural products such as organic acids,[93,96,103] amino acids,[104] sugars,[95,105] choline,[92,94] or urea,[92,95,96] to develop the so called Natural Deep Eutectic Solvents (NaDES). NaDESs fully match green chemistry principles,[106] and they represent an excellent opportunity as a new generation of green solvents.[106] NaDESs advantages are sustainability (not derived from petroleum), cheap and easy preparation, biodegradability and high purity.[88,90,106] NaDESs have been extensively applied as green solvents for the extraction of many bioactive compounds, such as secondary metabolites.[88] However, most of these solvents are hydrophilic and research into hydrophobic eutectic mixtures for the extraction of non-polar bioactive compounds is much less explored.[107]
One advantage of hydrophobic DESs is their water immiscibility, which allows their use in extractions and separations of compounds from aqueous phases.[97] The main areas of application of hydrophobic DESs are extraction of natural components from plants, CO_2 capture, organic synthesis, and water purification.[97]

1.5.1 TERPENES-BASED NADES

Terpenes are compounds which are widely diffused in nature, they constitute resins and essential vegetable oils. Their structure is based on repetition of isoprene units and they are characterized by a strong characteristic smell, they are volatile and insoluble in water. Chemical formula of terpenes is $(C_5H_8)_n$ where n is the number of isoprene units. Because of their very low solubility in water and relatively low price,[108] terpenes appear as good candidates to prepare sustainable and cheap hydrophobic solvents.[109] The terpenes used in this work are:

- menthol;
- thymol;

- geraniol;
- α-bisabolol.

Terpenes chemical structures are depicted in Figure 17.

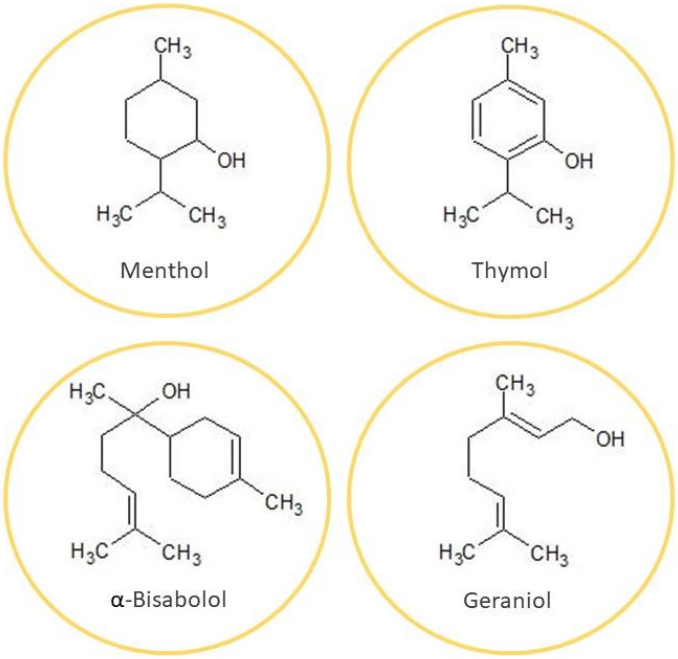

Figure 17 Chemical structure of menthol, thymol, α-bisabolol, geraniol.

Menthol and thymol are monoterpenoids used in various industrial processes and commercial products,[110] for example in the pharmaceutical field, where mixtures of thymol and menthol with active ingredients have been investigated as analgesic, antimicrobial, and anti-inflammatory vehicles.[109] Menthol is also used in toothpastes, shampoos, soaps and as ingredient in food, like candies, chewing gum, liqueurs.[111] Menthol is a white flaky crystalline substance, solid at room temperature. It has a peppermint smell and taste. It can be extracted from *Mentha* species, it is abundant, and cheap (~60 €/kg).[112]

Geraniol is a plant-based insect repellent and it exhibits various biochemical and pharmacological properties, such as antimicrobial properties.[113] It is a pale-yellow oily liquid which occurs in small quantities in geranium, lemon, and many other essential oils and it is the primary part of rose oil, palmarosa oil, and citronella oil.[114] Some studies show that both menthol and geraniol have anticancer and chemopreventive proprieties,[111] in particular geraniol is effective for the prevention of colon and pancreatic cancer.[113] Thymol is a translucent crystalline substance and it is a constituent of oil of thyme, a naturally occurring mixture of compounds in the thyme plant (*Thymus vulgaris*). It can be found in lime honey, cooking herbs, and oils of cranberry and mandarin orange. Thymol has antifungal properties and it is also a strong antioxidant, thanks to its potent free radical

scavenging activity.[115] α-bisabolol has antiinflammatory and skin-soothing properties: it can be found in creams, lotions, cleansers, sunscreens because it is able to strengthen the barrier function of the skin in order to prevent allergic and irritant skin reactions or repair the skin.[116]

Some physical, chemical and toxicological properties of terpenes mentioned above are reported in Table 6.

Table 6 Physical, chemical and toxicological properties of menthol, geraniol, alpha-bisabolol, thymol.

Terpene	Molecular Formula	Molecular Weight [g/mol]	Melting point [°C]	Water solubility [mg/L]	Toxicity to aquatic algae and cyanobacteria (EC50) [mmol/L]
Menthol	$C_{10}H_{20}O$	156.26	39	456 at 25°C	$1.04 \cdot 10^{-1}$
Geraniol	$C_{10}H_{18}O$	154.25	-15	100 at 25°C	$5.32 \cdot 10^{-3}$
α-Bisabolol	$C_{15}H_{26}O$	222.37	25	22 at 20°C	$3.42 \cdot 10^{-3}$
Thymol	$C_{10}H_{14}O$	150.22	51.5	800-980 at 20-25°C	$5.13 \cdot 10^{-2}$

The toxicity of the terpenes mentioned above is reported in literature as EC50 for algae and cyanobacteria. Toxicity is higher for terpenes with linear chemical structure (geraniol is the most toxic) and lower for cyclic terpenes (thymol and menthol). Since bisabolol is a mix between the two structures, it has an intermediate toxicity.

For their properties, menthol, geraniol, α-bisabolol and thymol seem good candidates to prepare sustainable, cheap and hydrophobic solvents using the formation of eutectic mixtures approach, with the aim of keeping algal cells alive during the extraction of lipophilic metabolites process.

Menthol, thymol and geraniol are considered safe substances for human use and they are listed by the U.S. Food and Drug Administration (FDA) as food additives for human consumption.

1.5.2 HYDROPHOBIC DESS

Hydrophobic DESs tested in this thesis are combination of:

- phenylacetic acid (PhAA) and thymol as HBD;
- trimethylglycine (TMG) and didecyldimethylammonium chloride (DDDACl) as HBA.

PhAA is an active auxin (a type of plant hormone) and it is a white/yellow crystal. It is found in some flowers, fruits and other plants, fermented drinks, tea and cocoa. It has a floral odor like geranium or rose and it can also be sweet smelling like honey when diluted. Phenylacetic acid is used in the manufacture of penicillin, other chemicals and perfumes.[114] TMG is an amino acid whose main property is to reduce cardiovascular diseases. As a matter of fact, it is a methylating agent that reduces plasma homocysteine (responsible for cardiovascular diseases). TMG is usually found in wheat bran, spinach, barley bran, shrimp, wheat bread.[117] DDDACl is a synthetic compound used as an antimicrobial and biocide. It is a light-yellow cream at room temperature. It causes cell membrane damage and it induces oxidative stress in lung epithelial cells.[118] Thymol, PhAA, TMG, DDDACl main physical and chemical properties are shown in Table 7. TMG can be considered highly soluble, while thymol, PhAA and DDDACl have low water solubility.[119]

Table 7 Physical and chemical properties of hydrophobic DESs tested in this work.

Chemical	Molecular Formula	Molecular Weight [g/mol]	Melting point [°C]	Water solubility [mg/L] at 25°C	Toxicity to aquatic algae and cyanobacteria (EC50) [mmol/L][120]
Thymol	$C_{10}H_{14}O$	150.22	51.5	$8.56 \cdot 10^{2}$	$5.13 \cdot 10^{-2}$
PhAA	$C_8H_8O_2$	136.15	76.5	$1.77 \cdot 10^{4}$	1.30
TMG	$C_5H_{11}NO_2$	117.15	94-100	$6.12 \cdot 10^{5}$	$1.02 \cdot 10^{1}$
DDDACl	$C_{22}H_{48}ClN$	362.1	293	$4.53 \cdot 10^{3}$	$1.71 \cdot 10^{-4}$

2 PURPOSE OF RESEARCH

Nowadays, several research groups are working on the use of green solvents for the algae extraction process.[36] The main purpose of this thesis is to find new green solvents which allow to achieve a high astaxanthin recovery rate and, at the same time, to keep algal cells alive.

This work first aims to compare hydrophobic NaDESs composed by edible components, natural carboxylic acids and terpenes, through Liquid−Liquid Extraction.

Some screening tests were carried out in order to:

1. find the best NaDESs in terms of cell viability maintenance and of extraction efficiency;
2. set the optimal extraction time;
3. set the optimal algal culture concentration.

The second step of this work consists in choosing the best NaDES as candidate for the extraction of astaxanthin through the milking test, in comparison with a vegetable oil, almond oil. The latter is currently the best green solvent identified for the milking of astaxanthin from *H. pluvialis*, exploitable to obtain an extract enriched in astaxanthin that can be directly used in cosmetic applications.

The third part consists in finding the best stress factor that accelerates *H. pluvialis* stress phase achievement and that improves astaxanthin production. The best candidate seems to be the antioxidant ascorbic acid, which was added to algal culture in different amount to find the best concentration for *H. pluvialis*.

The last part is focused on the effects that astaxanthin induces in animal cells, and it considers the differences between synthetic and natural astaxanthin. In particular, the antioxidant activity of astaxanthin, the influence of astaxanthin on lipid metabolism and on mitochondrial integrity are tested on cancer cell lines (Hep G2).

3 MATERIALS AND METHODS

3.1 *HAEMATOCOCCUS PLUVIALIS* CULTIVATION

The microalga *H. pluvialis* (strain H5A) was isolated from a freshwater sample collected in Ravenna (Italy) in July 2014 and maintained in culture in the Laboratory of Algal Biology at UniBo. For the purposes of this thesis, its cultivation started in April 2019. Microalgae grew in 1 L bottles or flasks under controlled conditions in a modified BBM medium inside a growth chamber at 20 ± 1°C, under an artificial light intensity of 80 µE/m^2/s and a 16 h light:8 h dark cycle, with a slight insufflation of air.

3.1.1 PREPARATION OF THE GROWING MEDIUM AND INDUCTION TO THE STRESS PHASE

In this section the medium composition for *H. pluvialis* optimal growth is shown. The protocol followed for the medium preparation was developed in collaboration with the Company *Micoperi Blue Growth*. It is called UFM_HP medium (Table 8), and it is a modified BBM medium[121]. After autoclaving bottles of distilled water, the necessary nutrients for *H. pluvialis* optimal growth were added under a sterile hood, in order to avoid contamination.

Table 8 Composition of UFM_HP medium for *H. pluvialis* cultivation.

Stock	Nutrients	Stock [g/L]	UFM_HP [mL/L]
1	$NaHCO_3$	100	1.68
2	KNO_3	200	1.5
4	$NH_4H_2PO_4$	200	1
5	$CaCl_2 \cdot 2\ H_2O$	100	0.25
6	$MgSO_4 \cdot 7\ H_2O$	200	0.375
7	$FeC_6H_5O_7 \cdot 3\ H_2O$	54	0.1
8	H_3BO_3	11.42	1
9	Micro	*	0.05
10	Vitamins	**	0.05

	Nutrients	Concentration [mg/L]	Qty. [g/L]
Stock 9*	$MnCl_2 \cdot 4\ H_2O$	1.5	1.68
	$ZnSO_4 \cdot 7\ H_2O$	1	1.5
	$CuSO_4 \cdot 5\ H_2O$	0.025	1
	$Na_2MoO_4 \cdot 2\ H_2O$	0.12	0.25
	$CoSO_4 \cdot 6\ H_2O$	0.00755	0.375
	Na_2SeO_3	0.00779	0.1

	Nutrients	Concentration [µg/L]	Qty. [g/L]
Stock 10**	Biotin (B7)	25	0.5
	Thiamine (B1)	17.5	0.35
	Cobalamin (B12)	15	0.3

An aliquot of fresh medium was periodically added to the algal culture to provide new nutrients and maintain the green phase, after the removal of a same volume of algal culture. A cotton cap was used to allow air exchange.

To induce *H. pluvialis* to the stress phase (in order to improve astaxanthin production), algal culture was brought out of the growth chamber and exposed to continuous high light intensity at 500 µE/m^2/s. Growth medium was not renewed and a dilution (1:3) was applied using sterile distilled water, in order to promote nutrient deficiency, without air insufflation. In Figure 18 *H. pluvialis* transition from the green to the red phase is depicted. In green-motile phase *H. pluvialis* flagellated cells can swim, they are small and are characterized by bright green colour due to the abundance of chlorophyll. In the transition phase there is a mixture of motile and non-motile cells. Non-motile cells appear larger, with a red centre caused by astaxanthin accumulation. In the red phase *H.*

pluvialis cells become cysts: non-motile cells with a red colour due to the accumulation of astaxanthin. Cysts are large and cell wall is thick.

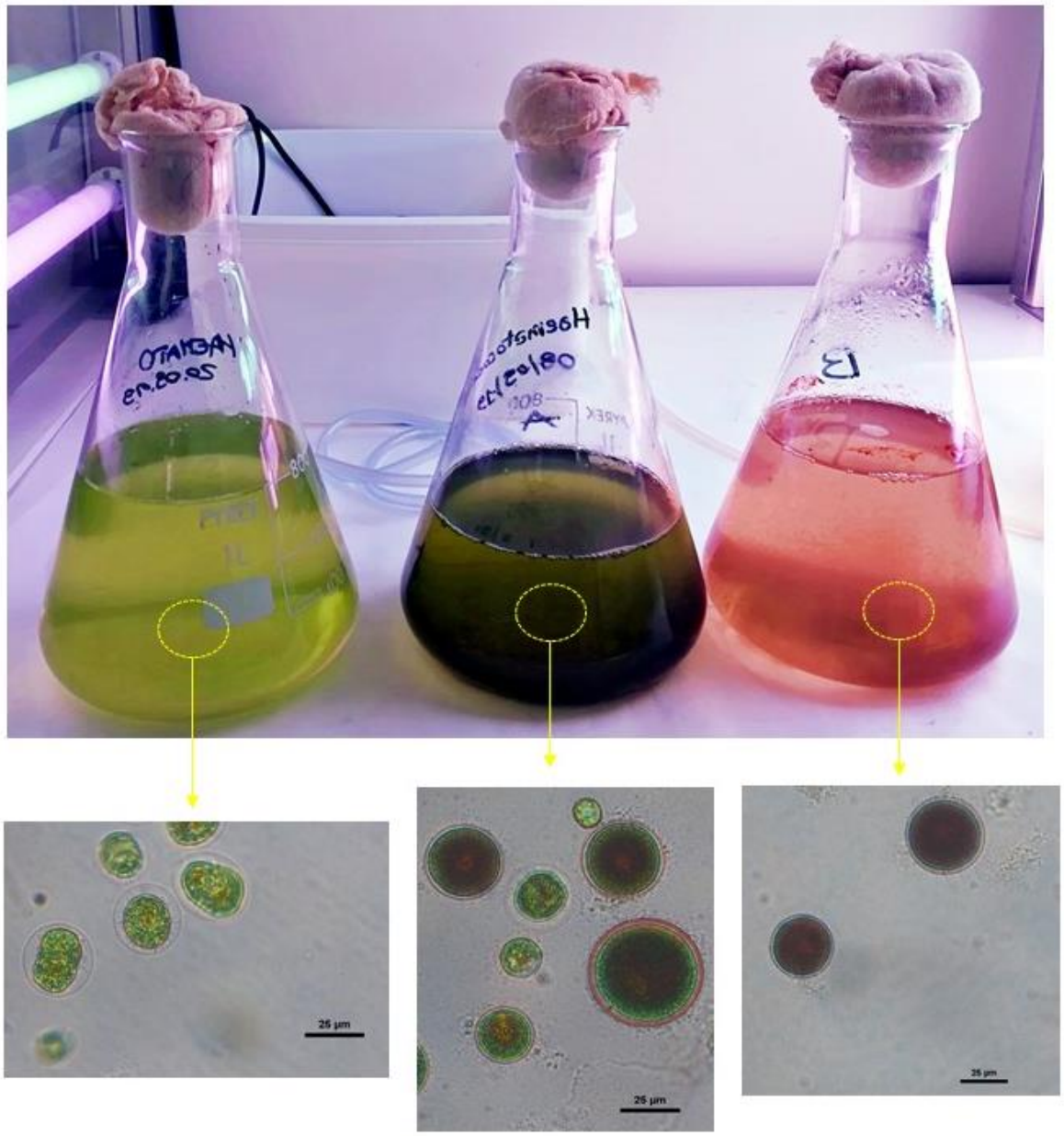

Figure 18 Three steps of *H. pluvialis* transition from green to red phase (from left to right): green motile-phase, the transition phase, red non-motile astaxanthin accumulated encysted phase. Scale bar: 25 µm.

3.2 IMPROVEMENT OF ASTAXANTHIN PRODUCTION

Different stress factors were tested in order to improve astaxanthin production and to accelerate stress phase achievement. The stress factors (in addition to nutrients starvation and high light, as mentioned before) were decrease of pH, increase of salinity and the addition of antioxidant ascorbic acid (like BHT, see section 1.2). Ascorbic acid (or vitamin C, Figure 19) is essential for the neutralization of free radicals; moreover it is used in food supplement for human immune defence. In the first test, two replicates for each stress condition using 200 mL of *H. pluvialis* culture in green motile phase were performed. Stress conditions were:

- pH = 6;
- 6 g/L of NaCl;
- 0.02 g/L of ascorbic acid.

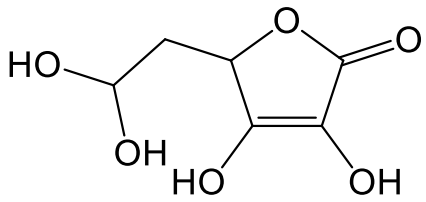

Figure 19 Ascorbic acid chemical structure.

Algal cell viability was monitored at day 2 and day 8 using the Pulse-Amplitude-Modulation (PAM) chlorophyll fluorimeter. At day 8 astaxanthin content was also spectrophotometrically analysed (Jasco V-650 spectrophotometer).

In the second test different concentrations of ascorbic acid were tested:

- 0.02 g/L;
- 0.05 g/L;
- 0.1 g/L;
- 0.2 g/L.

Both algal cell viability and astaxanthin content were analysed at day 7.

3.2.1 DRY WEIGHT ANALYSIS

Dry weight analysis of algal culture was done in order to know cell density and to monitor its growth. It consisted in collecting an aliquot of algal culture (3 or 5 mL) and filtrating it through glass microfiber filters (GF/C Whatman, diameter of 47 mm, porosity of 1.2 µm). Before the analysis, filters were washed with distilled water and dried in an oven at 105°C for at least 2 hours, wrapped in aluminium foil. To estimate filters tare with the analytical balance, they were placed in aluminium weighing vessels and placed inside a dryer with silica salts to avoid moistening them. The vacuum-seal filtration was done with a Büchner filter (Figure 20 A). After the filtration (Figure 20 B), filters were placed in the oven at 105°C in their aluminium weighing vessels for at least 1 hour. The final weight was measured. Net dry weight is expressed as:

$$\frac{\text{Algae plus filter weight [g]} - \text{Tare weight of filter[g]}}{\text{Filtered algal culture volume [L]}} \quad [1]$$

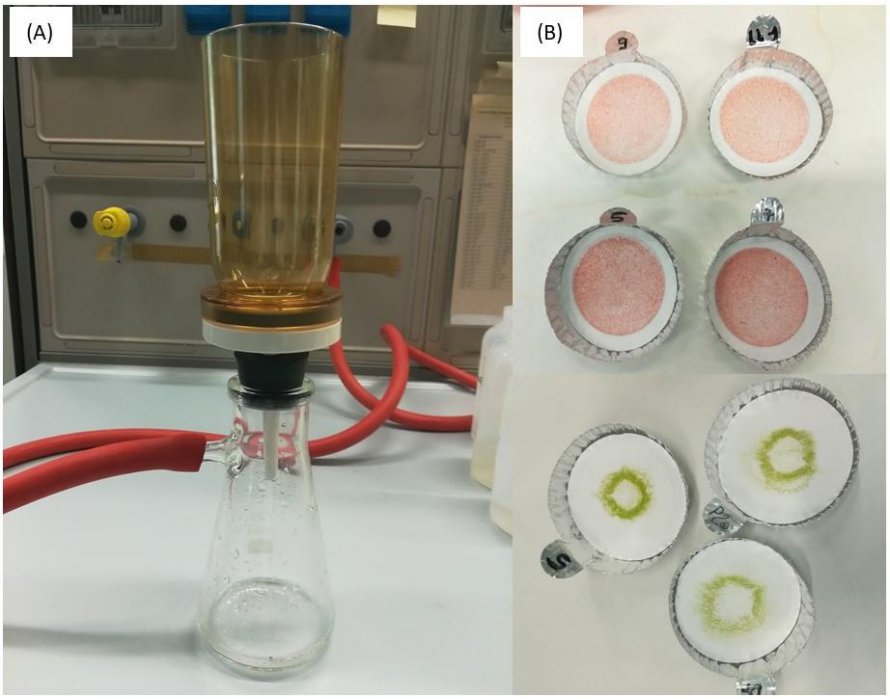

Figure 20 (A) Büchner filter used for dry weight analysis; (B) Filters with algal culture in their aluminium weighing vessels.

3.2.2 PHOTOSYNTHETIC EFFICIENCY ANALYSIS

H. pluvialis cells were analysed with an inverted light microscope (Zeiss Axiovert 135) at 320x magnification. Viability of *H. pluvialis* cells was analysed through photosynthetic efficiency measurement, with Pulse-Amplitude-Modulation (PAM) chlorophyll fluorimeter (101 with PDA-100, Walz, software WinControl). It is a fast, sensitive and non-invasive analysis.[122] PAM is a

suitable tool for estimating the viability of organisms capable of performing oxygen photosynthesis (which involves the chlorophyll A in the PSII photosystem). When an atom absorbs a photon, valence electrons enter in an excited state. After excitation, the atom returns to the ground state by emitting a photon with a characteristic energy, known as fluorescence.

In the absence of light, photosynthetic reaction centres are open and available for the absorption of a photon, therefore there is a decrease in fluorescence (quenching). The quencher (an electron-acceptor in the transport chain, called plastoquinone) oxidises and the fluorescence level becomes minimal (F_o). With a saturated light pulse (at least 800-1000 E/m^2/s for 0.6-0.8 s)[122], the reaction centres close and are no longer able to accept electrons. Fluorescence is emitted and photochemical quenching is completely suppressed. In this condition the plastoquinone is reduced and the fluorescence is maximum (F_m). The difference between F_m and F_o is the *maximum variable fluorescence* (F_v).

Constant actinic light (photosynthetically active and non-saturated) causes an immediate increase in fluorescence.[123] Actinic light leads to the closure of some reaction centres and fluorescence becomes constant (Ft). If actinic light is sufficiently intense, the quinones reduction rate is greater than its oxidation rate, so the fluorescence emitted is higher ($F_t > F_o$). With a saturating pulse, the fluorescence increases to the maximum value (F'_m) and then decays to F_t. Usually $F'_m < F_m$ because actinic light activates the mechanisms of energy dissipation, while $F_t > F_o$ since some reaction centres close with light.

There are two different types of quenching:

- non-photochemical quenching (NPQ), where energy is converted to heat (because of photoprotection or photoinhibition);
- photochemical quenching (PQ), which represents the effective fluorescence yield (that is the photosynthetic energy).

The fluorescence yield is enhanced when the yields of photochemistry and heat dissipation are decreased.

NPQ is expressed as:[124]

$$NPQ = \frac{F_m - F'_m}{F'_m} \qquad [2]$$

PQ is expressed as:

$$PQ = Y = \frac{F_v}{F_m} = \frac{F_m - F_o}{F_m} \qquad [3]$$

$\frac{F_v}{F_m}$ is the *optimal quantum yield* (Y_o) with PAR (Photosynthetically Active Radiation) $\neq 0$. It represents the maximum potential efficiency of light capture by the PSII and depends on the functional status of the photosynthetic system. Its optimal range is 0.5-0.8 but it depends on algal species and cultures. Lower levels indicate a stressful state of the algal culture.

PQ is also expressed with another parameter, the *effective quantum yield* (Y_e), Eq.4. It represents the quantum electronic transport efficiency to PSII at $PAR \neq 0$.

$$Y_e = \frac{F'_m - F_t}{F'_m} \qquad [4]$$

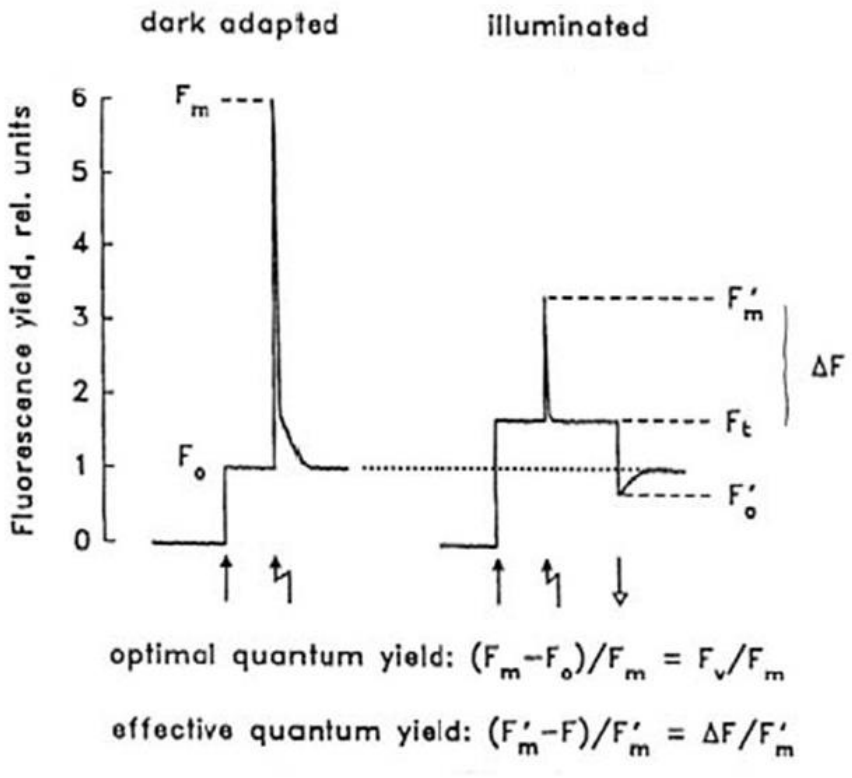

Figure 21 Time variations of variable fluorescence in photosynthetic organisms and terminology of fluorescence parameters (F_o F_m F_t F'_m). Source: image adapted from *Parametri fotosintetici da fluorescenza variabile modulate (PAM), ISPRA, 2010.*

In this thesis only the optimal quantum yield (Y_o) parameter was measured with PAM (Figure 22) in order to estimate cell viability after astaxanthin extraction. The results were expressed as percentages of the control.

The operating procedure consisted in collecting algal cells and placing them in a quartz cuvette with a light path of 1 cm. When algal culture density was too high, it was diluted with distilled water.

The cuvette was closed by the spherical sensor of PAR. The instrument was set up to send a pulse of saturated light (*Sat pulse*) after a few minutes of darkness.

Figure 22 PAM chlorophyll fluorimeter. Source: *Estrazione di astaxantina con approcci ecosostenibili da colture ottimizzate di Haematococcus pluvialis, M. Semeraro, 2018.*

3.3 *HAEMATOCOCCUS PLUVIALIS* CHARACTERIZATION

The following analyses were carried out on both *H. pluvialis* motile cells and red cysts. Chemicals were purchased from Sigma-Aldrich.

3.3.1 PROTEIN CONTENT ANALYSIS

To estimate the amount of proteins in algal biomass the method of *Lowry et al. (1951)* was followed; it is based on a colorimetric reaction given by the reagent of Folin and Ciocalteu, composed of a mixture of phosphomolybdate and phosphotungstate. The mixed acids in the Folin-Ciocalteu undergo a reduction proportional to the protein content, which gives a characteristic blue colour to the solution ($\lambda_{max} = 745 - 750$ nm).[125] The amount of proteins was measured with a spectrophotometer at a wavelength of 750 nm, based on a calibration curve obtained with bovine serum albumin (BSA) as standard. The procedure consists in weighing approximately 10 mg of the lyophilised algal culture and adding 3 mL of NaOH (0.5 M). To promote cell rupture, sample was mixed on vortex; later it was placed at 90°C for 8 minutes and then cooled for 2 minutes in ice. After cooling, sample was centrifuge at 2500 xg for 10 minutes at 4°C. The supernatant obtained from the extraction was collected in a test tube. To optimize the extraction the procedure was

repeated three times. At the end of the extractions, a sample consisting of 9 mL of supernatant was obtained. This sample was increased to a volume of 10 mL with the addition of NaOH buffer.
Subsequently three solutions were prepared:

- Solution 1: 5 g of Na_2CO_3 in 50 mL of NaOH (0,5 M)
- Solution 2: 0.5 g of $K_2C_4H_4O_6$ in 50 mL of H_2O
- Solution 3: 50 mg of $CuSO_4$ in 10 mL of Solution 2

Then the final solutions were prepared:

- Solution A: 20 mL of Solution 1 + 1 mL of Solution 3
- Solution B: 6 mL of Folin + 54 mL of distilled H_2O

These quantities are necessary for the preparation of 20 samples.
1 mL of sample with or without dilution (1:2) was put in a glass test tube, where 1 mL of Solution A was added. After mixing on vortex and waiting 10 minutes, 3 mL of Solution B was added to the sample. After a colorimetric reaction of 30 minutes, the sample was analysed with a Jasco V-650 spectrophotometer.

3.3.2 POLYSACCHARIDE CONTENT ANALYSIS

To estimate the amount of polysaccharides in algal biomass the method of *Myklestad et al. (1972*) was followed. This method is based on a colorimetric reaction given by phenol and sulphuric acid. Colour intensity of solution is proportional to polysaccharides content, analysed with the spectrophotometer at a wavelength of 490 nm, based on a calibration curve obtained with glucose as standard. The procedure consists in weighing approximately 10 mg of the lyophilised algal culture, adding 1 mL of H_2SO_4 (12 M), and putting the sample in a thermostatic bath at 37°C for 1 hour. Later the sample was increased to a volume of 11 mL with the addition of distilled H_2O, and it was mixed on vortex for 30 seconds. The sample was put in a thermostatic bath at 100°C for 2 hours, and in the ice for 30 minutes to get cool. Then it was centrifuged at 3000 xg for 10 minutes at 4°C and the supernatant was collected in order to estimate polysaccharides content. For the supernatant analysis the method of *Dubois et al. (1956*) was followed. The method consists in collecting 0,1 mL of supernatant and putting it in a glass test tube, in which 1 mL of phenol at 2.5%, and 2.5 mL of H_2SO_4 at 96% are added. After an exothermic colorimetric reaction of 30 minutes, the sample was analysed using a Jasco V-650 spectrophotometer.

3.3.3 LIPID CONTENT ANALYSIS

To estimate the total amount of lipids in algal biomass the method of *Bligh et Dyer (1959)* was followed though slightly modified. The procedure consists in weighing approximately 50 mg of lyophilised algal biomass in a glass test tube. 3 mL of a mixture of dichloromethane and methanol 2:1 volume ratio was added to the sample. Then the sample was heated at 60°C for 120 minutes. After cooling, the sample was centrifuged for 10 minutes to collect the supernatant containing lipids. To optimize the extraction, the procedure was repeated three times. The supernatant was collected and put into a glass test tube whose tare was previously estimated. Then it was dried under flow of nitrogen and finally weighed. Net lipid weight is expressed in relation to the weight of the lyophilised pellet used for extraction.

3.3.4 TOTAL FATTY ACIDS ANALYSIS

The Total Fatty Acid (TFA) content was analysed through gas chromatography coupled to mass spectrometry (GC-MS). The first part of the analysis was lipids derivatization: approximately 2 mg of the previously obtained dried extract (section 3.3.3) were taken and put in a glass vial. 0.4 mL of dimethyl carbonate (DMC), 0,1 mL of sodium hydroxide in methanol (NaOH in MeOH), 0.1 mL of dimethoxypropane were added to the sample and it was heated on a plate to 70° for 30 minutes. After cooling, 0.7 mL of boron trifluoride-methanol (MeOH - BF_3) and 0.05 mL of the internal standard methyl nonadecanoate (C19, 1000 ppm) were added to the sample. Then the sample was put on a plate to 70° for 30 minutes and, after cooling, 1 mL of cyclohexane, and 2 mL of Brine Solution (a high-concentration solution of salt in water) were added. After shaking, the cap was removed from the sample to promote the separation between the liquid and solid phases. The supernatant was analysed by GC-MS (Agilent Technologies, Waldbronn, Germany). The chromatographic column was HP-5MS, length 30 m, diameter 0.25 mm, film 0.25 μm. The parameters of the method used are shown in Table 9.

Table 9 Parameters of GC-MS method used for lipids derivatization.

Initial temperature [°C]	50
Final temperature [°C]	325

	Temperature [°C]	Rate [°C/min]
First step	50-220	50
Second step	220-265	5
Third step	265-325	50

Run time [min]	13.6
Post time [min]	1

The second part of the analysis was lipid silylation, in order to analyse Free Fatty Acid (FFA) content. Approximately 2 mg of the previously obtained dried extract (section 3.3.3) were put in a glass vial. 0.1 mL of acetonitrile, 0.05 mL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), 0.02 mL of pyridine, and 0.05 mL the internal standard methyl nonadecanoate (C19, 1000 ppm) were added to the sample. The sample was put on a plate to 70° for 60 minutes. 2 mL of acetonitrile were added to the sample and, after shaking, it was transferred to a GC mass vial and analysed by GC-MS (Agilent Technologies, Waldbronn, Germany). The chromatographic column was HP-5MS, length 30 m, diameter 0.25 mm, film 0.25 μm. The parameters of the method used are shown in Table 10.

Table 10 Parameters of GC-MS method used for lipids silylation.

Initial temperature [°C]	50
Final temperature [°C]	325

Initial time [min]	5

Temperature [°C]	Temperature gradient variation [°C/min]
50-325	10

Run time [min]	42.50
Post time [min]	1

3.3.5 TOTAL CAROTENOID CONTENT AND ASTAXANTHIN CONTENT

The total amount of carotenoids in algal biomass were determined by extracting approximately 50 mg of the lyophilised algal biomass with 2 mL of cyclohexane, 1 mL of ethanol and 1 mL of acetone. After 24 hours of extraction, the sample was centrifuged for 10 minutes and the supernatant was collected and dried under flow of nitrogen and finally weighed.

In order to estimate astaxanthin content in the carotenoid extract, approximately 2 mg of carotenoid extract were put into a new glass test tube. The sample was resuspended with dimethylsulphoxide (DMSO), appropriately diluted, and analysed with Jasco V-650 spectrophotometer at 530 nm. This analysis is based on a calibration curve obtained with synthetic astaxanthin.

3.4 SCREENING TESTS

3.4.1 NADESS PREPARATION FOR THE SCREENING TESTS

The NaDES tested in this thesis were prepared through the combination of nontoxic and biodegradable chemicals from renewable resources;[110] specifically, four different terpenes were tested as HBAs, and five different carboxylic acids were tested as HBDs.

Firstly, menthol was chosen as HBA to prepare a range of hydrophobic DESs using different fatty acids as HBDs at 2:1 HBA/HBD molar ratio. Three menthol-based NaDESs were prepared successfully as homogenous liquids by mixing menthol and HBD in a certain molar ratio at room temperature on a magnetic plate until a homogeneous liquid was formed, without any crystal precipitation (see Table 11). NaDESs composed of myristic acid and palmitic acid were not used in the experiment because they were did not form a homogeneous mixture (Figure 23). Geraniol was chosen as the second terpene and it was successfully mixed with two different fatty acids, lauric acid and oleic acid, at 2:1 HBA/HBD molar ratio (Table 11). The last two terpenes, α-bisabolol and thymol, were mixed with oleic acid.

Chemical structures of HBAs and HBDs are depicted in Figure 24.

Table 11 Overview of terpene-based hydrophobic DESs that were tested in this work.

HBA	HBD	Molar ratio (HBA:HBD)	Aspect at room temperature
Menthol	Caprylic acid (C8:0)	2:1	Transparent liquid
	Lauric acid (C12:0)	2:1	Transparent liquid
	Myristic acid (C14:0)	2:1	Solid liquid mixture
	Palmitic acid (C16:0)	2:1	Solid liquid mixture
	Oleic acid (C18:1)	2:1	Transparent liquid
Geraniol	Lauric acid (C12:0)	2:1	Transparent yellow liquid
	Oleic acid (C18:1)	2:1	Transparent yellow liquid
α-Bisabolol	Oleic acid (C18:1)	2:1	Transparent liquid
Thymol	Caprylic acid (C8:0)	1:1	Transparent yellow liquid
	Lauric acid (C12:0)	1:1	Solid liquid mixture
	Oleic acid (C18:1)	1:1	Transparent liquid

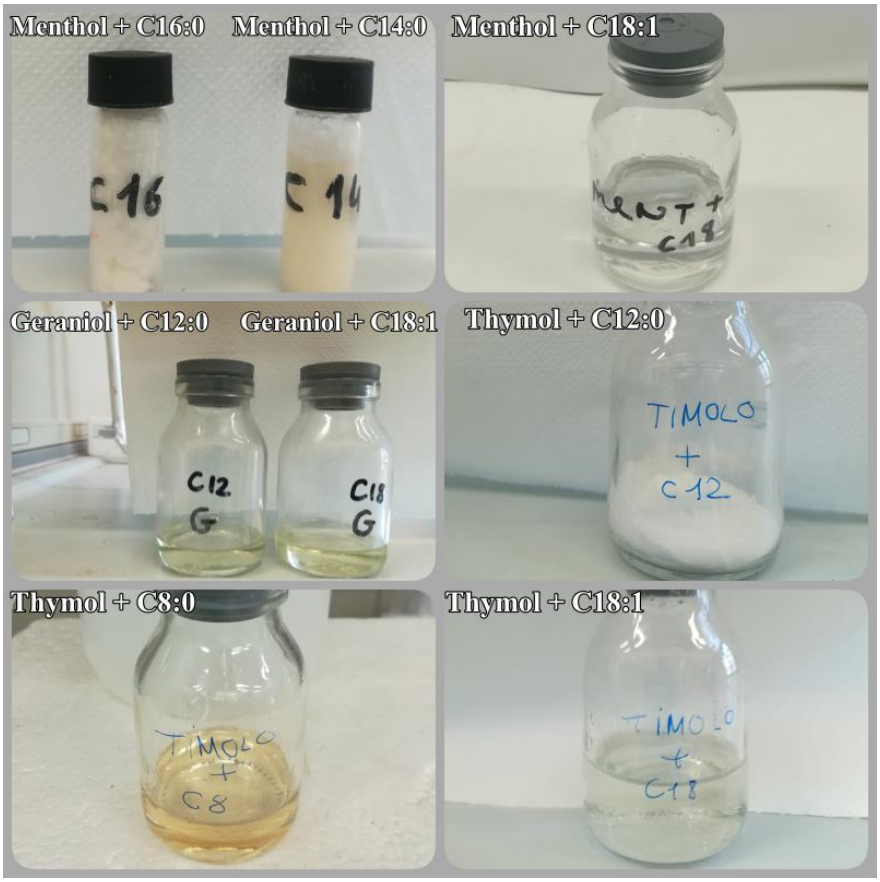

Figure 23 From top to bottom: menthol-based DESs, geraniol-based DESs and thymol-based DESs.

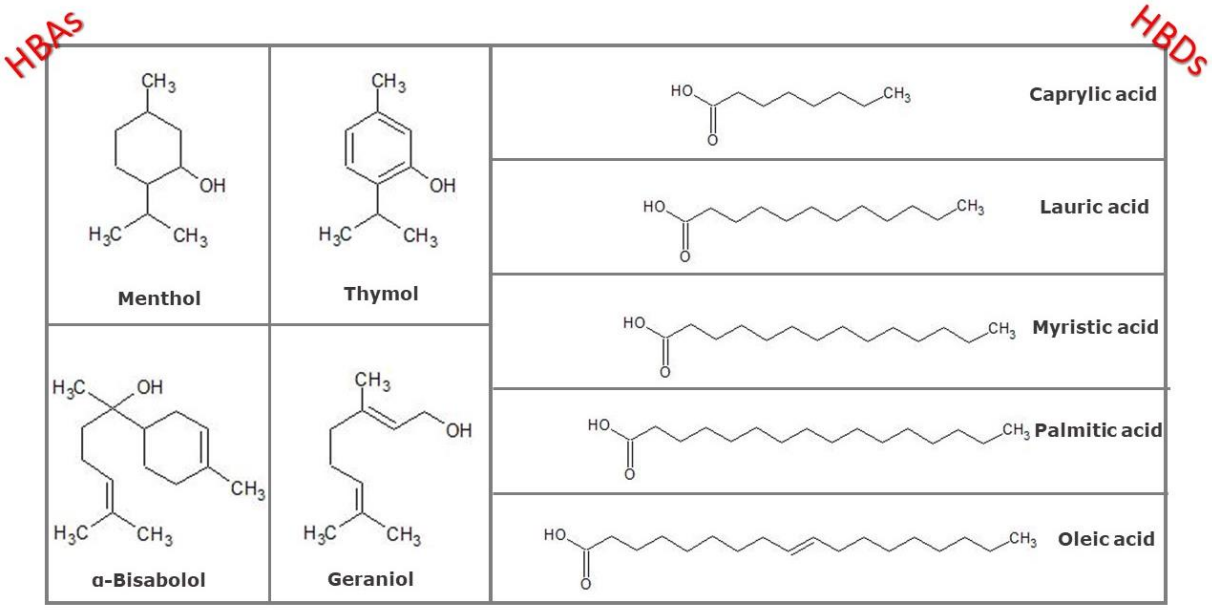

Figure 24 DESs chemical structures tested in this work.

3.4.2 HYDROPHOBIC DESS PREPARATION FOR SCREENING TESTS

The hydrophobic DESs were generously provided by Professor M. Tiecco of the University of Chieti-Pescara "G. D'Annunzio".

All hydrophobic DESs were very dense at room temperature. For this reason, they were heated at 50°C for 30-50 minutes. However, it was difficult to collect them and to mix with algal culture through magnetic stir bar. DDDACl: thymol and DDDACl: PhAA were the less fluid DESs (Table 12). TMG-based chemicals are NaDESs because of the natural source of their components.

Chemical structures of HBAs and HBDs are depicted in Figure 25.

Table 12 Overview of hydrophobic DESs that were tested in this work**.**

HBA	HBD	Molar ratio (HBA:HBD)[119]	Aspect at room temperature	Density [g/mL] at 25°C[119]
TMG	Thymol	1:3	Transparent yellow liquid	0.96 ± 0.03
	PhAA	1:2	Solid liquid mixture	1.16 ± 0.03
DDDACI	Thymol	1:2	Transparent liquid	0.88 ± 0.22
	PhAA	1:2	Transparent liquid	0.94 ± 0.02

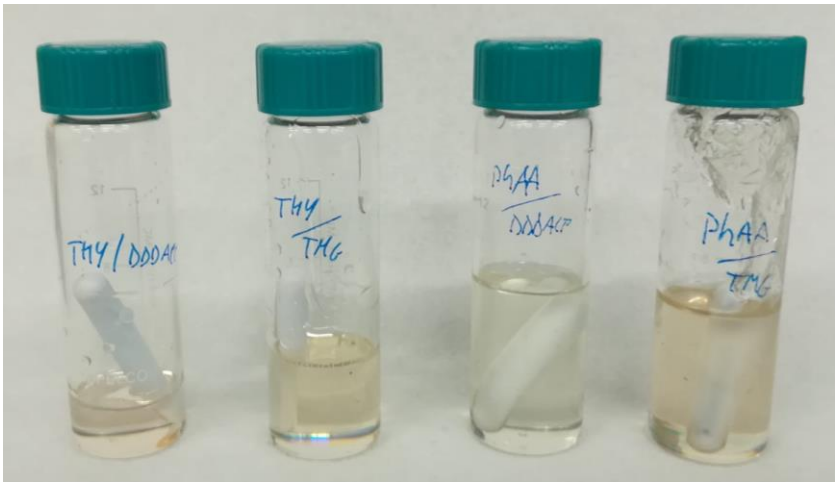

Figure 25 The four hydrophobic DESs prepared for screening tests. From left to right: thymol: DDDACl, thymol: TMG, PhAA: DDACl, PhAA: TMG.

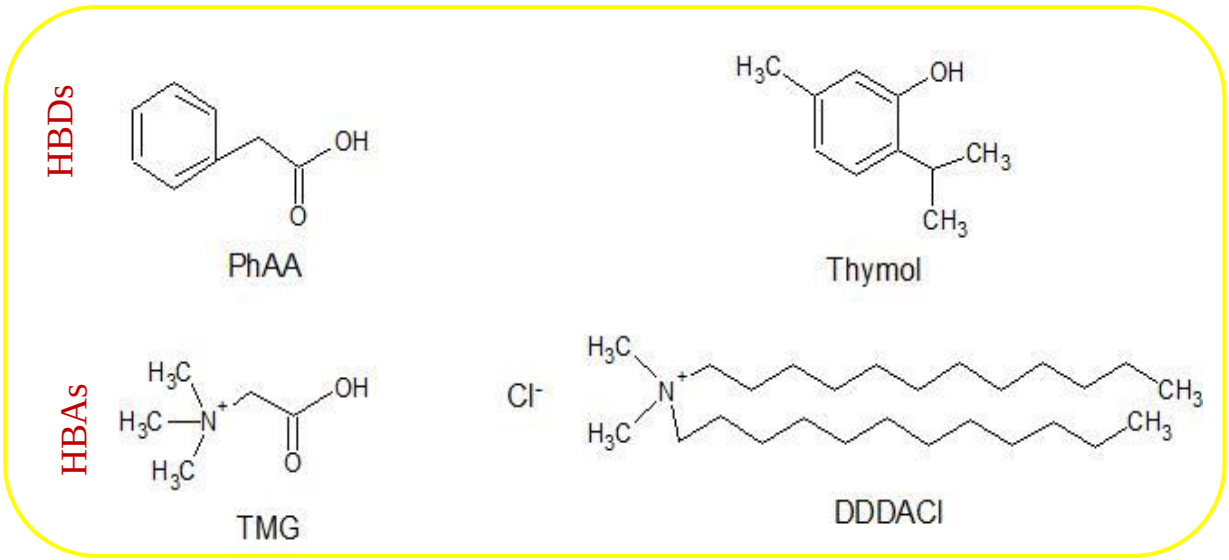

Figure 26 Hydrophobic DESs chemical structures tested in this work.

3.4.3 SCREENING TESTS PROCEDURE

LLE were carried out using the prepared hydrophobic NaDESs as extractants at room temperature.[126]

H. pluvialis culture used in most of the screening tests has been shifted towards a red-cyst phase since January 2019 (10 months old culture).

The extraction procedure is depicted in Figure 27.

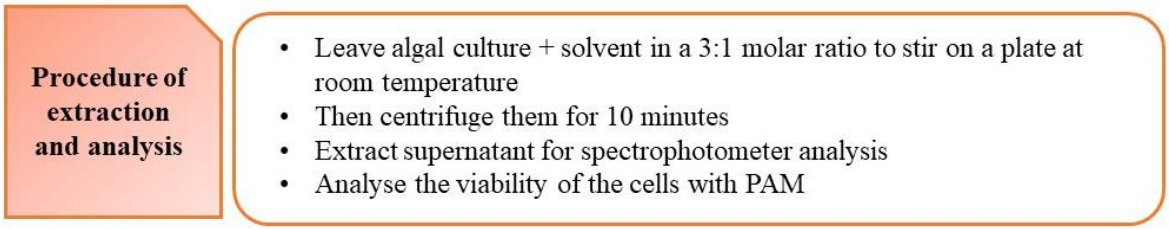

Figure 27 Procedure of LLE extraction and determination of astaxanthin content and of cell viability.

In addition to the NaDESs, three types of almond oils were tested as solvents (Figure 28):

- almond oil of *Bìolis Nature*, sweet almond body oil 98% natural ingredients;
- almond oil of *I Provenzali*, 100% natural pure sweet almond oil;
- almond oil of *Omnia Botanica*, 100% natural pure sweet almond oil.

Figure 28 Almond oils tested in this work: *Bìolis Oil* (98% natural ingredients), *I Provenzali* (100% natural pure sweet oil), *Omnia Botanica* (100% natural pure sweet oil).

The boundary conditions of the experiment were varied in terms of:

- algal biomass concentration;
- extraction with almond oil under high light, 500 µE/m^2/s (the same light as in the stress phase);
- use of an old almond oil (which has been open for more than a year) and a new almond oil;
- extraction from *H. pluvialis* which was in stress phase for more than 8 months, and from young *H. pluvialis*, in stress phase for 1 month.

3.4.4 NMR ANALYSIS

^{1}H NMR analysis was performed on the hydrophobic NaDESs in order to observe their solubility in water, thus their potential migration into algal media. Each NaDES was mixed with deuterated water on a magnetic plate. The solution was centrifugated and NMR analysis was carried out for the water phase. The spectrum of each NaDES allowed to observe whether one or both components solubilized in water. In water, the hydrogen bond(s) responsible for the DES formation are broken and the compound(s) solubilise into the water phase according to their individual water solubility.[126]

3.5 MILKING TEST

Three milking tests were performed at different stress times of algal culture, corresponding to a different age of the red cysts, to test the extraction and viability trend related to cellular aging:

- after 1 month of stress (Time 1, T1)
- after 2 months of stress (Time 2, T2)
- after 10 months of stress (Time 3, T3)

All tested solvents were hydrophobic to form a multiphase system with water. The result is the separation of two phases of different densities: algal culture on the bottom and solvent on the top. The latter forms a viscous surface layer containing astaxanthin and it can be easily removed.

Two hydrophobic solvents are proposed in milking test (based on the results obtained in the screening test):

- Menthol: oleic acid
- Almond oil

The extraction performance of the selected solvents has been evaluated as astaxanthin recovery from red phase *H. pluvialis* culture. For the milking test the same procedure of LLE extraction was followed (Figure 27): solvent was combined with algae culture concentrated 1 g/L in a 1:3 molar ratio (generally 3 mL of solvent was add to 9 mL of algal culture). For each test three replicates were made. Each sample was shaken on a magnetic stirrer and extraction times were respectively 1 hour for menthol-based NaDES and 48 hours for almond oil. After centrifugation (10 min), the supernatant was collected and astaxanthin content was measured with a spectrophotometer (Jasco V-650) at 530 nm (calibration curve obtained with synthetic astaxanthin). If necessary, the sample was diluted with DMSO for menthol-based NaDES, and with a solution of dichloromethane and methanol (2:1 volume ratio) for almond oil. The results are expressed as percentage of astaxanthin recovery on total astaxanthin dry biomass content (% DW). Cell viability was evaluated in terms of precipitate (pellet) photosynthetic activity with PAM, and the results are expressed as percentage of live algal cells on the control viability (which is considered 100%). Once the milking test was concluded, *H. pluvialis* cells were washed and re-grown under optimal conditions, then subjected to stress and re-extracted in a new milking test.

Figure 29 Milking test steps: (A) milking test execution (with magnetic stirrers); (B) end of the extraction; (C) separation of two phases after centrifugation; (D) algal culture re-cultivation with fresh medium under optimal conditions; (E) transition phase; (F) green phase; (G) stress phase again. The cycle is finished, and a new extraction can take place.

3.6 EFFECT OF ASTAXANTHIN ON ANIMAL CELLS

3.6.1 CELL CULTURE

In this work hepatocellular carcinoma cell line (Hep G2, Figure 30) was used. Hep G2 was derived from a liver hepatocellular carcinoma of a 15 years old Caucasian male.

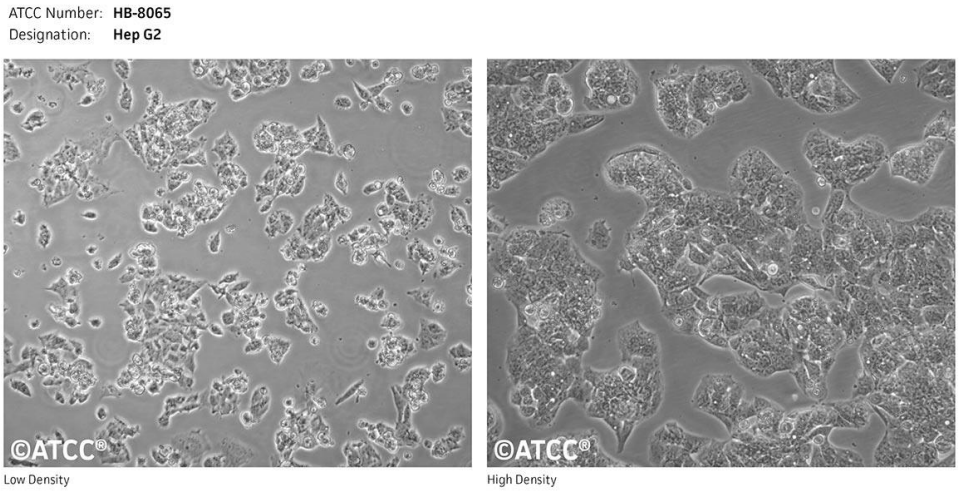

Figure 30 Hep G2 cells. Source: ATCC Cell Guide.

The medium used to grow cells was Dulbecco's Modified Eagle Medium (DMEM) high glucose (BioWhittaker®) with 10% FBS (Fetal bovine serum). Hep G2 were incubated in T25 flasks at 37°C in DMEM with 5% CO_2 (Thermo® Forma series II).

When the cells reach a confluence of about 70-80% they were split generally in a 1:10 ratio. The procedure followed for subculturing Hep G2 consist in removing culture medium, washing with 2 mL of PBS, followed by the addition of 2 mL of Trypsin-EDTA solution (BioWhittacker®) and 5 minutes of incubation at 37°C. Trypsin-EDTA is a protease whose function is to break down proteins which enable the cells to adhere to the vessel. Then trypsin was inactivated by adding 400 µL of CALF. Then cells were centrifuged at 300 RPM for 3 minutes, and, after removing the supernatant, 1 mL of DMEM was added by gently pipetting cells. Medium was renewed every two days. In order to avoid contamination, all actions were carried out under a laminar air flow cabinet (Faster BH-EN 2004).

3.6.2 ASTAXANTHIN ANTIOXIDANT PROPERTIES

In order to test astaxanthin antioxidant property, evaluation of Reactive Oxygen Species (ROS) though the fluorogenic probe 2',7'-dichlorofluorescin diacetate (H_2DCFDA) was conducted.

The cell-permeant H_2DCFDA passively diffuses into cells and it is retained in the intracellular level after being deacetylated by intracellular esterases. Upon oxidation by ROS, the nonfluorescent H_2DCF is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) The level of oxidative stress is directly correlated with the intensity of DCF fluorescence.[127]

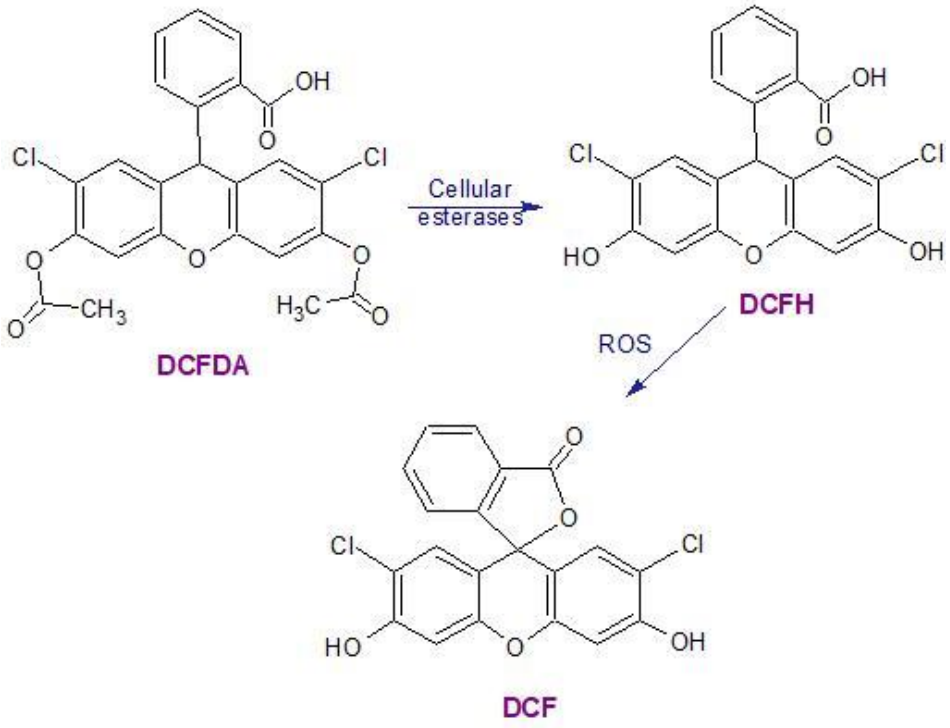

Figure 31 Chemical structure and mode of action of DCFDA

Hep G2 cells used for this test were seeded in 96-Well Multiwell plate. After attachment, cells were treated with natural astaxanthin extracted from *Haematococcus pluvialis* or with synthetic astaxanthin, at two different concentrations (0.1 μM and 1 μM) for 24 hours. After this time, culture medium was removed, and cells were washed with DMEM. 10 μM DCFDA was added to DMEM and 200 μL of DMEM + DCFDA were put to each well (except unstained well). Multiwell plate was incubated at 37°C for 30 minutes.

To test the ability of astaxanthin to counteract an acute oxidative stress cells were treated with a radical generator, tert-butyl hydroxyperoxide (TBH) at the concentration of 150 μM in Hank's Balanced Salt Solution (HBSS). After 30 minutes cells were washed two times with HBSS and analysed with the *EnSpire Multimode Plate Reader* at the excitation wavelength of 485 nm and emission wavelength of 535 nm.

3.6.3 NILE RED

The lipid content analysis of Hep G2 cells was conducted in order to verify astaxanthin ability to interfere with lipid metabolism. Nile red dye was used to identify and to quantify intracellular lipid droplets. Nile red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one, Figure 32) is a compound intensely fluorescent in a lipid-rich environment.

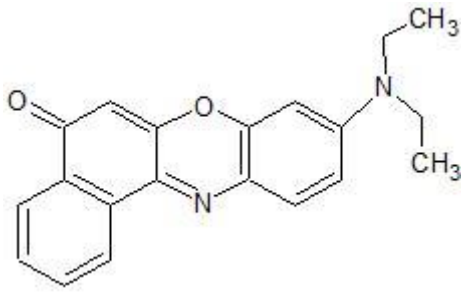

Figure 32 Nile red chemical structure.

In 6-Well Multiwell plate a coverslip was put in each well and Hep G2 cells 40% confluent were seeded. After incubating at 37°C 5% CO_2 for 24 hours, cells were treated with natural and synthetic astaxanthin (0.1 μM and 1 μM). After incubating for 24 hours, cells were fixed with 1 mL of 4% paraformaldehyde/PBS. After 10 minutes, paraformaldehyde was quenched with 2 mL of 3% FCS/PBS for 30 minutes. Nile red dye was added to PBS to effect a 1:100 diluition and cells were incubated for 10 minutes. After each step wells were washed with 1.5 mL of PBS. The last step consists in fixing coverslips on a microscope slide with a few drops of mounting oil. Cells were observed under confocal microscopy with red filter and 60x magnification. Laser wavelength was 543 nm and detection at 650 nm. Confocal microscopy allows to obtain high-resolution images through a laser beam at a specific wavelength sent through the sample.[61] Lipid droplets distribution and number were processed with ImageJ program.

3.6.4 MEASUREMENT OF OXYGEN CONSUMPTION

The effect of astaxanthin on mitochondrial oxygen consumption rate was measured using an oxygraph device equipped with Clark electrod. Oxygraph device is equipped with two chambers and uses polarographic oxygen sensors to measure oxygen concentration and calculate oxygen consumption. The technique provides information on mitochondrial quality and integrity, and on maximal mitochondrial respiratory electron transport system capacity.[128] Clark electrode consist of a platinum cathode and a silver anode, both in contact with an electrolyte solution (KCl). When a potential difference of 0.8V is imposed between the two electrodes oxygen is reduced to water and the current detected by the device is proportional to the oxygen concentration.

Oxidation-reduction reaction:[129]

At the anode	$4\,Ag + 4\,Cl^- \leftrightarrow 4\,AgCl + 4\,e^-$
At the cathode	$4\,H^+ + 4\,e^- + O_2 \leftrightarrow 2\,H_2O$
Overall	$4\,Ag + 4\,Cl^- + 4\,H^+ + O_2 \leftrightarrow 4\,AgCl + 2\,H_2O$

This method allows to measure the following respiratory states in intact cells:

- basal cellular oxygen consumption in the presence of endogenous substrates;
- basal cellular oxygen consumption in the presence of oligomycin, inhibitor of ATP synthase and consequently of mitochondrial respiration;
- Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), for an uncoupled respiration. FCCP is a transmembrane proton transporter that allows to reactivate mitochondrial respiration in cells treated with oligomycin.

Hep G2 cells 80% confluent were treated with natural and synthetic astaxanthin (0.1 μM). After 24 hours from the treatment, about $2x10^6$ cells in DMEM were added into an oxygraph chamber containing a magnetic stirrer bar (700 rpm) at 30 °C and the oxygen consumption was recorded for 20 minutes. Oligomycin and FCCP were added to the sample as indicated in Table 13. Data were normalized on number of cells.

Table 13 Method of mitochondrial respiration test.

Chemical	Concentration [μM]	Time [minutes]	Respiratory state
-	-	5	Basal respiration (endo)
Oligomycin	1	4	Stop of respiration (oligo)
FCCP	0.5	3	Maximum respiratory rate
FCCP	0.5	3	Maximum respiratory rate

3.7 STATISTICAL ANALYSIS

Some results were analysed by one-way ANOVA using the GraphPad Prism program, in order to verify the significance of the data. In some cases, outliers were also identified with the same program.

4 RESULTS AND DISCUSSION

4.1 *HAEMATOCOCCUS PLUVIALIS* CHARACTERIZATION

Algal biomass characterization was performed in order to quantify the amount of proteins, polysaccharides, lipids, carotenoids in:

- *H. pluvialis* culture in stress for 1 month (used for milking test T1);
- *H. pluvialis* culture in stress for 2 months (used for milking test T2);
- *H. pluvialis* culture in green phase.

As Figure 33 shows, *H. pluvialis* composition in the red phases was similar, even if carotenoid and lipid content was slightly higher in T2, while polysaccharides were less. This probably happens because T2 stress period was longer. According to studies reported in literature (section 1.2.2), carotenoids and polysaccharides content was lower in the green phase (respectively 21.4% and 30.4% in the green phase, 26-31% and 61-57% in the red phase), while protein level was higher (22.2% in the green phase and 14-16% in the red phase).

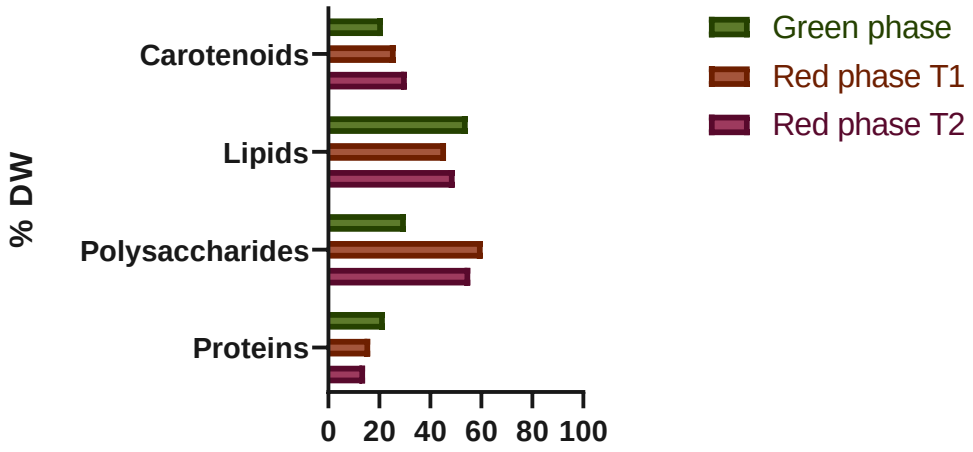

Figure 33 Proteins, polysaccharides, lipids, carotenoids content in *H. pluvialis* red phase T1, red phase T2, and green phase.

Table 14 shows astaxanthin content in total carotenoids and in total biomass. In the green phase astaxanthin content in total biomass was almost null (0.2%). Astaxanthin content in *H. pluvialis* under stress conditions increased with the stress period extension.

Table 14 Astaxanthin content in *H. pluvialis* under the stress phases (T1, T2, T3) and in the green phase.

	Astaxanthin in total carotenoid content [%]	**Astaxanthin content in total biomass [DW%]**
Red phase T1	7.4	2.0
Red phase T2	6.8	2.1
Red phase T3	-	2.5
Green phase	0.7	0.2

Total Fatty Acid (TFA) content is shown in Figure 34. The qualitative and quantitative composition of TFA varied greatly between the green and the red phases. In the green phase C16 fatty acids (C16:0 and C16:3) predominated, while in both red phases a mix of C18:1, C18:2, C18:3 composed more than 60% of the TFA (in accordance with *Zhekisheva et al.* study)[32]. This percentage was slightly higher in the red phase T2, probably because the stress period was longer. The other difference among the red phases was the higher percentage of C16:0 in T1. In conclusion, the analysis seemed to show that the production of C18:1 (absent in the green phase) was directly related to the production of astaxanthin during the stress phase.

Table 15 compares Total Fatty Acid (TFA), Free Fatty Acid (FFA), Bond Fatty Acid (BFA) content in the different phases. TFA was higher in the red phases, in particular in T1. Most of the fatty acids present were in the form of esters (BFA), while FFA were between 0.5 and 1%.

Finally, a qualitative analysis was conducted on chromatograms obtained with silylation (Addendum 1) to identify which compounds were present in each phase. In the green phase sterols were more abundant than in the red ones. Sterols play an essential role in the physiology of organisms.

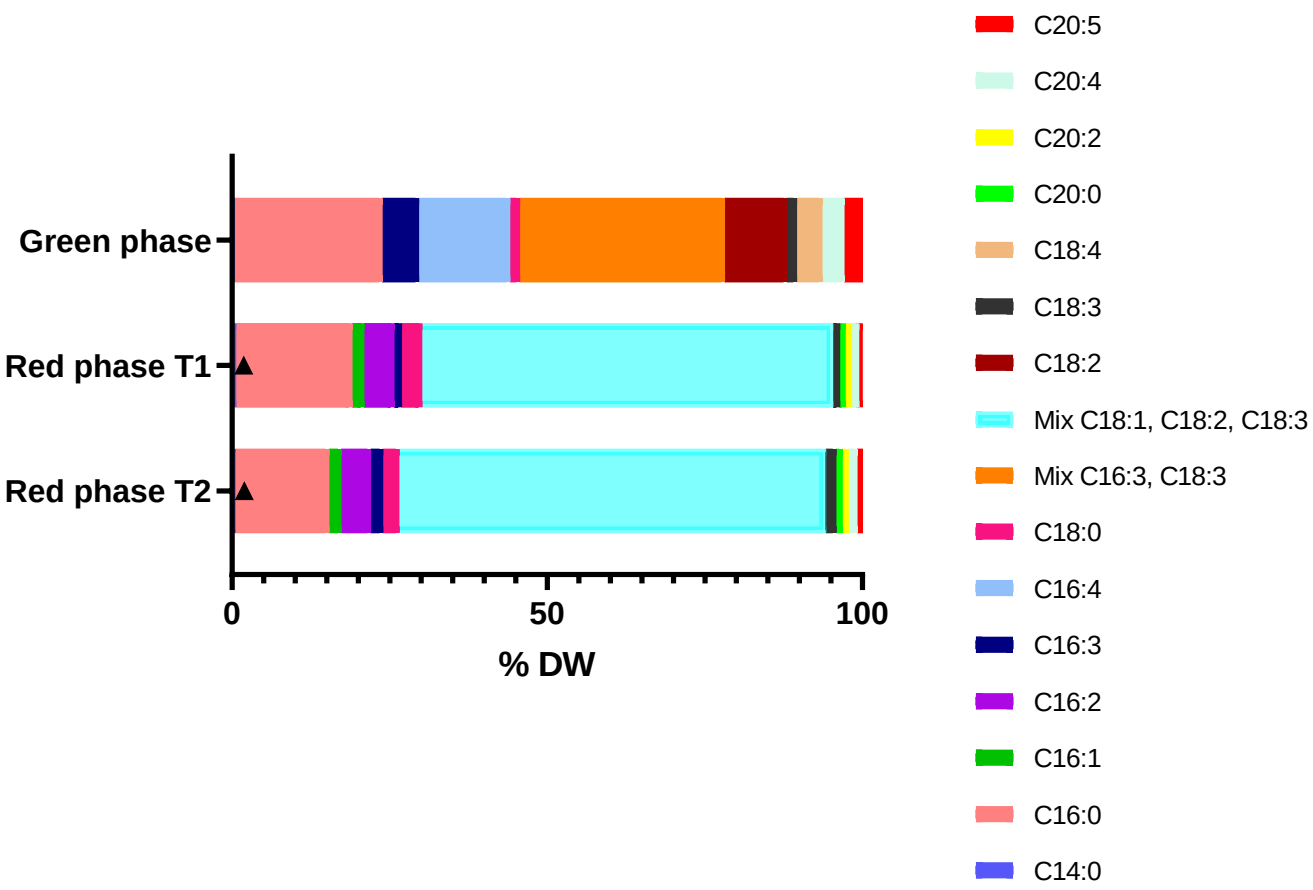

Figure 34 Total Fatty Acid (TFA) content in *H. pluvialis* red phase T1, red phase T2, and green phase.

Table 15 Total Fatty Acid (TFA), Free Fatty Acid (FFA), Bond Fatty Acid (BFA) content in *H. pluvialis* red phase T1, red phase T2, and green phase. The results are expressed as percentage of the total lipid content.

	% TFA	% FFA	% BFA
Green phase	11.0	0.8	10.2
Red phase T1	19.3	1.0	18.3
Red phase T2	16.3	0.5	15.8

4.2 IMPROVEMENT OF ASTAXANTHIN PRODUCTION

Haematococcus pluvialis photosynthetic efficiency and astaxanthin content were measured after 8 days in cultures exposed to various stress conditions: acidic pH, salinity, addition of an antioxidant (vitamin C). As Figure 35 shows, cell viability of algal biomass treated with ascorbic acid 0.02 g/L was approximately 100%, however astaxanthin content was lower. Both viability and astaxanthin content were lower than control for cells grown under acidic pH and high NaCl concentration.

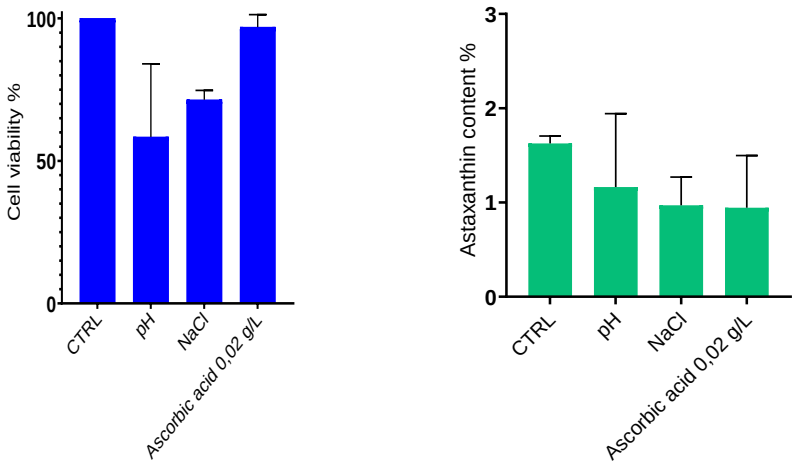

Figure 35 Viability (left) and astaxanthin content (right) of *H. pluvialis* cells cultured in acidic medium, in presence of NaCl and of ascorbic acid.

In the second test *H. pluvialis* was treated with different concentration of ascorbic acid. Cell viability and astaxanthin content were analysed on the 7° stress day. Cell viability was similar among treatments except for algae treated with ascorbic acid at 0.2 g/L, in which percentage was 100% (Figure 36).

Astaxanthin content was very high in all treatments, especially for ascorbic acid 0.2 g/L (2.4%).

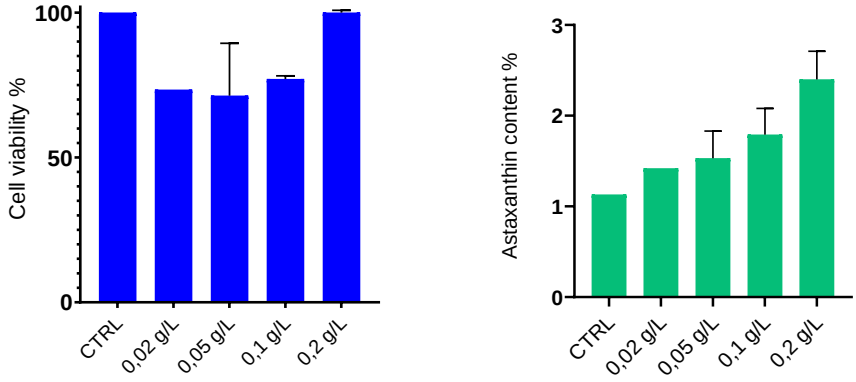

Figure 36 Viability (left) and astaxanthin content (right) of *H. pluvialis* cells treated with different concentrations of ascorbic acid.

In conclusion, ascorbic acid seems to be the best stress factor among those tested, in addition to nutrient deficiency and high light radiation. As a matter of fact, ascorbic acid (or vitamin C) is an essential antioxidant that neutralizes free radicals by protecting microalga from high light. Both its power to maintain cell viability and the astaxanthin content in the algal biomass grows as its concentration increases. In Figure 37 and Figure 38 *H. pluvialis* cells treated with the different stress factors (pH, NaCl, different concentrations of ascorbic acid) are depicted. Visually, algal cells treated with pH have less astaxanthin (Figure 37 C, D). There is a central accumulation of astaxanthin and a green periphery chlorophyll enriched. Only few algal cells treated with low ascorbic acid concentrations were turned in red cysts (Figure 38 B, C). Cysts transition is evident in *H. pluvialis* cells treated with 0.1 g/L and 0.2 g/L of ascorbic acid (Figure 38 D, E, F).

Figure 38 E, F shows that cells tend to aggregate with high concentrations of ascorbic acid, probably due to the effect of this compound which could act as a flocculant, reducing the surface charge of cells.

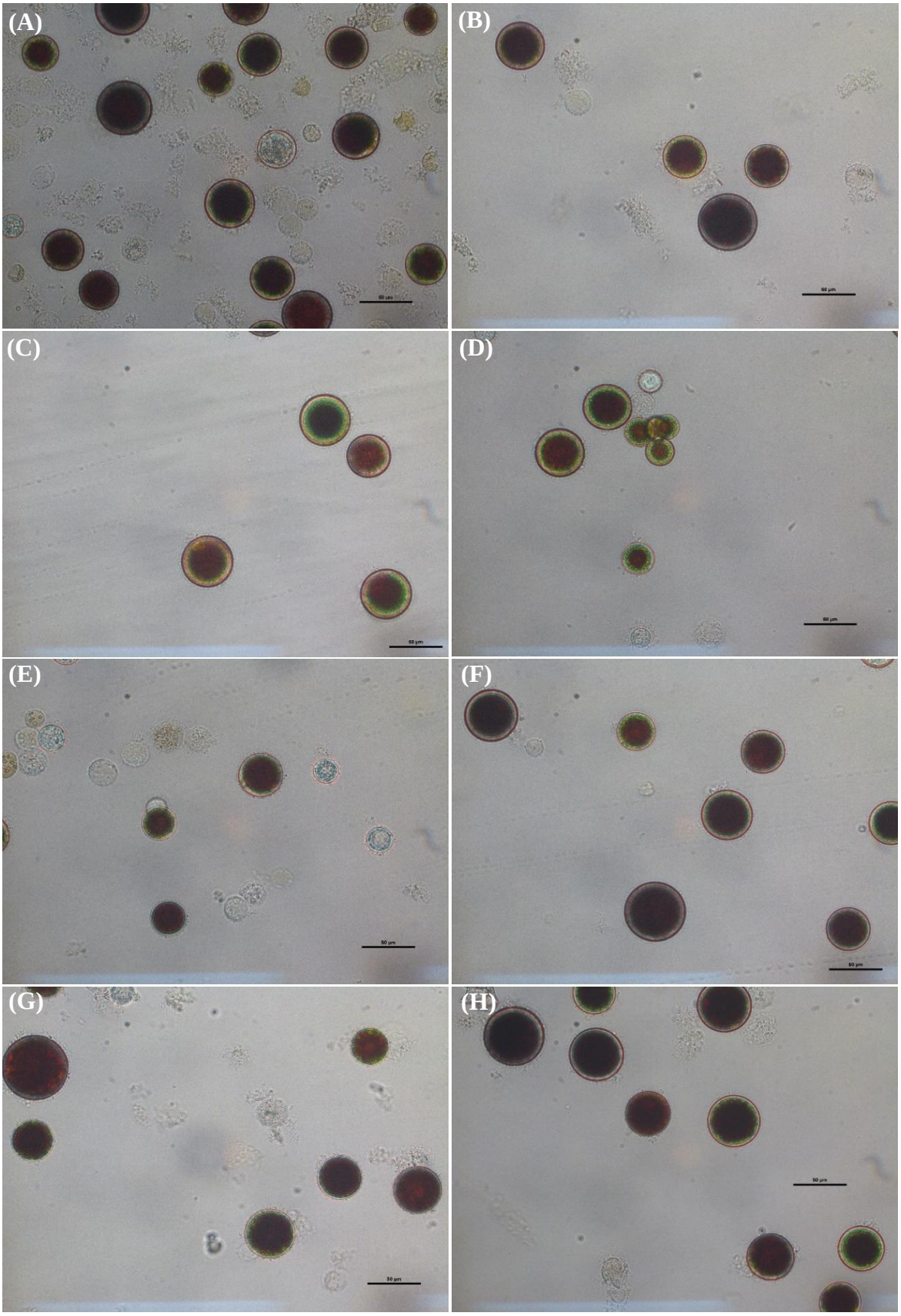

Figure 37 (A), (B) Control; (C), (D) pH; (E), (F) NaCl; (G), (H) Ascorbic acid.

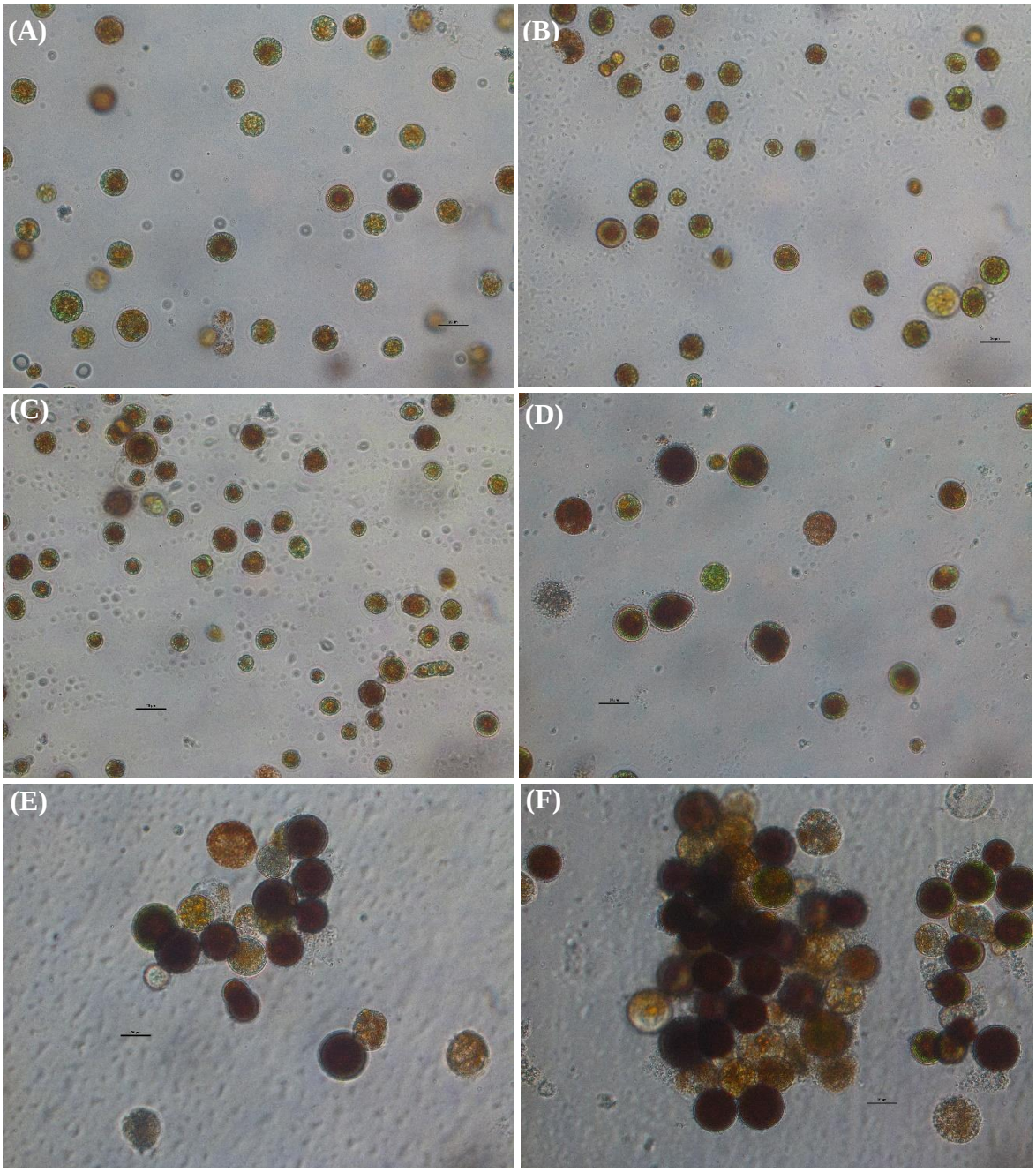

Figure 38 Microscope photos (32x magnification): (A) Control; (B) Ascorbic acid 0.02 g/L; (C) Ascorbic acid 0.05 g/L; (D) Ascorbic acid 0.1 g/L; (E), (F) Ascorbic acid 0.2 g/L. Scale bars: 25 μm.

4.3 SCREENING TESTS

The eleven successfully prepared DESs were screened to test their efficiency in keeping algal cells alive and in extracting astaxanthin from *Haematococcus pluvialis*. DESs were tested at different extraction times and different operative conditions. *H. pluvialis* culture used for this test was kept under stress for 10 months. This section can be divided into four parts:

1. comparison of different terpenes (menthol, geraniol, α-bisabolol, thymol) mixed with different carboxylic acids;
2. comparison among different almond oils;
3. comparison of NaDESs in terms of astaxanthin extraction efficiency (extraction time of 48 hours);
4. comparison among other hydrophobic DESs made with thymol, PhAA, TMG, DDDACl.

4.3.1 OPTIMAL ALGAL CULTURE CONCENTRATION

A screening test aimed to establish the optimal biomass concentration for LLE extraction. An extraction with almond oil was done for 24 hours at a 3:1 algal culture/oil molar ratio with 4 different biomass concentrations:

- 1 g/L
- 0.5 g/L
- 0.33 g/L
- 0.2 g/L

The results suggest that the best extraction was with concentrated biomass 1g/L. The same can be said for cell viability (Table 16). This could be due to a negative and lethal effect of the solvent (oil) on the algal cells when their density is low.

Table 16 Cell viability and astaxanthin recovery after treatment of algal cultures with almond oil at different concentrations.

Biomass concentration [g/L]	**Cell viability %**	**Astaxantin recovery %**
1	81.6	33.7
0.5	33.4	32.5
0.33	2.4	23.0
0.2	4.4	17.6

4.3.2 TERPENE-BASED NADESS

The best DES in terms of algal cell viability was menthol: oleic acid (89.2%) after 1 hour of extraction. It was followed by α-bisabolol (85.5%), menthol: lauric acid (35.5%) and thymol: oleic acid (34.8%, see Table 17). As expected, cell viability decreased dramatically with extraction time. By comparing menthol-based and geraniol-based DESs, geraniol resulted more toxic since it damaged algal cells (Table 17). This was probably due to its linear chemical structure, that could favour the interaction with cellular membranes and therefore their damage (see Figure 24). Contrarily, thymol-based DESs seemed to be the most toxic (except for geraniol-based DESs). However, this result disagrees with the toxicity of thymol (Table 6), which is much lower than that of α-bisabolol. After short extraction times (10, 20 and 30 minutes), the percentage of cell viability is higher than after 1 h, as expected. The highest percentage was obtained after extraction with thymol: oleic acid (63.8%) after 10 minutes and it decreased with the passage of time (Table 17). Thymol: caprylic acid DES was toxic just after 10 minutes of extraction (11.0%).

For astaxanthin extraction efficiency the first screening test was conducted without calculating algal biomass dry weight and the total astaxanthin content, thus only relative comparisons could be done. After 1 hour, menthol: oleic acid was the best NaDES for astaxanthin extraction (9.73 µg/mL, Table 18). After 24 hours menthol: lauric acid DES was the best solvent (569.28 µg/mL of extracted astaxanthin), while menthol: oleic acid DES was the worst (278.57 µg/mL).

In the second screening test, astaxanthin recovery rate after 1 hour of extraction was higher in menthol: lauric acid DES (9.6%, see Table 19). However, there was not a big difference between menthol: lauric acid, thymol: oleic acid (9.4%) and geraniol: lauric acid DESs (8.7%). Menthol: oleic acid and geraniol: oleic acid extraction efficiency was very low (respectively 2.6% and 4.2%). As expected, extraction percentage increased with time. After 4 hours of extraction, geraniol: lauric acid was the best in terms of astaxanthin recovery (42.6%) and it was followed by geraniol: oleic acid (30.2%, Table 19). Bisabolol-based DES cannot be considered an efficient solvent for the extraction of astaxanthin. Even if algal cells were alive, the concentration of astaxanthin was very low after 1 hour, probably below the detection limits of the instrument. After 48 hours 34.4% of astaxanthin was recovered, but algal cells were damaged (Table 17). Moreover, bisabolol-DES was particularly dense and viscous, by making it difficult to collect (Figure 39). After 1 hour, thymol-based DES seemed to be toxic to algal cells (Table 17) and it had a low extraction capacity (9.4%). At shorter times (10,

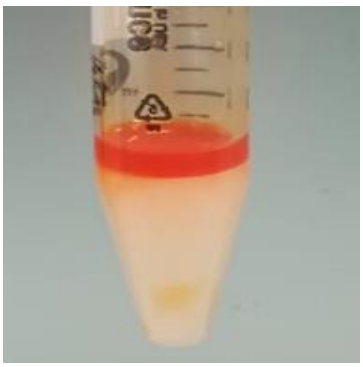

Figure 39 Extraction of 48 hours with α-bisabolol: oleic acid.

20, 30 minutes) astaxanthin extraction was particularly low with both thymol-based DESs (Table 19).

Table 17 Cell viability with the eight prepared NADESs after different times of extraction. Cell viability is expressed as a relative percentage of the control.

HBAs	HBDs	Cell viability %							
		10 min	20 min	30 min	1h	2h	4h	24h	48h
Menthol	Caprylic acid (C8:0)	-	-	-	5.9	-	-	0	-
	Lauric acid (C12:0)	-	-	-	35.5	11.8	2.1	0.7	-
	Oleic acid (C18:1)	-	-	-	89.2	78.2	62.9	15.9	-
Geraniol	Lauric acid (C12:0)	-	-	-	0	0	0	-	-
	Oleic acid (C18:1)	-	-	-	0	0	0	-	-
α-bisabol	Oleic acid (C18:1)	-	-	-	85.5	-	-	-	12.9
Thymol	Caprylic acid (C8:0)	11.0	7.65	-	-	-	-	-	-
	Oleic acid (C18:1)	63.8	46.9	43.2	34.8	-	-	-	-

Table 18 Astaxanthin content in NADES, expressed in µg, after different time of extraction.

HBAs	HBDs	Astaxanthin content in NADES [µg/mL]	
		1h	24h
Menthol	Caprylic acid (C8:0)	2.0	329.4
	Lauric acid (C12:0)	7.4	569.2
	Oleic acid (C18:1)	9.7	278.6

Table 19 Astaxanthin recovery % in the eight prepared NADESs after different time of extraction. Astaxanthin recovery is expressed as a recovery percentage of the total astaxanthin content.

HBAs	HBDs	Astaxanthin recovery %							
		10 min	20 min	30 min	1h	2h	4h	24h	48h
Menthol	Lauric acid (C12:0)	-	-	-	9.6	8.1	22.5	-	-
	Oleic acid (C18:1)	-	-	-	2.6	3.7	16.7	-	-
Geraniol	Lauric acid (C12:0)	-	-	-	8.7	3.0	42.6	-	-
	Oleic acid (C18:1)	-	-	-	4.2	9.4	30.2	-	-
α-bisabol	Oleic acid (C18:1)	-	-	-	-	-	-	-	34.4
Tymol	Caprylic acid (C8:0)	1.2	3.0	-	-	-	-	-	-
	Oleic acid (C18:1)	0.9	1.6	2.7	9.4	-	-	-	-

In the third screening test menthol: oleic acid and thymol: oleic acid were compared after an extraction time of 30 minutes and 1 hour (Figure 40). For this test a different *H. pluvialis* culture was used (in stress for 2 months). In terms of cell viability menthol: oleic acid was the best NADES (91.7%) and the percentage did not change with time. Viability of cell treated with thymol-based

DESs slightly decreased with time (25.5% and 20.3%, respectively after 30 minutes and 1 hour of extraction). In terms of astaxanthin recovery, thymol: oleic acid was the best NaDES and the recovery increased with time (17.7% and 21.6%, respectively after 30 minutes and after 1 hour of extraction). Menthol-based DES recovery was only 8.9% and 13.7% of total astaxanthin content (Figure 40).

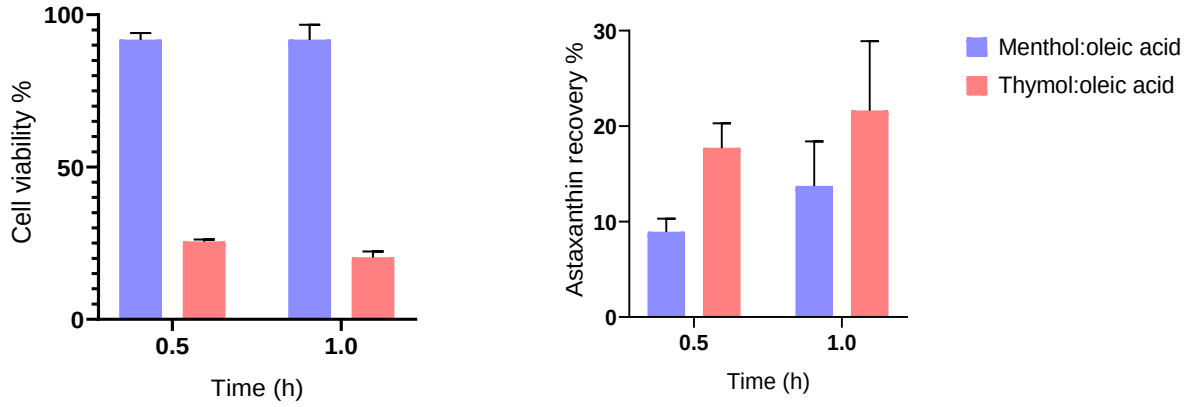

Figure 40 Comparison between menthol: oleic acid and thymol: oleic acid in terms of algal cell viability (left) and astaxanthin extraction efficiency (right).

Figure 41 shows pictures of *H. pluvialis* cells, made with optical microscope, after extractions with different NaDESs. Since the extraction rates are generally low, most of the cells are still rich in astaxanthin. However, there are also cells partially or totally green, due to the removal of astaxanthin avoiding cell death.

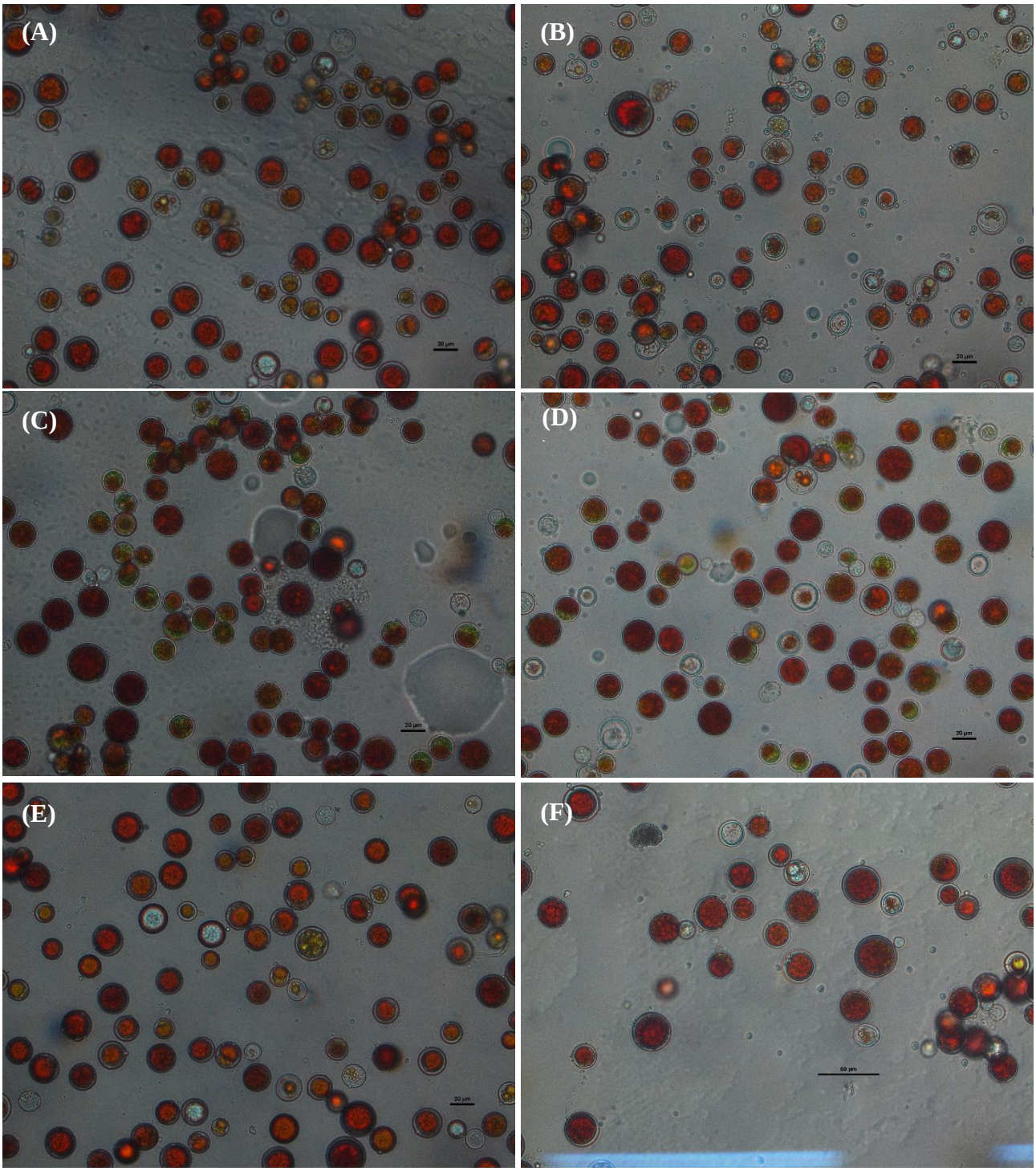

Figure 41 Pictures from optical microscope, 32x magnification. (A) Geraniol: oleic acid after 1 hour of extraction; (B) geraniol: oleic acid after 4 hours of extraction; (C) menthol: oleic acid after 1 hour of extraction; (D) menthol: oleic acid after 4 hours of extraction; (E) menthol: lauric acid after 1 hour of extraction; (F) thymol: oleic acid after 1 hour of extraction. Scale bar is 50 µm for (F), and 20 µm for the other pictures.

4.3.3 ALMOND OILS

Almond oil is widely used in cosmetic formulations as a moisturizer and emollient,[82] because it is considered as one of the best carrier oils in cosmetic preparations.[130] It is used also for its nutritional benefits because it is rich in mono and polyunsaturated fatty acids (60%, mainly oleic acid, and 30% respectively) and in the antioxidant α-tocopherol (0.02 - 0.05 wt%).[82]

Pure almond oil (*I Provenzali*) and 98% almond oil (*Bìolis Oil*) extraction efficiency was firstly compared under two different light conditions: high light (500 µE/m^2/s) to maintain *H. pluvialis* under stress conditions, and low light (21 µE/m^2/s). The extraction time was 48 hours. All cell viability percentages were particularly low, except for pure oil under normal light condition (73.3 ± 10.30)% (Table 20). Under high light, the samples appeared milky and dense, so it was difficult to analyse them with PAM. Both almond oils used in this test proved to be rancid since they had been open for a year, so they had lost their properties. High light probably caused almond oils degradation. Astaxanthin recovery with *Bìolis Oil* was high under low light (62.1%) and lower under high light, probably for oil degradation (11.5%). Astaxanthin recovery of pure oil was impossible to analyse with the spectrophotometer because it appeared too dense and milky.

Table 20 Comparison between pure almond oil and 98% almond oil under different light intensity.

Almond oil	Cell viability %	Astaxanthin recovery %
Pure oil	73.3 ± 10.3	-
High light pure oil	0.1 ± 0.1	-
98% oil	2.8 ± 2.9	62.1 ± 17.2
High light 98% oil	0.01 ± 0	11.5 ± 2.2

Another test was conducted in order to compare *Bìolis Oil* with two different brands of pure almond oil, *I Provenzali* and *Omnia Botanica.* New oils and young algal biomass were used to obtain comparable results to those of the milking tests. As Figure 42 shows, almond oils ability in keeping cells alive was similar but it was higher for pure oil (72.8% with *Bìolis Oil*, 80.3% and 81.8% respectively with *I Provenzali* and *Omnia Botanica*), probably because of the additives present in *Bìolis Oil*. In terms of extraction the three oils ability did not show a big difference: astaxanthin extraction percentage was approximately 16-18%.

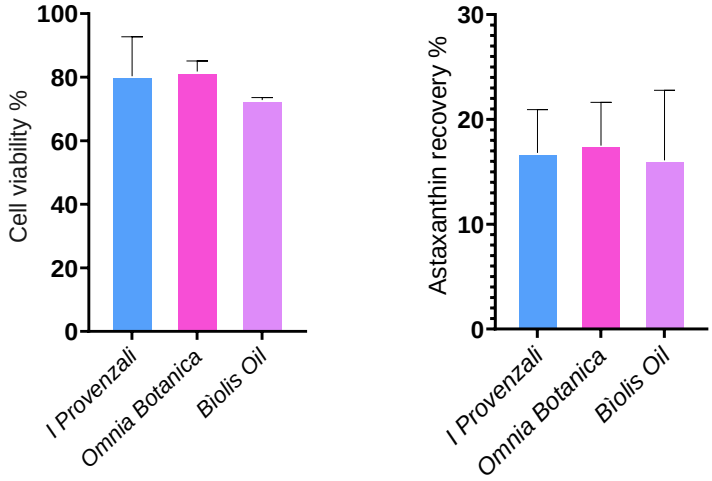

Figure 42 Comparison between *I Provenzali* (pure almond oil), *Omnia Botanica* (pure almond oil), *Biolis Oil* (98% almond oil), in terms of cell viability (left) and extraction efficiency (right).

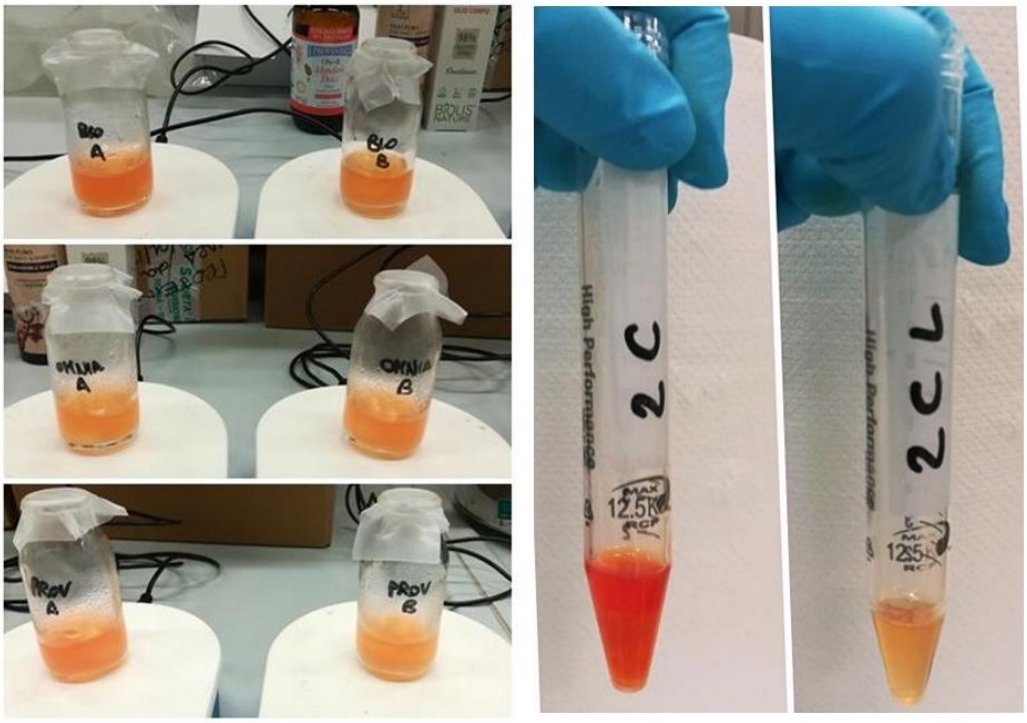

Figure 43 (A) Liquid-Liquid Extraction with new almond oils (*I Provenzali, Omnia Botanica*, 98% almond oil); (B) comparison between almond oil under low (2C) and high light (2C L).

4.3.4 NaDES EXTRACTION EFFICIENCY AFTER 48 HOURS

The purpose of the last test was to maximize the yield of astaxanthin recovery using a selection of the NaDESs previously tested. It consisted in comparing menthol: lauric acid, menthol: oleic acid, geraniol: lauric acid, geraniol: oleic acid, thymol: oleic acid after an extraction of 48 hours. PAM analysis was done to evaluate cell viability, however, as expected, algal cells were dead. There was not a significant difference among NaDESs extraction capacity (Addendum 2). However, geraniol: oleic acid and menthol: lauric acid were the best (respectively 68.0% and 63.4%, Figure 44; they were followed by geraniol: lauric acid, thymol: oleic acid and menthol: oleic acid (respectively 62.3%, 57.8% and 55.9%).

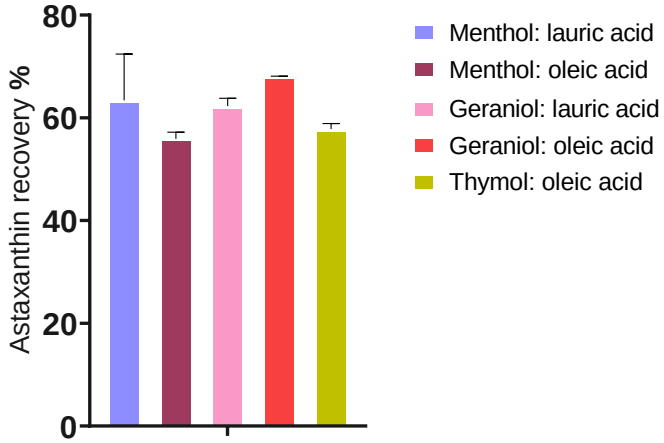

Figure 44 Comparison among the main DESs previously tested after 48 hours of extraction.

4.3.5 HYDROPHOBIC DESs

The extraction was made with 3 mL of algal culture in stress for 2 months, which was mixed with 1 mL of each DES for 1 hour.

All tested hydrophobic DESs were not able to keep algal cells alive (Table 21). For thymol: TMG the reason is probably the high solubility of TMG in water, while, among the other DESs, DDDACl is the most toxic chemical (Table 7). In terms of astaxanthin recovery only PhAA: DDDACl could be acceptable. Even if astaxanthin recovery after 1 hour of extraction was higher than that of previously tested NaDESs, these hydrophobic DESs cannot be considered for the milking test (whose priority is the maintenance of the algal crop viability). PhAA: TMG was not analysed because it formed a solid precipitate impossible to recover (Figure 45).

Table 21 Comparison between thymol: DDDACl, thymol: TMG, PhAA: DDDACl in terms of cell viability and astaxanthin recovery.

DESs	Cell viability %	Astaxanthin recovery %
Thymol: DDDACl	1.6	11.7
Thymol: TMG	3.5	13.0
PhAA: DDDACl	5.1	52.8

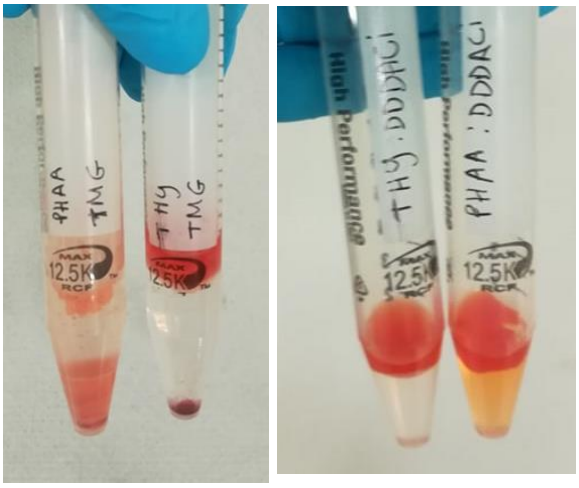

Figure 45 From left to right: PhAA: TMG, thymol: TMG, thymol: DDDACl; PhAA: DDDACl. In PhAA: DDDACl aqueous phase appears coloured, probably for algal cells lysis.

4.3.6 NMR ANALYSIS

The results of ^{1}H NMR analysis suggested that the hydrogen bond acceptors (HBAs) were mainly solubilized in water. In detail:

- in menthol: lauric acid, geraniol: lauric acid, and thymol: oleic acid, only terpenes (HBAs) were solubilized in water;
- in menthol: oleic acid and geraniol: oleic acid, both terpenes and, partially (and surprisingly), oleic acid were solubilized in water.

NMR spectra are shown in Addendum 3.

Since terpenes tend to solubilize, the phenomenon of algal cells mortality could be exclusively related to the toxicity of each individual terpene. However, some limitations must be considered in this analysis:

- NaDESs behaviour in the solvent used for the NMR analysis (deuterated water) could be different than in the culture medium, where salts and nutrient are presents;
- the analysis was made only from a qualitative and not a quantitative point of view, therefore the HBAs amount solubilized in water is unknown.

4.4 MILKING TESTS

Milking tests were done with *Haematococcus pluvialis* culture in stress for 1 month (T1), 2 months (T2), and 10 months (T3), in order to observe possibly changes related to algal cells aging. Two solvents were used for milking test: almond oil and menthol: oleic acid (the latter is the best NaDES in terms of cell viability). Extraction time lasted respectively 48 hours for oil, and 1 or 2 hours for the NaDES. In Figure 46 the comparison between 98% almond oil and pure almond oil is shown. According to the screening tests (section 4.3.3), the highest percentage of viability occurred with pure almond oil (55.2% for T1 and 43.7% for T2). There was not any significant difference among almond oils in terms of extraction efficiency.

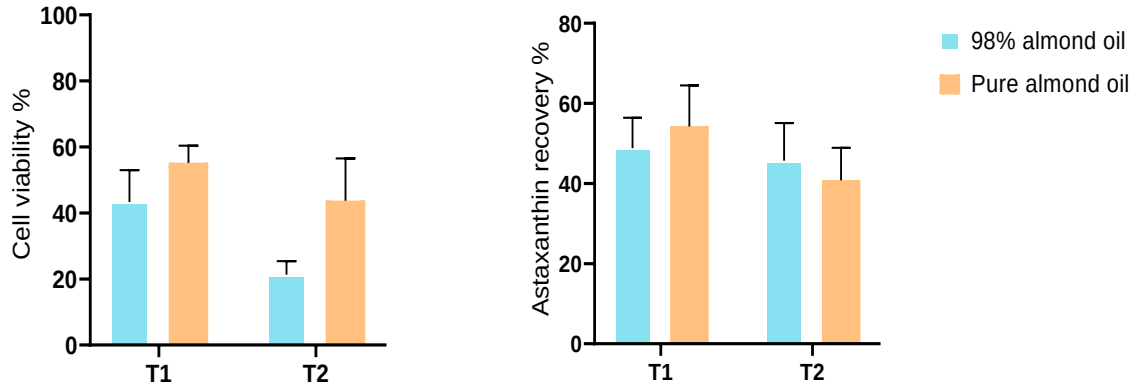

Figure 46 Milking test T1 and T2 with 98% almond oil and pure almond oil: cell viability (left) and astaxanthin recovery (right).

In Figure 47 menthol: oleic acid DES and almond oil efficiency for milking test is compared. Menthol: oleic acid DES efficiency in keeping cell viability is higher than almond oil (94.4%, 80.3%, 70.6% versus 55.2%, 43.7%, 48.9%). In milking test with menthol: oleic acid cell viability decreased with cell aging. This was not the same with almond oil. Astaxanthin recovery was higher with almond oil, but very low with menthol: oleic acid (1.9%, 2.1% and 0.5%). A downward trend

can be identified for extraction with almond oil (54.2% for younger cells, 40.8% and 31.1% for older cells). The differences between almond oil and menthol are significant (Addendum 4).

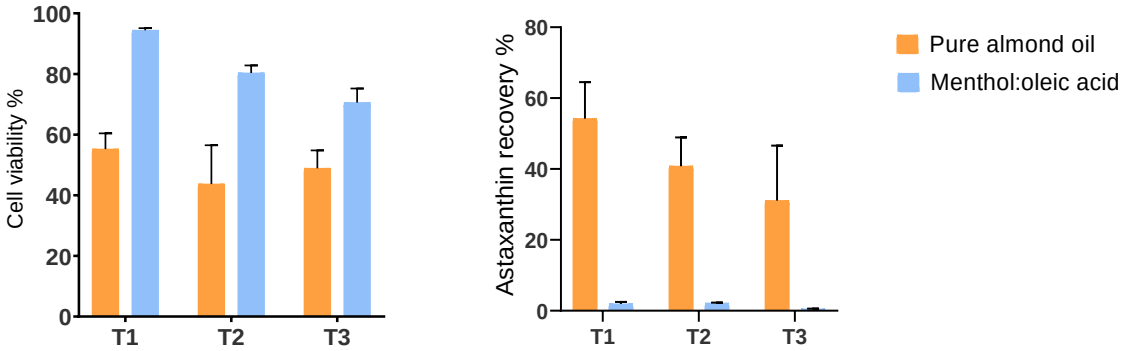

Figure 47 Milking test T1, T2, T3 with pure almond oil and menthol: oleic acid DES: cell viability (left) and astaxanthin recovery (right).

Note that the recovery rate in milking test with pure almond oil decreased with algal cells aging (Figure 47), although the level of astaxanthin increased (Table 14). As a matter of fact, the recovery rate was 54.2% in T1, 40.8% in T2 and 31.1% in T3. This probably happens because cell walls become thicker with cell aging. It is therefore more difficult for the solvent to cross the cell wall.

Another milking test with menthol: oleic acid DES was conducted by using young culture stressed for 1 and 2 months. In this test, extraction times were 1 and 2 hours. Younger cells viability (T1) did not change with time extraction, and it was about 100% (Figure 48). Older cells viability (T2) decreased significantly with extraction time (from 80.3% to 29.4%). As expected, astaxanthin extraction increased with extraction time, especially for older cells (T2, from 2.1% to 8.1%). Both graphs highlight that the difference between T1 and T2 are higher after 2 hours of extraction.

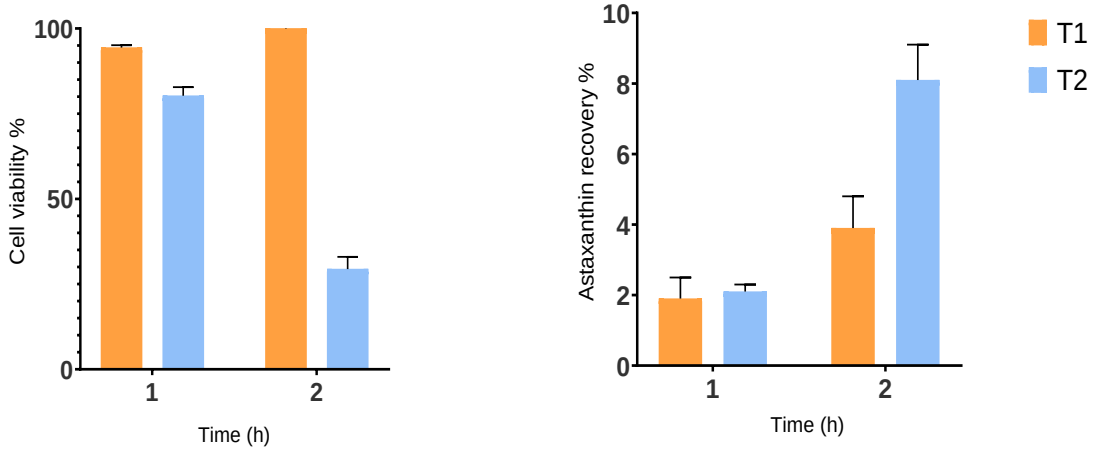

Figure 48 Milking test T1 and T2 with menthol: oleic acid for 1 and 2 hours: cell viability (left) and astaxanthin recovery (right).

4.4.1 RE-CULTIVATION OF *HAEMATOCOCCUS PLUVIALIS* AFTER MILKING

After each milking test, *H. pluvialis* cultures were re-cultivated under optimal conditions in order to verify their possible regenerative growth. Algal cells were washed with distilled H_2O, fresh medium was added, and they were put under light intensity of 80 µE/m^2/s (see section 3.1).

Semeraro (2018) demonstrates that after milking test *H. pluvialis* culture can be re-cultivated switching to the green phase, and it can synthetize new astaxanthin once back in stress phase.[87]

Table *22* shows timing of algal cells transitions from red phase to green phase (R→V) with the different solvents used in milking tests. All solvents allowed a new cycle of growth and stress, but with different times. Transition R→V of algal cells treated with menthol: oleic acid was slower than that with almond oil, respectively about 20 days and 10 days. Transition times did not seem to depend on algal culture age.

Table 22 Transition R→V of algal cells treated with menthol: oleic acid, almond pure oil, and 98% almond oil.

Solvent	Start of transition (R→V)	Complete transition (R→V)
Menthol: oleic acid	8 days	20 days
Almond pure oil	6 days	8 days
98% almond oil	6 days	8 days

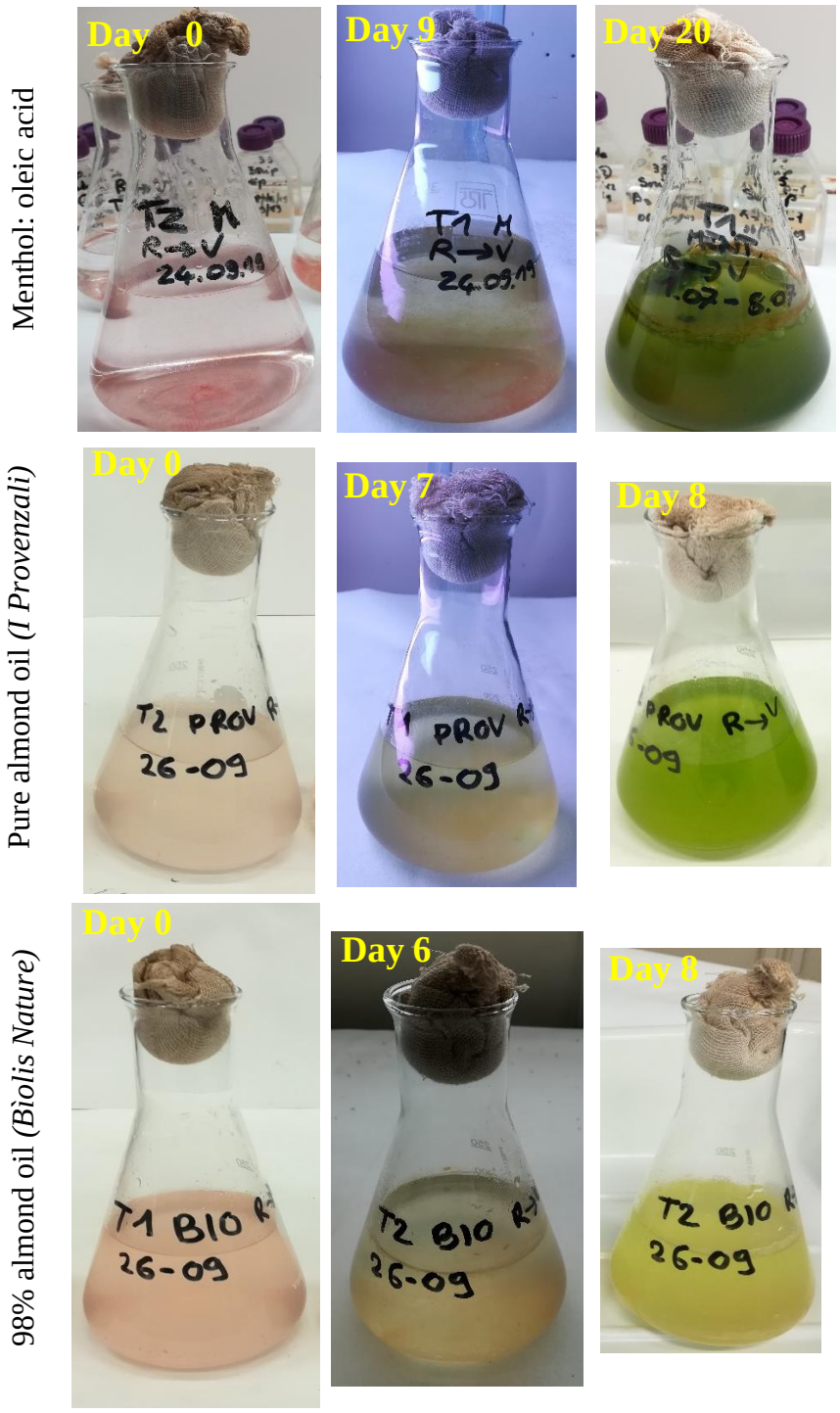

Figure 49 *H. pluvialis* transitions form red phase (after milking test) to green phase. In the first row (top) is represented a milking test with menthol:oleic acid; in the second row milking test is with pure almond oil; in the third row milking test is with 98% almond oil.

4.5 EFFECT OF ASTAXANTHIN ON ANIMAL CELLS

4.5.1 ASTAXANTHIN ANTIOXIDANT PROPERTIES

The results of the test on astaxanthin antioxidant properties are shown in Figure 50, which allows to compare the antioxidant activities of two different concentrations of natural and synthetic astaxanthin (0.1 and 1 µM). Moreover, two different scenarios are compared: quenching of basal ROS and induced ROS (by TBH).

The results show that astaxanthin has no effect on the basal ROS levels while it is a strong antioxidant able to recover the acute oxidative stress induced by TBH treatment of Hep G2 cells. The antioxidant power is not dose dependent at least in the concentration range used (0.1-1 µM) suggesting that its antioxidant activity is in the nanomolar range. Moreover, both natural and synthetic astaxanthin showed the same strong antioxidant power. According to the ANOVA, the differences between astaxanthin 0.1 µM and 1 µM as well as natural versus synthetic were not significant (Addendum 5).

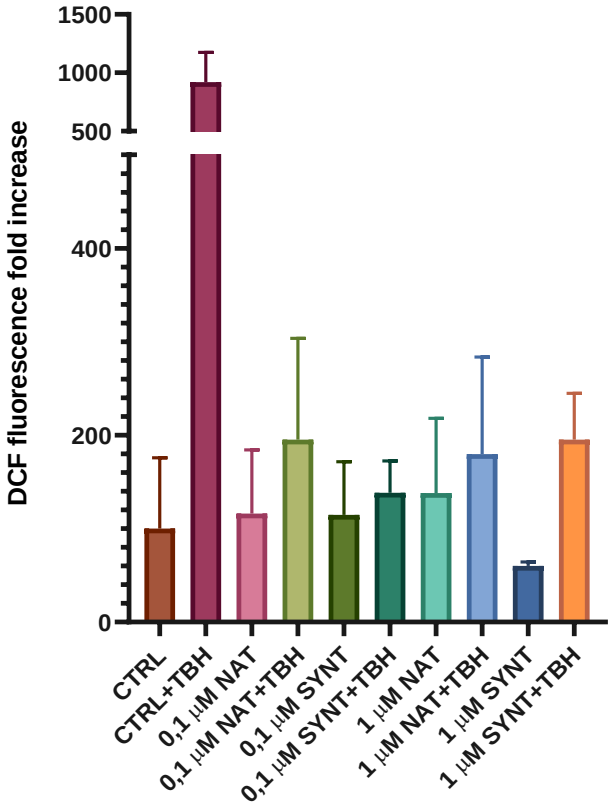

Figure 50 ROS levels in Hep G2 cells treated with natural and synthetic astaxanthin 0.1 and 1 µM.

4.5.2 LIPID CONTENT ANALYSIS: NILE RED

The size of lipid droplets was analysed on Hep G2 cells treated with natural and synthetic astaxanthin. An upward trend was identified as astaxanthin concentration increases only for synthetic astaxanthin (Figure 51). On the contrary, differences among lipid droplets size were not significant for natural astaxanthin (Addendum 6). Figure 52 shows that the size of lipid droplets in cells treated with synthetic astaxanthin was much larger than cells treated with natural astaxanthin. This means that only synthetic astaxanthin seemed to interfere with cellular lipid metabolism, even if the mechanism is not yet clear.

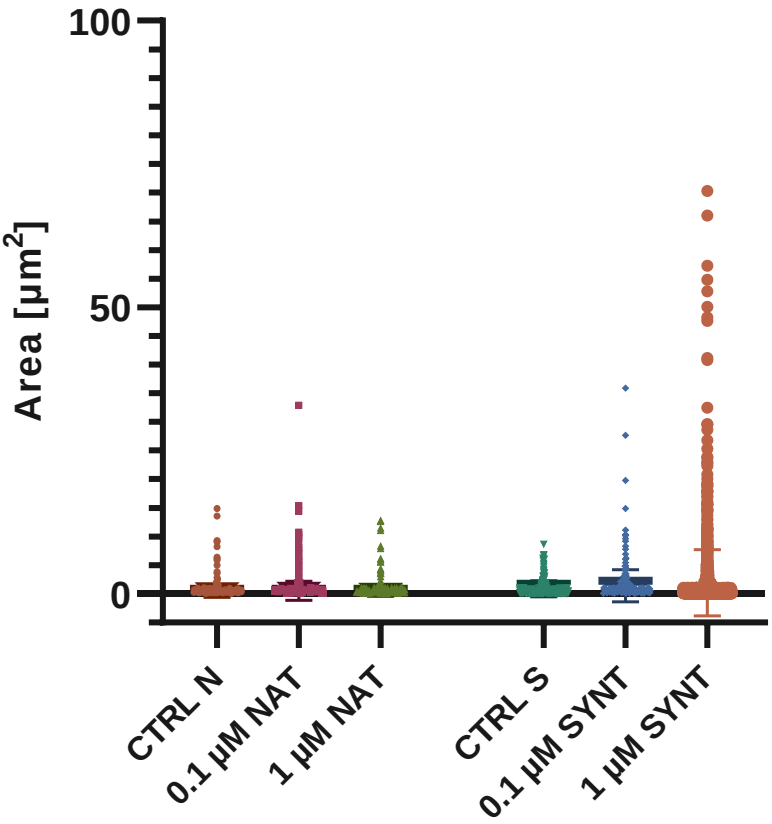

Figure 51 Size of lipid droplets in Hep G2 cells treated with natural and synthetic astaxanthin. CTRL N: control of cells treated with natural astaxanthin; CTRL S: control of cells treated with synthetic astaxanthin.

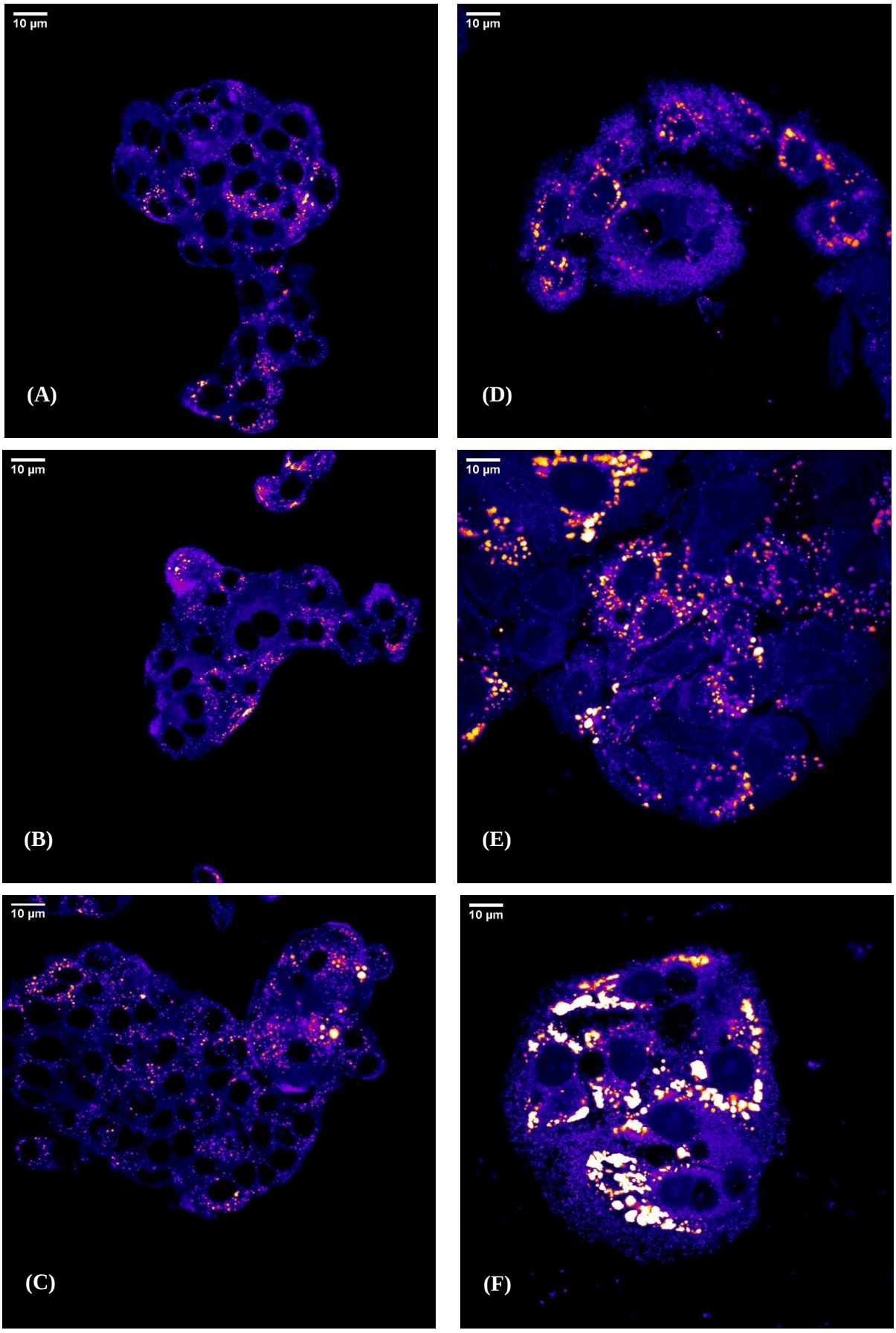

Figure 52 Confocal microscope pictures of Hep G2 cells treated with astaxanthin: (A) Control 1; (B) Natural astaxanthin 0,1 µM; (C) Natural astaxanthin 1 µM; (D) Control 2; (E) Synthetic astaxanthin 0,1 µM; (F) Synthetic astaxanthin 1 µM. Scale bars: 10 µm.

4.5.3 MEASUREMENT OF OXYGEN CONSUMPTION

Mitochondrial function was measured in terms of oxygen consumption per minute in the respiratory chain. Treatment with both synthetic and extracted astaxanthin did not mark any difference. There was a slight respiratory capacity increase in cells treated with synthetic astaxanthin, however it cannot be considered statistically significant (Figure 53).

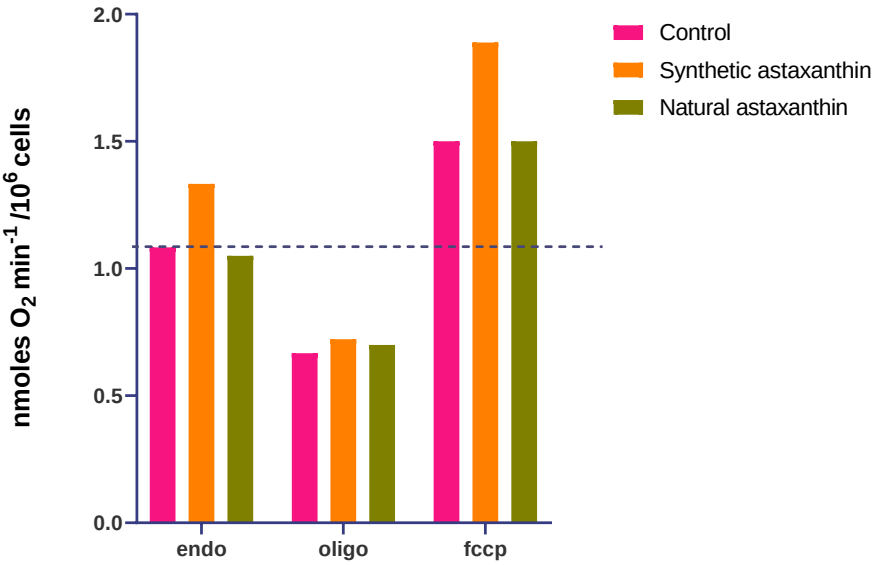

Figure 53 Oxygen consumption per minute in Hep G2 cells treated with natural and synthetic astaxanthin 0.1 μM. Endo: basal respiration; oligo: stop of respiration; fccp: maximum respiration.

5 CONCLUSIONS

To date, few studies have been conducted on the sustainable extraction of astaxanthin from *Haematococcus pluvialis.* The *Haematococcus pluvialis* microalga accumulates astaxanthin under stress conditions. After having tested different stress factors, such as acidic pH, salinity and ascorbic acid in combination with high light and nutrient deprivation, ascorbic acid was found to be the best factor for promoting astaxanthin production. As a matter of fact, in a range between 0,02-0,2 g/L, astaxanthin accumulation increased with acid concentration.

The main purpose of this research was to develop a sustainable extraction of astaxanthin from *H. pluvialis* by reaching the right compromise between good extraction efficiency and high viability of the algal culture. In fact, among the main limitations of *H. pluvialis* cultivation there are slow growth and low density. The application of milking extractions in which algal culture is kept alive, could solve this problem and reduce energy and economical costs associated to a new cultivation necessary after traditional extractions. The milking extractions were done using naturally derived solvents, suitable for human applications, like NaDESs and vegetable oils, in order to make the process sustainable for both the environment and human health. The best candidates for the milking test were menthol: oleic acid and almond oil, whereby an extract enriched in astaxanthin can be obtained for cosmetic, nutraceutical and food purposes. The best compromise between astaxanthin recovery and cell viability was achieved through a Liquid-Liquid Extraction with almond oil, whereby cell viability was 55 ± 5,2% and astaxanthin recovery was 54,2 ± 10,3%. Instead, menthol: oleic acid was able to maintain a very high viability (94.4 ± 0,7%) but the astaxanthin recovery rate was very low (1,9 ± 0,6%). Both solvents allow to re-cultivate algal culture to be subjected to new extractions. The transition time from green to red resulted shorter with almond oil (8 days against 20 days for menthol).

In future experiments, it should be recommended to find more insoluble acceptors/donors to be used as solvents in culture medium. As a matter of fact, by reducing cell contact with solvent, cell mortality should decrease. However, it should be also considered that, besides cellular mortality, the astaxanthin recovery rate could also decrease. Moreover, tests scale should be enlarged in order to observe any changes in yields; studies on *H. pluvialis* cell wall structure should be conducted to detect how much algal culture age and repeated extractions affect yields of the milking tests.

About the effects of astaxanthin on animal cells, the results show a high antioxidant power, however without any significant differences between synthetic and natural astaxanthin and even between the different concentrations tested. The reason could be found in astaxanthin

concentrations (0,1 and 1 μM) which were too low to give significant results. Therefore, higher concentrations of astaxanthin should be tested in the future. The results of animal cell tests (in particular of lipid metabolism) suggested that only synthetic astaxanthin seemed to have significant effects. This phenomenon was contradictory with the above-mentioned *in vivo* studies and this may suggest that the mechanism of astaxanthin absorption *in vitro* is different. *M. Mimoun-Benarroch et al. (2016)* study states that astaxanthin absorption may also depend from its isomer form, since genes activation of lipid carriers seems to be structure-dependent.[131] For this reason, further studies *in vitro* should be conducted in order to understand astaxanthin uptake into cells and the mechanism of action on lipid metabolism.

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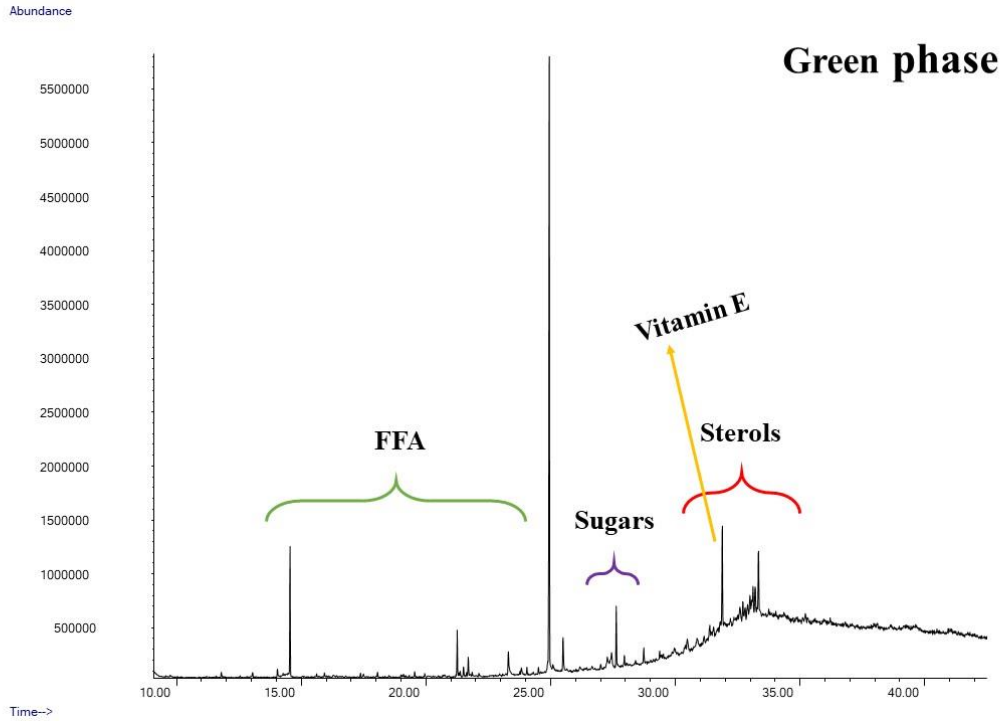
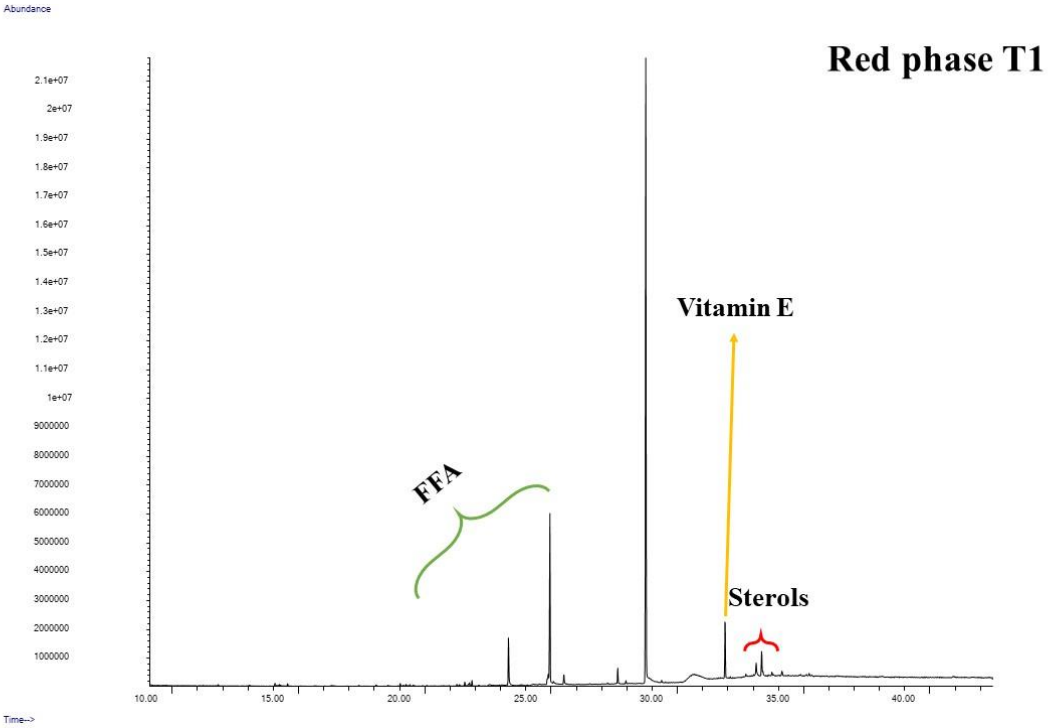
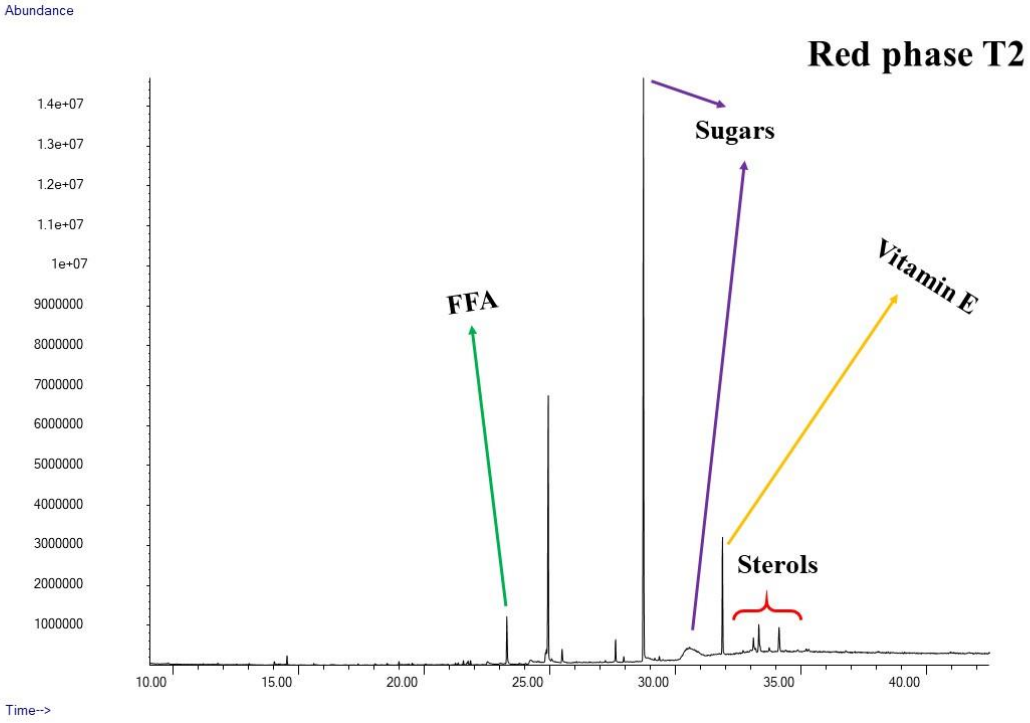
7 ADDENDA

Addendum 1

Chromatograms of silylated lipidic extracts (green phase, red phase T1, and red phase T2).







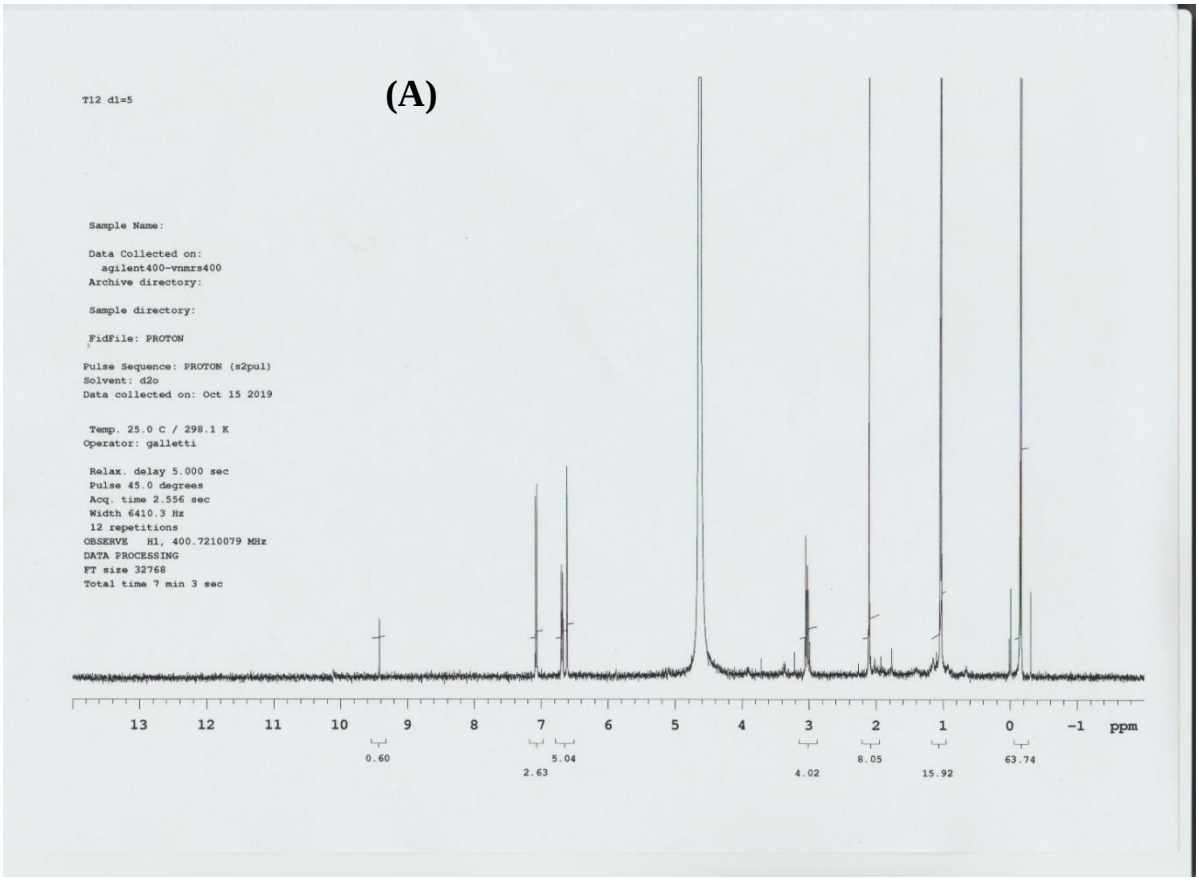
Addendum 2

One-way ANOVA on NaDESs astaxanthin extraction efficiency after 48 hours.

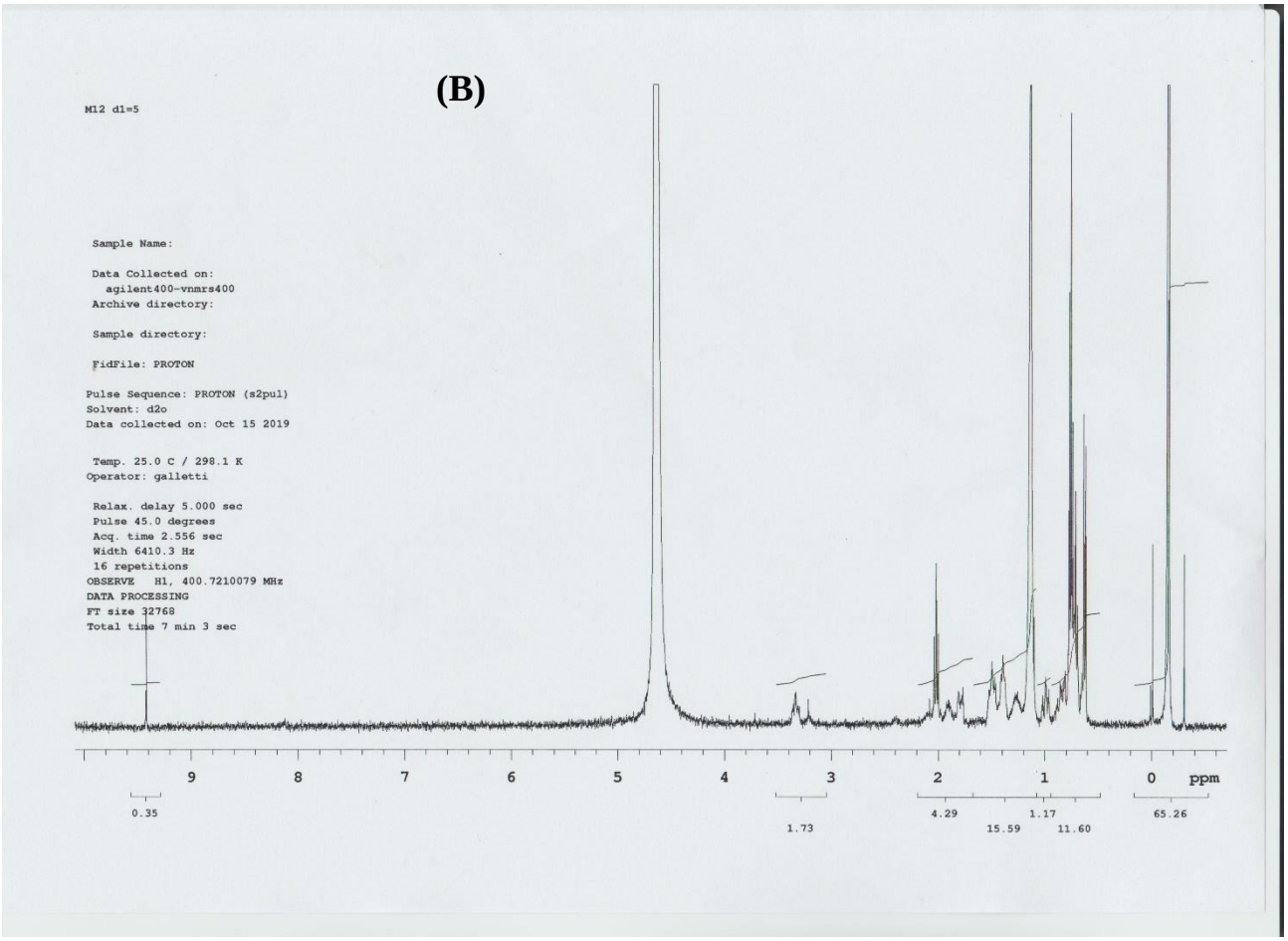
Number of families	1				
Number of comparisons per family	10				
Alpha	0,05				
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant ?	Summary	Adjusted P Value	
Menthol:(C12:0) vs. Menthol:(C18:1)	7,526	-9,199 to 24,25	No	ns	0,4591	A-B
Menthol:(C12:0) vs. Geraniol:(C12:0)	1,111	-15,61 to 17,84	No	ns	0,9985	A-C
Menthol:(C12:0) vs. Geraniol:(C18:1)	-4,630	-21,35 to 12,09	No	ns	0,7963	A-D
Menthol:(C12:0) vs. Thymol:(C18:1)	5,662	-11,06 to 22,39	No	ns	0,6742	A-E
Menthol:(C18:1) vs. Geraniol:(C12:0)	-6,415	-23,14 to 10,31	No	ns	0,5834	B-C
Menthol:(C18:1) vs. Geraniol:(C18:1)	-12,16	-28,88 to 4,568	No	ns	0,1477	B-D
Menthol:(C18:1) vs. Thymol:(C18:1)	-1,864	-18,59 to 14,86	No	ns	0,9892	B-E
Geraniol:(C12:0) vs. Geraniol:(C18:1)	-5,741	-22,47 to 10,98	No	ns	0,6646	C-D
Geraniol:(C12:0) vs. Thymol:(C18:1)	4,551	-12,17 to 21,28	No	ns	0,8051	C-E
Geraniol:(C18:1) vs. Thymol:(C18:1)	10,29	-6,432 to 27,02	No	ns	0,2353	D-E

Addendum 3

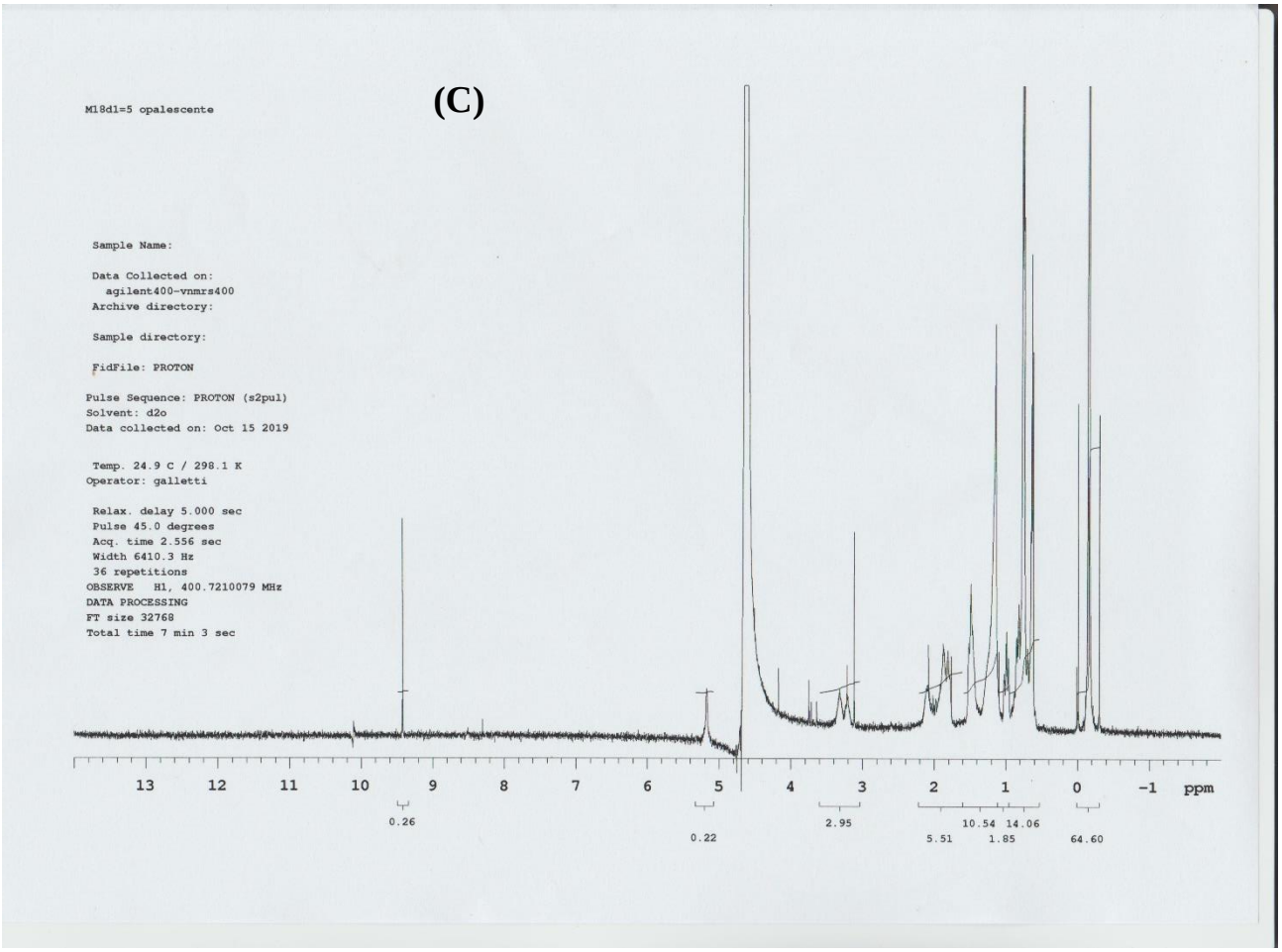
NMR spectra of NaDEs after contact with D_2O: (A) Thymol: oleic acid; (B) Menthol: lauric acid; (C) Menthol: oleic acid; (D) Geraniol: lauric acid; (E) Geraniol: oleic acid.
NMR spectra of astaxanthin: (F) Natural astaxanthin; (G) Synthetic astaxanthin.



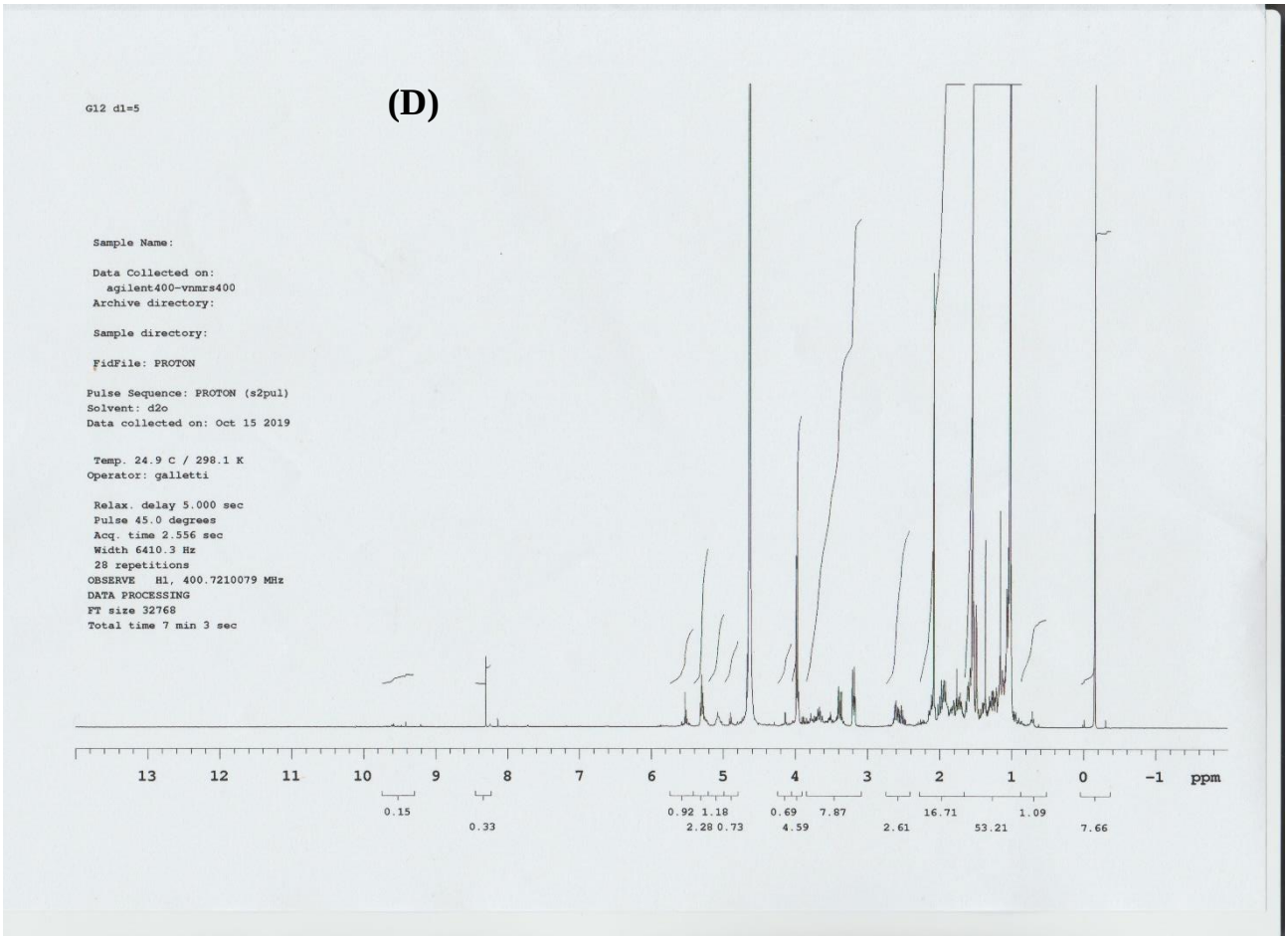
(B)


M12 d1=5
Sample Name:
Data Collected on:
agilent400-vnmrs400
Archive directory:
Sample directory:
FidFile: PROTON
Pulse Sequence: PROTON (s2pul)
Solvent: d2o
Data collected on: Oct 15 2019
Temp. 25.0 C / 298.1 K
Operator: galletti
Relax. delay 5.000 sec
Pulse 45.0 degrees
Acq. time 2.556 sec
Width 6410.3 Hz
16 repetitions
OBSERVE H1, 400.7210079 MHz
DATA PROCESSING
FT size 32768
Total time 7 min 3 sec
9 8 7 6 5 4 3 2 1 0 ppm
0.35
1.73
4.29
15.59
1.17
11.60
65.26

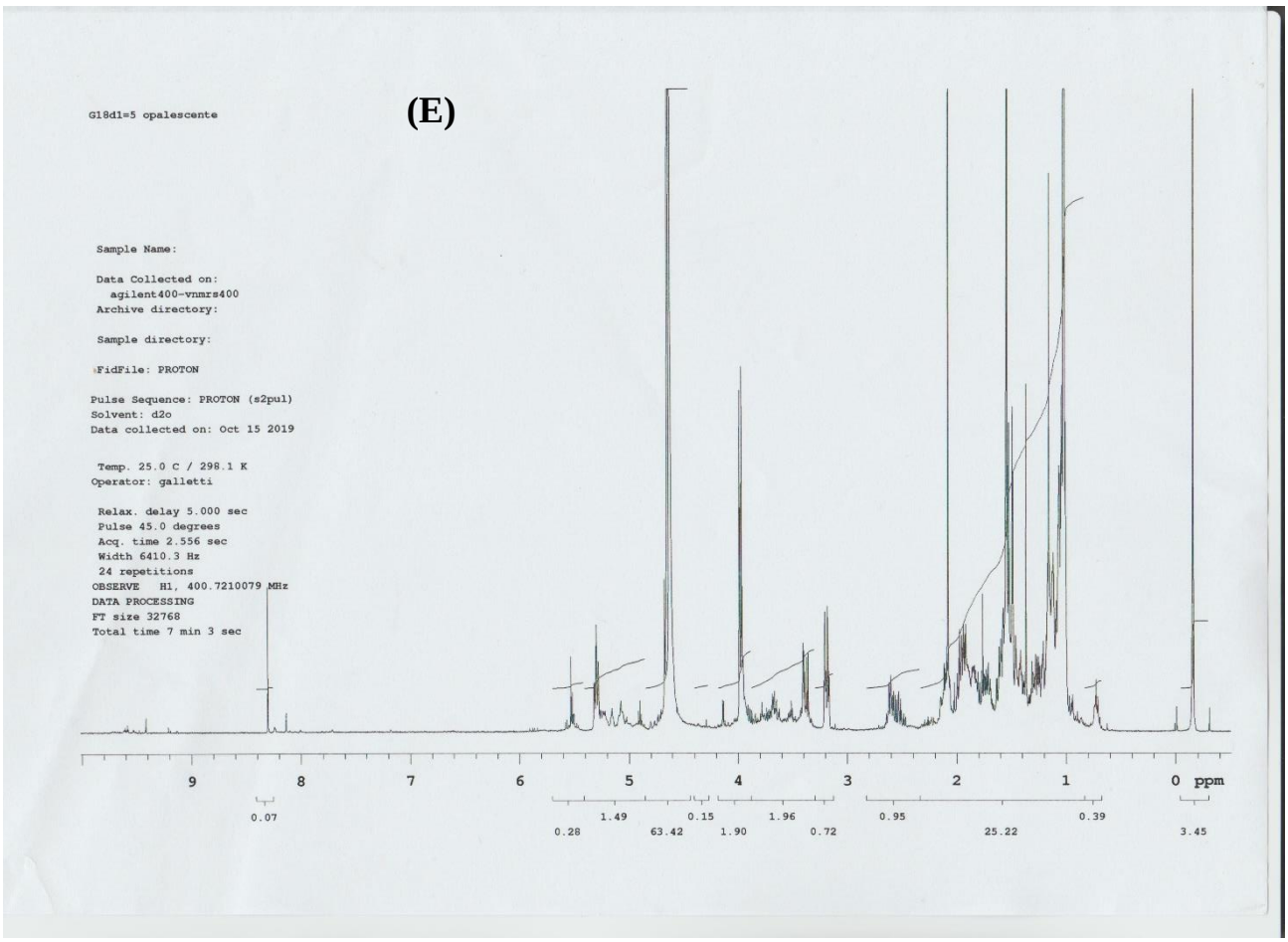
(C)


M18d1=5 opalescente
Sample Name:
Data Collected on:
agilent400-vnmrs400
Archive directory:
Sample directory:
FidFile: PROTON
Pulse Sequence: PROTON (s2pul)
Solvent: d2o
Data collected on: Oct 15 2019
Temp. 24.9 C / 298.1 K
Operator: galletti
Relax. delay 5.000 sec
Pulse 45.0 degrees
Acq. time 2.556 sec
Width 6410.3 Hz
36 repetitions
OBSERVE H1, 400.7210079 MHz
DATA PROCESSING
FT size 32768
Total time 7 min 3 sec
13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 ppm
0.26
0.22
2.95
5.51
10.54
1.85
14.06
64.60

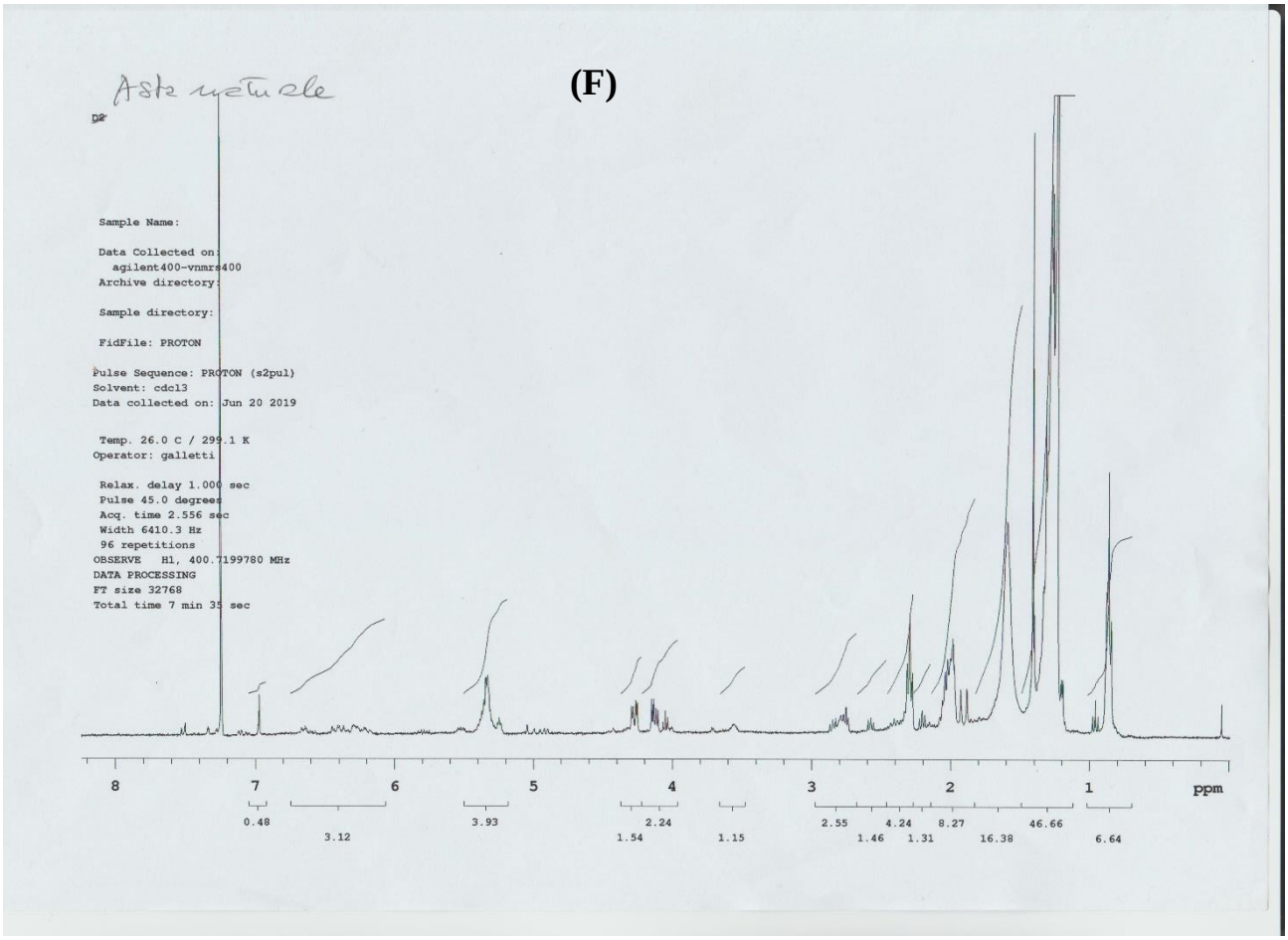
(D)


G12 d1=5
Sample Name:
Data Collected on:
agilent400-vnmrs400
Archive directory:
Sample directory:
FidFile: PROTON
Pulse Sequence: PROTON (s2pul)
Solvent: d2o
Data collected on: Oct 15 2019
Temp. 24.9 C / 298.1 K
Operator: galletti
Relax. delay 5.000 sec
Pulse 45.0 degrees
Acq. time 2.556 sec
Width 6410.3 Hz
28 repetitions
OBSERVE H1, 400.7210079 MHz
DATA PROCESSING
FT size 32768
Total time 7 min 3 sec
13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 ppm
0.15
0.33
0.92 1.18
2.28 0.73
0.69 7.87
4.59
2.61
16.71
53.21
1.09
7.66

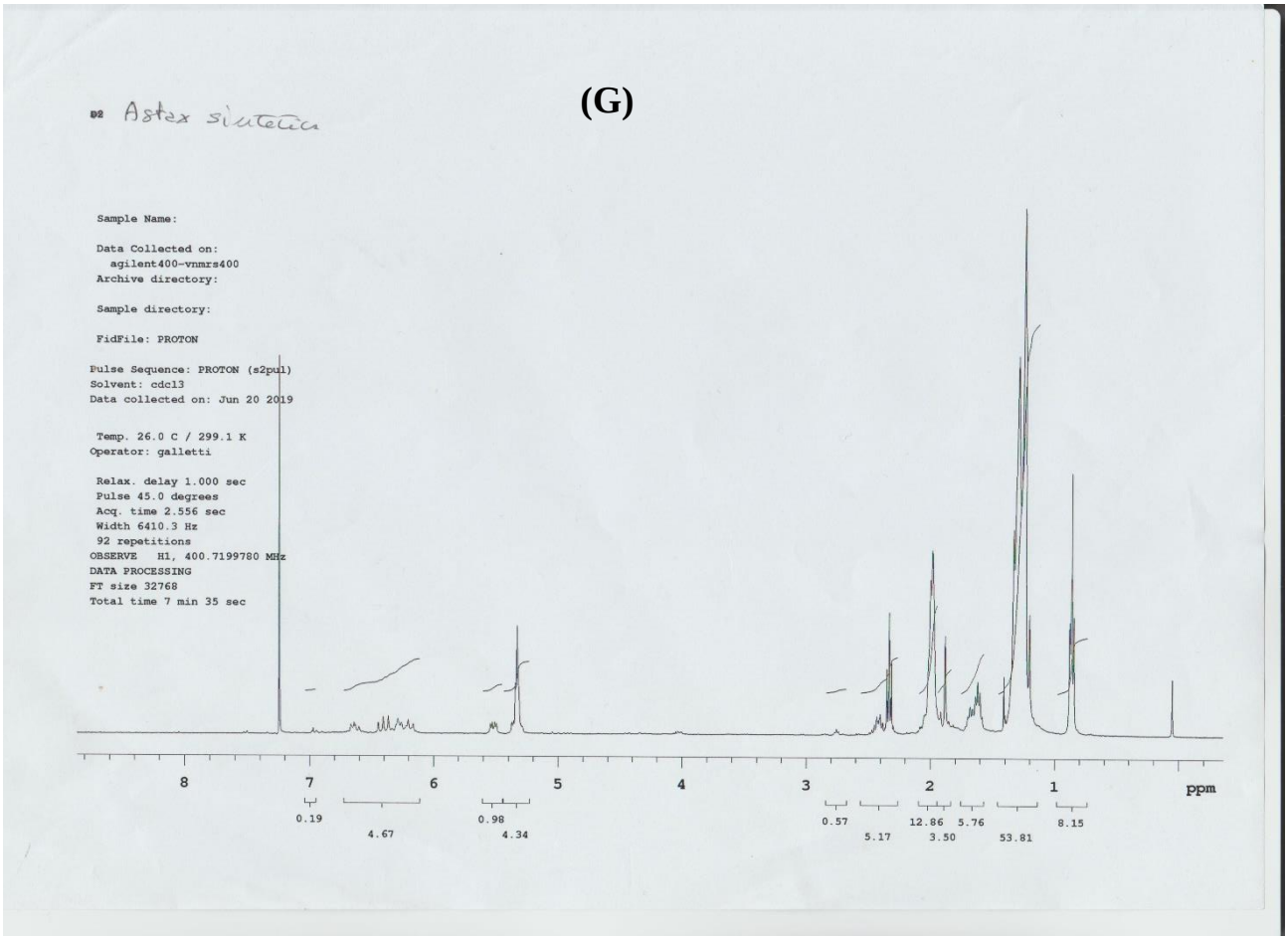
(E)


G18d1=5 opalescente
Sample Name:
Data Collected on:
agilent400-vnmrs400
Archive directory:
Sample directory:
FidFile: PROTON
Pulse Sequence: PROTON (s2pul)
Solvent: d2o
Data collected on: Oct 15 2019
Temp. 25.0 C / 298.1 K
Operator: galletti
Relax. delay 5.000 sec
Pulse 45.0 degrees
Acq. time 2.556 sec
Width 6410.3 Hz
24 repetitions
OBSERVE H1, 400.7210079 MHz
DATA PROCESSING
FT size 32768
Total time 7 min 3 sec
9 8 7 6 5 4 3 2 1 0 ppm
0.07
0.28
1.49
63.42
0.15
1.90
1.96
0.72
0.95
25.22
0.39
3.45

(F)


Asta naturale
Sample Name:
Data Collected on:
agilent400-vnmrs400
Archive directory:
Sample directory:
FidFile: PROTON
Pulse Sequence: PROTON (s2pul)
Solvent: cdcl3
Data collected on: Jun 20 2019
Temp. 26.0 C / 299.1 K
Operator: galletti
Relax. delay 1.000 sec
Pulse 45.0 degrees
Acq. time 2.556 sec
Width 6410.3 Hz
96 repetitions
OBSERVE H1, 400.7199780 MHz
DATA PROCESSING
FT size 32768
Total time 7 min 35 sec
8
7
6
5
4
3
2
1
ppm
0.48
3.12
3.93
2.24
1.54
1.15
2.55
1.46
4.24
1.31
8.27
16.38
46.66
6.64

(G)


Astax sintetica
Sample Name:
Data Collected on:
agilent400-vnmrs400
Archive directory:
Sample directory:
FidFile: PROTON
Pulse Sequence: PROTON (s2pul)
Solvent: cdcl3
Data collected on: Jun 20 2019
Temp. 26.0 C / 299.1 K
Operator: galletti
Relax. delay 1.000 sec
Pulse 45.0 degrees
Acq. time 2.556 sec
Width 6410.3 Hz
92 repetitions
OBSERVE H1, 400.7199780 MHz
DATA PROCESSING
FT size 32768
Total time 7 min 35 sec
8
7
6
5
4
3
2
1
ppm
0.19
4.67
0.98
4.34
0.57
5.17
12.86
3.50
5.76
53.81
8.15

Addendum 4

One-way ANOVA on the milkng tests with almond oil and menthol: oleic acid: (A) algal cell viability; (B) astaxanthin recovery.

Number of families	1
Number of comparisons per family	15
Alpha	0,05

(A)

Almond oil T1 vs. Almond oil T2	11,52	-6,316 to 29,36	No	ns	0,3175	A-B
Almond oil T1 vs. Almond oil T3	6,243	-11,60 to 24,08	No	ns	0,8400	A-C
Almond oil T1 vs. Menthol T1	-39,23	-57,07 to -21,40	Yes	****	<0,0001	A-D
Almond oil T1 vs. Menthol T2	-25,11	-42,95 to -7,276	Yes	**	0,0050	A-E
Almond oil T1 vs. Menthol T3	-15,41	-33,25 to 2,425	No	ns	0,1061	A-F
Almond oil T2 vs. Almond oil T3	-5,280	-23,12 to 12,56	No	ns	0,9112	B-C
Almond oil T2 vs. Menthol T1	-50,76	-68,59 to -32,92	Yes	****	<0,0001	B-D
Almond oil T2 vs. Menthol T2	-36,64	-54,48 to -18,80	Yes	***	0,0002	B-E
Almond oil T2 vs. Menthol T3	-26,94	-44,77 to -9,098	Yes	**	0,0029	B-F
Almond oil T3 vs. Menthol T1	-45,48	-63,31 to -27,64	Yes	****	<0,0001	C-D
Almond oil T3 vs. Menthol T2	-31,36	-49,20 to -13,52	Yes	***	0,0008	C-E
Almond oil T3 vs. Menthol T3	-21,66	-39,49 to -3,818	Yes	*	0,0149	C-F
Menthol T1 vs. Menthol T2	14,12	-3,718 to 31,96	No	ns	0,1559	D-E
Menthol T1 vs. Menthol T3	23,82	5,983 to 41,66	Yes	**	0,0075	D-F
Menthol T2 vs. Menthol T3	9,701	-8,137 to 27,54	No	ns	0,4855	E-F

(B)

Almond oil T1 vs. Almond oil T2	13,45	-9,335 to 36,23	No	ns	0,4040	A-B
Almond oil T1 vs. Almond oil T3	23,10	0,3115 to 45,88	Yes	*	0,0463	A-C
Almond oil T1 vs. Menthol T1	52,37	29,58 to 75,15	Yes	****	<0,0001	A-D
Almond oil T1 vs. Menthol T2	52,11	29,33 to 74,90	Yes	****	<0,0001	A-E
Almond oil T1 vs. Menthol T3	53,70	30,92 to 76,49	Yes	****	<0,0001	A-F
Almond oil T2 vs. Almond oil T3	9,646	-13,14 to 32,43	No	ns	0,7145	B-C
Almond oil T2 vs. Menthol T1	38,92	16,13 to 61,70	Yes	**	0,0010	B-D
Almond oil T2 vs. Menthol T2	38,66	15,88 to 61,45	Yes	**	0,0011	B-E
Almond oil T2 vs. Menthol T3	40,26	17,47 to 63,04	Yes	***	0,0008	B-F
Almond oil T3 vs. Menthol T1	29,27	6,487 to 52,05	Yes	**	0,0100	C-D
Almond oil T3 vs. Menthol T2	29,02	6,234 to 51,80	Yes	*	0,0106	C-E
Almond oil T3 vs. Menthol T3	30,61	7,825 to 53,39	Yes	**	0,0072	C-F
Menthol T1 vs. Menthol T2	-0,2536	-23,04 to 22,53	No	ns	>0,9999	D-E
Menthol T1 vs. Menthol T3	1,338	-21,45 to 24,12	No	ns	>0,9999	D-F
Menthol T2 vs. Menthol T3	1,591	-21,19 to 24,38	No	ns	0,9999	E-F

Addendum 5

One-way ANOVA on Hep G2 cells treated with natural and synthetic astaxanthin 0,1 and 1 µM, and with TBH.

Alpha	0,05					
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value	
CTRL vs. CTRL+TBH	-820,8	-888,7 to -752,8	Yes	****	<0,0001	A-B
CTRL+TBH vs. 0,1 µM SYNT+TBH	782,3	714,4 to 850,3	Yes	****	<0,0001	B-D
CTRL+TBH vs. 1 µM SYNT+TBH	725,5	651,1 to 799,9	Yes	****	<0,0001	B-F
CTRL+TBH vs. 0,1 µM NAT+TBH	725,5	666,4 to 784,6	Yes	****	<0,0001	B-H
CTRL+TBH vs. 1 µM NAT+TBH	741,0	681,0 to 801,0	Yes	****	<0,0001	B-J
0,1 µM SYNT+TBH vs. 1 µM SYNT+TBH	-56,84	-124,8 to 11,10	No	ns	0,1945	D-F
0,1 µM SYNT+TBH vs. 0,1 µM NAT+TBH	-56,82	-107,5 to -6,090	Yes	*	0,0146	D-H
0,1 µM SYNT+TBH vs. 1 µM NAT+TBH	-41,30	-93,03 to 10,42	No	ns	0,2520	D-J
1 µM SYNT+TBH vs. 0,1 µM NAT+TBH	0,02594	-59,11 to 59,16	No	ns	>0,9999	F-H
1 µM SYNT+TBH vs. 1 µM NAT+TBH	15,54	-44,45 to 75,53	No	ns	0,9982	F-J
0,1 µM NAT+TBH vs. 1 µM NAT+TBH	15,51	-23,92 to 54,95	No	ns	0,9641	H-J

Addendum 6

One-way ANOVA on Hep G2 cells treated with nile red.

Number of families	1					
Number of comparisons per family	15					
Alpha	0,05					
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value	
0.1 NAT vs. 1 NAT	0,1567	-0,2032 to 0,5166	No	ns	0,8165	A-B
0.1 NAT vs. CTRL NAT	0,07521	-0,3490 to 0,4994	No	ns	0,9960	A-C
0.1 NAT vs. 0.1 SYNT	-0,8819	-1,378 to -0,3857	Yes	****	<0,0001	A-D
0.1 NAT vs. 1 SYNT	-1,412	-1,791 to -1,034	Yes	****	<0,0001	A-E
0.1 NAT vs. CTRL SYNT	-0,3325	-0,9520 to 0,2871	No	ns	0,6450	A-F
1 NAT vs. CTRL NAT	-0,08149	-0,4533 to 0,2904	No	ns	0,9892	B-C
1 NAT vs. 0.1 SYNT	-1,039	-1,491 to -0,5864	Yes	****	<0,0001	B-D
1 NAT vs. 1 SYNT	-1,569	-1,888 to -1,250	Yes	****	<0,0001	B-E
1 NAT vs. CTRL SYNT	-0,4892	-1,074 to 0,09581	No	ns	0,1620	B-F
CTRL NAT vs. 0.1 SYNT	-0,9571	-1,462 to -0,4522	Yes	****	<0,0001	C-D
CTRL NAT vs. 1 SYNT	-1,488	-1,878 to -1,097	Yes	****	<0,0001	C-E
CTRL NAT vs. CTRL SYNT	-0,4077	-1,034 to 0,2189	No	ns	0,4305	C-F
0.1 SYNT vs. 1 SYNT	-0,5306	-0,9980 to -0,06317	Yes	*	0,0154	D-E
0.1 SYNT vs. CTRL SYNT	0,5494	-0,1279 to 1,227	No	ns	0,1891	D-F
1 SYNT vs. CTRL SYNT	1,080	0,4832 to 1,677	Yes	****	<0,0001	E-F