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Review Article

Astaxanthin and its Formulations as Potent Oxidative Stress Inhibitors

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ABSTRACT
Astaxanthin is one of the most potent antioxidant agents with a carotenoid chemical structure present in nature. This compound is common in some marine organisms and is abundantly present in *Haematococcus pluvialis* microalgae. Astaxanthin may exist in its "free" form or as part of esters derived from fatty acids and protein-derived complexes. There are studies that demonstrate the differences between the biological properties of astaxanthin and some of its derivatives. Astaxanthin may be relatively unstable or difficult to assimilate due to the intrinsic reactivity of the compound. Therefore, several studies have shown methods to obtain and evaluate astaxanthin formulations. This article describes studies that show the antioxidant properties of astaxanthin and some of its esters, both *in vitro* and *in vivo*. In addition, the article discusses some formulations based on emulsions and encapsulated micro- and nanoscale astaxanthins and the antioxidant properties of such formulations.
Key words: Antioxidant properties, Astaxanthin formulations, Encapsulates, Emulsions, Reactive oxygen species.

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INTRODUCTION

Astaxanthin is a red carotenoid that is present in some fish, crustaceans and microalgae. The most important natural source of astaxanthin is the microalga *Haematococcus pluvialis*. Astaxanthin is significant because it is a powerful antioxidant. Consequently, this compound can be useful in the prevention and control of chronic degenerative diseases, such as diabetes and cancer. In addition, it has been determined to be a safe antioxidant because it has no pro-oxidant properties analogous to β-carotene. Astaxanthin is mainly used in nutraceuticals, food and pharmaceuticals. In relation to the latter, Yamashita (2015) carried out a detailed analysis explaining why astaxanthin should be considered a drug.[1]

Extracts rich in astaxanthin derivatives, mainly fatty acid esters from natural sources such as *H. pluvialis*, are more active than synthetic astaxanthin. This could be attributed to astaxanthin derivatives being more stable than free astaxanthin. Additionally, the polyunsaturated fatty acid fragments present in these esters may have the ability to inhibit free radicals and thereby combat oxidative stress.

Astaxanthin is unstable and susceptible to degradation, especially when extracted from its biological matrix and exposed to environmental conditions. Therefore, various methods have been developed to obtain formulations that can prolong the half-life of this compound.[2] The stability of astaxanthin has been controlled in different storage conditions and using processes such as micro- and nano-encapsulation and emulsification.[3,4] The stability of astaxanthin depends primarily on pH, temperature, light and air.[2]

The objectives of this article are to analyse the structural characteristics of astaxanthin and its antioxidant properties under different conditions, as well as discuss formulations based on this compound from micro- and nano-scale encapsulation methods.

Chemical structure

The name astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) is derived from the crustacean *Astacus astacus* and belongs to the family of xanthophyll carotenoids. This molecule has two ionone rings linked through a polyunsaturated hydrocarbon chain of nine alternating double bonds, Figure 1. The presence of a terminal hydroxyl group and ketones in the ionone rings allows the possibility of esterification, high antioxidant activity and greater polarity in relation to other carotenoids. The thirteen conjugated double bonds present in astaxanthin give it its red colour. These bonds also contribute to the antioxidant activity of this compound by donating electrons and reacting with free radicals to convert them into a more stable product, which ends the free radical chain reaction in a wide variety of living organisms.[5] The two stereogenic carbon atoms at positions C-3 and C-3' in the ionone rings attached to the hydroxyl groups cause astaxanthin to exist in nature as a mixture of two enantiomers (3*R*,3'*R*- and 3*S*,3'*S*-astaxanthin) and a *meso* compound (3*R*,3'*S*), Figure 1.[6] Synthetic astaxanthin consists of a racemic

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mixture of the two enantiomers and the *meso* form.[7] This tetraterpene is sensitive to high temperatures, light and oxidative conditions that can cause isomerization of astaxanthin with *E* configuration to astaxanthin with *Z* configuration. *Z*-astaxanthin has less antioxidant activity than *E*-astaxanthin.[8] The *Z* isomers are thermodynamically less stable than the *E* isomers, Figure 2. Most carotenoids found in nature are predominantly all-*E* isomers.[9]

Natural sources

Astaxanthin and its esters are distributed widely and naturally in marine organisms, including crustaceans, such as shrimp and crabs and fish, such as salmon and sea bream, as well as microalgae. Astaxanthin exists in a free or esterified state, forming mono-esters and di-esters of fatty acids, Figure 3.[10] Derivatives of astaxanthin may also be carotene-proteins and carotene-lipoproteins. Commonly, astaxanthin is red, but when it forms complexes with several proteins, light absorption changes. This causes the colours of crustaceans containing astaxanthin-protein complexes in their shells, to vary from green, yellow and blue to brown. When these crustaceans are cooked, the they turn red because these proteins become denatured and release astaxanthin from their protein group. Astaxanthin was one of the first lobster carotenoids to be isolated and identified.[11] In crustaceans, astaxanthin accumulates mainly in the shell, with red-orange coloration that is released only after heat or solvent treatment. The primary stereoisomer of astaxanthin found in the Antarctic krill, *Euphausia superba*, is (3*R*,3'*R*) that mainly contains the esterified form, while in the Atlantic wild salmon the configuration is (3*S*,3'*S*) and exists in its free form.[12]

The astaxanthin in microalgae is produced at different concentrations, depending primarily on the species and the environmental conditions where they grow, Table 1.

Astaxanthin is the main carotenoid in *H. pluvialis* microalgae, forming complex esters with various fatty acids (mono- and di-esters). These microalgae could serve as continuous sources of astaxanthin after cultivation in large-scale bioreactors. Both microalgae and crustaceans have similar concentrations of astaxanthin. Red yeast *P. rhodozyma* synthesizes and accumulates astaxanthin as its main carotenoid, but at lower concentrations than in microalgae and crustaceans.[4] Unlike β-carotene, astaxanthin has no provitamin A activity.[24] Natural sources of astaxanthin are numerous, but astaxanthin is present in very low concentrations. The main source of astaxanthin is the green microalgae *H. pluvialis*, which is also known as *H. lacustris* or *Sphaerella lacustris*, Figure 4.

Antioxidant activity

In 1991 the antioxidant activity of astaxanthin, which proved to be very potent, was first reported.[25] This led to the increasing acceptance of astaxanthin as a nutritional supplement. Recently, there has been a sharp increase worldwide in both research and demand for natural astaxanthin in applications for human health. Astaxanthin is a unique antioxidant because it has three simultaneous distinctions: it is powerful; it is safe and it has a strategic position within the cell membrane because it bonds with the membrane from the inside out, Figure 5.[1,26] The inhibitory activity of astaxanthin in peroxy radical-mediated lipid peroxidation, with an effective dose (ED_{50}) of 200 nM, is more than 100 times greater than that of α-tocopherol, ED_{50} = 2940 nM, in the mitochondrial homogenate of rats.[4] Among the most common hydrophilic and lipophilic antioxidants, such as phenols, tocopherols, carotenoids, ascorbic acid, coenzyme Q10 and α-lipoic acid, astaxanthin has been shown to have the strongest singlet oxygen extinction activity ($^1O_2$) when used under the same test conditions.[24] The ability to eliminate hydroxyl radicals from astaxanthin encapsulated in liposomes has also been shown to be superior to that of α-tocopherol.[27] Astaxanthin is classified as a "pure antioxidant", since it does not possess any pro-oxidative property similar to β-carotene and lycopene.[28] Carotenoids, such as lycopene and β-carotene, induce disorder of the membrane bilayer enriched with polyunsaturated fatty acids and cause a sharp increase in the levels of lipid hydroperoxides (LOOH), while astaxanthin retains the structure of the membrane and exhibits significant antioxidant activity (40% decrease in LOOH levels).[29] Astaxanthin also has higher photostability in human dermal fibroblasts than canthaxanthin and β-carotene. In addition, astaxanthin efficiently blocks UVA rays.[30]

Astaxanthin, having a base structure of carotenoid, acts as an antioxidant capable of inactivating singlet oxygen and free radicals.[31] The antioxidant activity of astaxanthin is about 10 times higher than the antioxidant activity of other carotenoids, such as zeaxanthin, lutein and β-carotene. Table 2 shows the antioxidant capacity in trolox equivalents (TEAC) of some carotenoids, including astaxanthin.

Studies of the antioxidant activity of astaxanthin *in vitro*

Yi *et al*. (2018) carried out an evaluation of the antioxidant properties of astaxanthin in oil-in-water emulsions (*O*/*W*) containing neutral, anionic and cationic emulsifiers under conditions of photosensitization with chlorophyll.[34] The emulsions were irradiated with visible light for 24 h. The results of this study showed that 100 μM astaxanthin acts as an antioxidant in *O*/*W* emulsions with neutral and anionic emulsifiers through singlet oxygen inactivation.

Goto *et al*. (2001) evaluated the effects of astaxanthin and β-carotene on lipid peroxidation induced by ADP (adenosine diphosphate) and Fe^{2+} in liposomes and found that astaxanthin was about twice as effective as β-carotene.[35] The authors attributed this high antiperoxidative activity to not only the conjugated polyunsaturated chain, but also the trapping of a free radical by the C3 atom of astaxanthin. This discovery was made based on calculations of the relative stability of free radicals. Additionally, the authors theorized the formation of intramolecular and intermolecular hydrogen bonds with polar behavior and interconversion between the two hydrogen bond formations, Figure 6. In this way astaxanthin is able to trap free radicals both on the surface and inside the lipid membrane.

Study of the antioxidant activity of astaxanthin *in vitro* with cells

Kim *et al*. (2009) evaluated the antioxidant properties of astaxanthin in proximal tubular epithelial cells subjected to oxidative stress due to high concentrations of glucose, inflammation and apoptosis.[36] The evaluation of the antioxidant effect of astaxanthin was performed from key markers and the measurement of lipid peroxidation; as well as the concentration

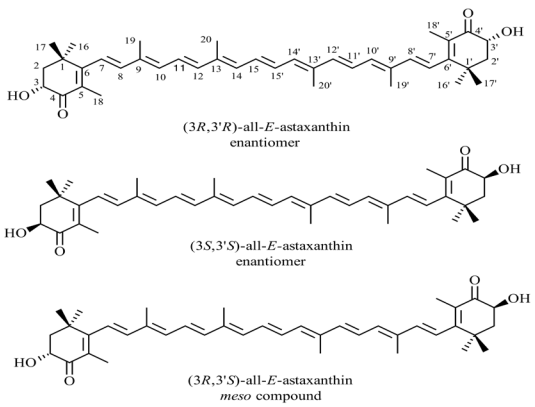

Figure 1: Stereoisomers of all-*E-astaxanthin* in its free form.

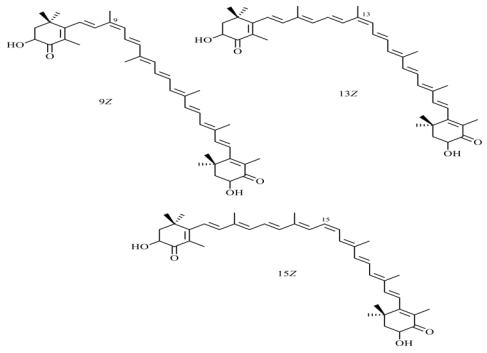

Figure 2: Structures of 9Z-, 13Z- and 15Z-astaxathin.

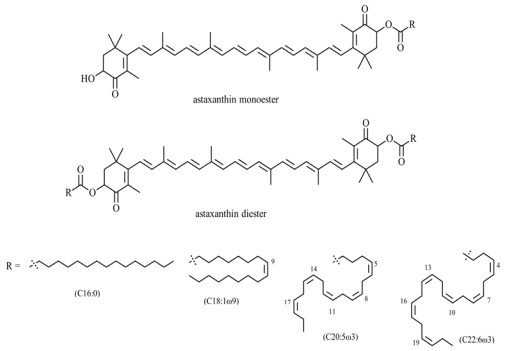

Figure 3: General structure of the mono- and di-esters of all-*E-astaxanthin*.

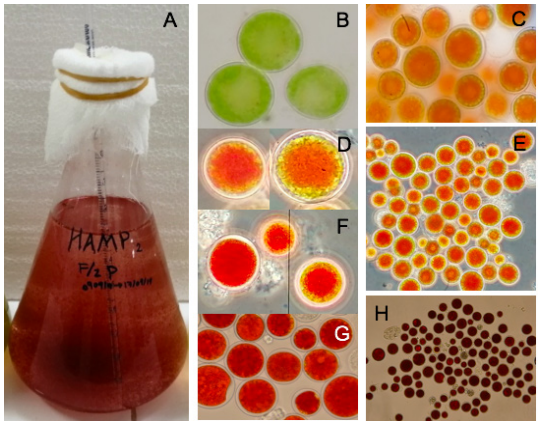

Figure 4: Cell morphology variation of *Haematococcus pluvialis* in different stages using fluorescent mercury lamp, A) reddish color culture, B) palmella green coccoid cells, C-E) immature aplanospores accumulating astaxanthin, F-G) haematocyst cells, mature cyst (aplanospore), H) cyst cell Lugol fixed.

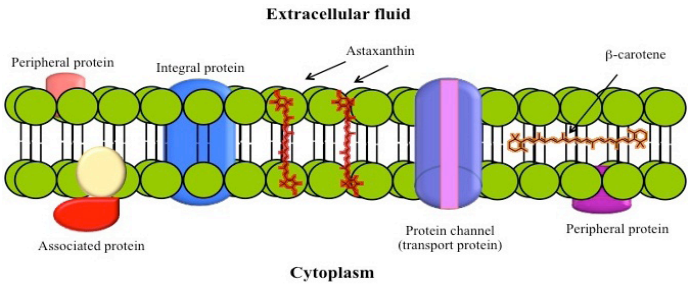

Figure 5: Schematic diagram of membrane bilipid structure and astaxanthin and β-caroteno distribution.

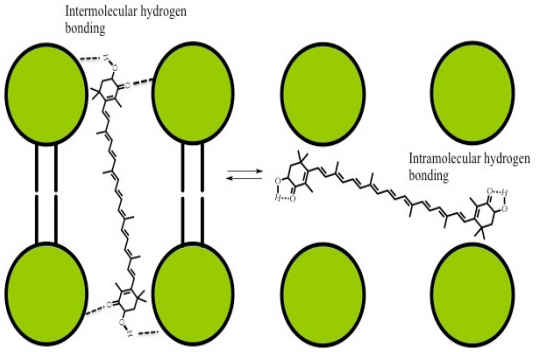

Figure 6: Possible positions of astaxanthin molecules with inter- and intra-molecular hydrogen bonds in the phospholipid membrane.[35]

of total reactive species, superoxide, nitric oxide and peroxynitrite. Expressions of inflammatory proteins, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear translocation factor-kappa B (NF-kB) and levels of Bcl-2/Bax protein were also measured. The results of this study showed that astaxanthin suppresses lipid peroxidation and the aforementioned reactive oxygen species. Also, it reduces the levels of Bax pro-apoptotic proteins and increases the Bcl-2 antipathotic.

Li *et al.* (2013) studied the cytoprotective effect of astaxanthin in ARPE-19 cells against oxidative stress caused by H_2O_2 and the possible mechanism involved in protection. ARPE-19 is a retinal pigment epithelial cell line (RPE).[37] These cells form stable monolayers which exhibit morphological and functional polarity. The results showed that astaxanthin decreased the loss of cellular viability induced by H_2O_2, cellular apoptosis and intracellular ROS generation. In addition, treatment with astaxanthin activated the Nrf2-ARE pathway. The Nrf2-ARE pathway is an essential cell defense mechanism against oxidative stress and can be evaluated *in vitro* through a western blot test.[38] Nrf2 is a transcription factor that induces the expression of a large number of cytoprotective and detoxifying genes that can be linked to the antioxidant response element (ARE) to induce the expression of phase II enzymes. Consequently, the phase II enzymes NQO1, HO-1, GCLM and GCLC mRNA and proteins were increased. Astaxanthin inhibited the expression of cleaved caspase-3 protein induced by H_2O_2. Activation of the phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) pathway was involved in the protective effect of astaxanthin in ARPE-19 cells. The PI3K/Akt pathway has an important role in modulating Nrf2-ARE dependent on protection against oxidative stress in RPE cells.

Regnier *et al.* (2015) conducted an exhaustive study of the antioxidant effect of extracts of *Haematococcus pluvialis* rich in esters derived from astaxanthin in human endothelial cells.[39] In that study several methods were applied to evaluate the antioxidant activity, among them the cellular antioxidant activity. This method involves using dichloro-dihydro-fluorescein diacetate (DCFCH-DA) as an oxidation sensitive indicator to measure the production of reactive oxygen species (ROS) in human umbilical vein endothelial cells (HUVEC). DCFCH-DA in the presence of oxidizing agents, becomes 2',7'-dichlorofluorescein, a fluorescent compound. The study showed that astaxanthin esters, derivatives of palmitic (C16:0), palmitoleic (C16:1ω7), oleic (C18:1ω9), linoleic (C18:2ω6), γ-linolenic (C18:3ω6) and stearidonic (C18:4ω3) acids, obtained from extracts of *H. pluvialis* exhibited antioxidant activity 90 times greater than synthetic astaxanthin. The *H. pluvialis* extracts evaluated in that study consisted of 62.6% monoesters and 36.9% diesters of astaxanthin.

Bolin *et al.* (2010) evaluated the effect of the use of astaxanthin *in vitro* to limit the production of oxygen radicals and behavioural lesions in human lymphocytes.[40] Active lymphocytes treated with 5 μM

astaxanthin showed an immediate decrease in $O_2^{\bullet-}/H_2O_2$ production, while intracellular $NO^{\bullet}$ and Ca^{2+} levels simultaneously increased. In long-term treatments (> 24 h), the cytotoxicity test for astaxanthin resulted in LC_{50} = 11.67 ± 0.42 μM. Higher superoxide dismutase and catalase activities in lymphocytes treated with 5 μM astaxanthin were associated with increased rates of minor oxidative lesions. These results suggest that astaxanthin has anti-inflammatory effects by preserving the structures of human lymphocytes sensitive to redox reactions.

Dose *et al*. (2016) determined the antiradical activity of synthetic astaxanthin by ESR and spin entrapment and the oxygen extinction activity by photon counting.[41] The cellular antioxidant activity of astaxanthin was examined through several bioassays, including transactivation of PON1, cellular levels of GSH, transactivation of Nrf2 and expression of its target genes and lipid peroxidation in cultured cell lines. The study showed that synthetic astaxanthin increased levels of cellular GSH in a dose-dependent manner in HepG2 cells. Consequently, the study authors suggested that an elevated GSH concentration may be a sign of lower levels of oxidative stress as a result of treatment with synthetic astaxanthin.

Wolf *et al*. (2010) studied the effect of astaxanthin on redox processes in human cervical cancer cells (HeLa) through a green fluorescent protein sensitive to redox processes (roGFP1).[42] The HeLa cells used in this study express the roGFP1 protein in both the cytosol and mitochondria. The results showed that astaxanthin decreased physiological oxidative stress and protected cultured cells against strong oxidative stress induced with a respiratory inhibitor. In addition, astaxanthin improved the conservation of a high mitochondrial membrane potential and stimulated respiration. Astaxanthin at nanomolar concentrations was effective in maintaining the mitochondria of HeLa cells in a reduced state under conditions of oxidative stress.

Farrugia *et al*. (2015) conducted a study of the effect of astaxanthin in bone marrow macrophages of wild mice, mice deficient in the nuclear factor related to factor 2 (Nrf2) and/or peritoneal macrophages and splenocytes of obese mice.[43] The results showed that astaxanthin has an anti-inflammatory effect because it inhibits the nuclear translocation of NF-κB p65 and prevents the accumulation of reactive oxygen species in Nrf2-dependent and independent mechanisms. NF-κB is a key transcription factor for the induction of proinflammatory genes.[44]

In vivo studies

Studies of the antioxidant effect of astaxanthin have also been performed *in vivo*. For example, Al-amin *et al*. (2015) studied the regiospecific and age-dependent antioxidant effects of astaxanthin in the brains of mice.[45] The mice, grouped by age, received supplements of 2 mg of astaxanthin/kg of body weight/day, for four weeks. To assess the antioxidant activity, the levels of non-enzymatic oxidative markers, malondialdehyde (MDA) and nitric oxide (NO), the product of advanced protein oxidation (APOP), were measured. In addition, measurements were made of the concentration of glutathione (GSH) and the enzymatic antioxidant activity of catalase (CAT) and superoxide dismutase (SOD) from isolated regions of the brain. The results demonstrated that astaxanthin reduces levels of MDA, APOP and NO in the cortex, striatum, hypothalamus, hippocampus and cerebellum in both young and old mice. Astaxanthin increases the activity of CAT and SOD enzymes and improves the level of GSH in the brain. The activity of astaxanthin is age-dependent, being greater in young mice than in older mice.

Che *et al*. (2018) carried out a comparative analysis of the effect of astaxanthin versus the astaxanthin ester derived from docosahexanoic acid (astaxanthin-DHA ester) in doubly transgenic APP/PS1 mice over a period of two months.[46] These mice are products of the cross between mice that overexpress at high levels the human mutant of the Amyloid Precursor Protein (APP) and mice that express the presenilin-1 protein (PS1). PS1 raises levels of β-amyloid 42 isoform, which has been identified in patients with Alzheimer's disease. APP/PS1 mice are characterized by β-amyloid deposits in a life span of six months. Senile β-amyloid plaques play a critical role in the pathogenesis of Alzheimer's disease.[47] Che *et al*. (2018) found that both astaxanthin and astaxanthin-DHA ester decrease the effects of Alzheimer's disease in varying degrees.[46] The astaxanthin-DHA ester was more effective than astaxanthin in improving learning and memory skills as shown by behavioural experiments with the mice studied. Additionally, mechanical research indicated that the astaxanthin-DHA ester was better able than astaxanthin to inhibit the generation of β-amyloid, regulate oxidative stress and suppress neuroinflammation, among others.

Takahashi *et al*. (2004) found that astaxanthin accumulates primarily in microsomal and mitochondrial fractions of yeast-fed chicken liver (*Phaffia rhodozyma*) which is rich in astaxanthin.[48] This induces a protective effect by astaxanthin against oxidative stress, leading to an improvement in the metabolic profile and a reduction in liver inflammation.[49]

Karppi *et al*. (2007) studied the effect of administration of astaxanthin supplements in capsules on lipid peroxidation in healthy, non-smoking Finnish men, aged 19 to 33 years, for three months in a double-blind randomized study design.[50] They also evaluated the absorption of astaxanthin from the capsules in the bloodstream and their safety. The intervention group received two capsules of 4 mg astaxanthin (Astaxin®) daily and the control group had two identical-looking placebo capsules. The results of this study showed that concentrations of 12- and 15-hydroxy fatty acids, products of fatty acid oxidation, in plasma were significantly reduced in the group that received astaxanthin, but not in the placebo group.

Choi *et al*. (2011) evaluated the effect of astaxanthin at doses of 5 mg and 20 mg/day for three weeks in a population of 23 people (twenty men and three women) who were overweight ($25.0 < BMI \leq 29.9$ kg/m^2) and obese (BMI ≥ 30.0 kg/m^2).[51] To assess the effect, these researchers employed four biomarkers: malondialdehyde (MDA), 15-isoprostane F2t (ISP), superoxide dismutase (SOD) and total antioxidant capacity (TAC). MDA is an aldehyde that is produced during the breakdown of lipid hydroperoxides and is commonly used as an indicator of lipid peroxidation. ISP is a prostaglandin that is used to measure the degree of damage from lipid peroxidation because it is caused by free radical-mediated lipoprotein peroxidation. SOD catalyzes the dismutation of superoxide anion to H_2O_2 and molecular O_2. Finally, TAC refers to the antioxidant activity of all constituents present in the medium that act against free radicals. The results obtained by Choi *et al*. (2011) showed a strong improvement in the four biomarkers for the two groups studied at the end of the three weeks of the study.

Encapsulation-based astaxanthin formulations

Astaxanthin is a highly unsaturated molecule which easily decomposes when exposed to heat, light and oxygen. In addition, its poor water solubility, stability and bioavailability limits its proper oral administration and administration *in vivo*. Consequently, there is a need to develop formulations that minimize these disadvantages.

The combination of astaxanthin with dietary oils increases its activity. For example, astaxanthin combined with fish oil increases the hypolipidemic/hypocholesterolemic effects in plasma as well as the phagocytic activity of activated neutrophils, compared to astaxanthin alone.[52] This combination also improves the immune response and decreases the risks of infectious and cardiovascular diseases. It also decreases the proliferative activity of B and T lymphocytes with a consequent decrease in production levels of superoxide anion ($O_2^{\bullet-}$),

H_2O_2 and nitric oxide ($NO^{\cdot}$) and increase of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase.[53] Astaxanthin is able to prevent structural changes in cells through the suppression of oxidative stress caused by fish oil. On the other hand, it has been shown that the dosage of *H. pluvialis* biomass, dispersed in olive oil, to rats increases the bioavailability and antioxidant properties of astaxanthin.[54] Consequently, it becomes important to review the methods used to obtain astaxanthin-based formulations.

General characteristics of the encapsulates

Encapsulation is a process for trapping a substance within another substance, producing particles with diameters from a nanometric scale to a millimetric scale, Figure 7. This process guarantees the stabilization and conservation of bioactive compounds through structured systems, as well as their release or supply under the desired conditions.[55] There are various encapsulation methodologies that can be applied to active compounds with a lipid nature, such as astaxanthin. Encapsulation can be achieved using physical, physical-chemical and chemical methods. Among the most commonly used physical encapsulation methods are spray drying, lyophilization, techniques based on supercritical fluids and solvent evaporation. Among the physical-chemical methods of encapsulation are coacervation, liposomes and ionic gelation. The chemical encapsulation methods are interfacial polymerization and molecular inclusion complexation.[56,57]

Materials used to encapsulate bioactive compounds must be GRAS (Generally Recognized as Safe). Among them, polysaccharides, lipids and proteins are the most relevant to produce encapsulates.[58] Some applications may require encapsulating materials with different properties implying the combination of materials of different chemical natures to provide a multifunctional system. The right combination of these materials can improve the properties of the delivery systems in terms of mechanical, thermal and barrier resistance, encapsulation efficiency, stability and bioavailability of bioactive compounds, compared to those obtained with a single material.

The capsules can be classified, according to their size, into macrocapsules (> 5000 µm), microcapsules (1 to 5000 µm) and nanocapsules (<1 µm).[59] The capsules, depending on their shape and construction, can be divided into microcapsules and microspheres; this is also applicable to nanoscale capsules. The microcapsules are particles that consist of an inner core that contains the active substance, covered with a layer of polymer that constitutes the membrane of the capsule. Mononuclear and polynuclear microcapsules can be distinguished by the division of the nuclei. Microspheres are matrix systems in which the nucleus disperses and/or dissolves uniformly in a network of polymers.[60] When the capsule structure is unknown, they are generically referred to as encapsulates.

Astaxanthin micro-encapsules

Jiang and Zhu (2019) carried out astaxanthin microencapsulation with a zein-oligochitosan complex under mild conditions by using a simple phase separation, achieving encapsulation efficiencies of approximately 95% to form encapsulates with a particle size of 9.0 ± 0.9 µm.[61] Zein, one of the encapsulating agents, is a prolamine protein found in corn. The second encapsulant, oligochitosan, is an amino oligosaccharide composed of randomly distributed units of β-(1-4) *D*-glucosamine (deacetylated units) and *N*-acetyl-*D*-glucosamine (acetylated units). In this study the encapsulation of astaxanthin was verified through FT-IR and morphological observation. The forces that allowed the formation and stabilization of astaxanthin capsules in zein-oligochitosan were hydrogen bridges, hydrophobic interactions and electrostatic interactions. This method of encapsulating astaxanthin significantly improved the stability of astaxanthin during its exposure to UV light. Additionally, these capsules are homogeneously dispersed in alcoholic and acidic solutions. However, a release of astaxanthin was observed in the alcoholic solutions stored for four weeks.

Astaxanthin nano-encapsules

Nano-capsules offer several advantages over microcapsules. These advantages include greater total contact surface, greater solubility, absorption and controlled release, among others. The nanoencapsulation systems are divided into five groups: nano-carriers, special equipment-based techniques, biopolymer nanoparticles and two other techniques.[59] In the literature there are reports of studies related to producing astaxanthin nano-encapsules. For example, Tachaprutinum *et al*. (2009) performed the encapsulation of polymer nano-spheres by solvent displacement.[62] The method consisted of dialyzing a solution made of the polymeric material and astaxanthin in dimethylformamide, against water. These authors found that when using poly-(ethylene oxide)-4-methoxycinnamoylphthaloyl-chitosan (PCPLC), encapsulation efficiencies of 98% were obtained. This study also evaluated the thermal stability of the nano-spheres at temperatures of 70°C for periods of 2 h without finding significant changes. The tests of controlled release of astaxanthin in acetone showed that after 60 h in the solution, 80% of the astaxanthin had been released from the nano-spheres.

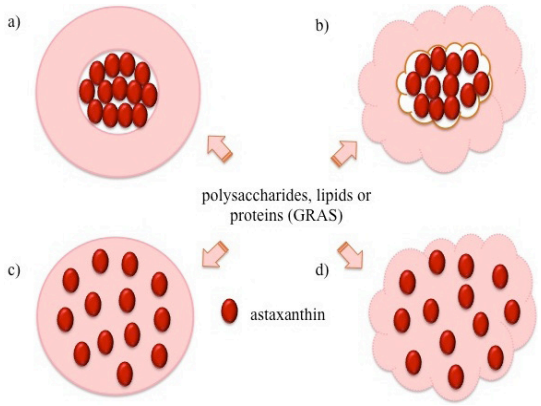

Figure 7: a) Spherical or b) Irregular microcapsules (white interior represents the core material of microcapsules); and c) spherical or d) irregular micro-spheres.

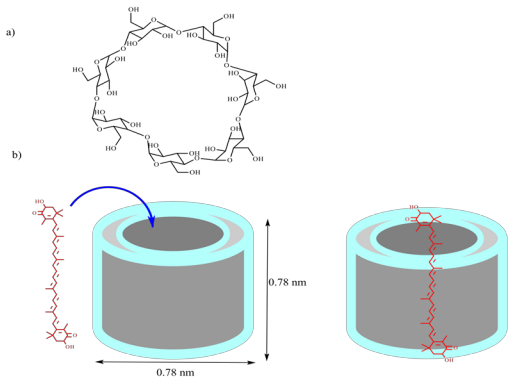

Figure 8: a) β-Cyclodextrin molecule. b) Representation of astaxanthin in the inclusion complex.

Table 1: Production of astaxanthin in microalgae.

Microalgae strain	Growing conditions	Yield (%, on dry basis)	Source
Chlorella zofingiensis CCAP211/14	Photoautotrophic, batch	0.37	Del Campo *et al.* 2004[13]
Chlorella zofingiensis SAG211/14	Photoautotrophic, batch	0.65	Orosa *et al.* 2001[14]
Chlorella zofingiensis ATCC 30412	Heterotrophic, batch	1.3	Ip and Chen, 2005[15]
Chlorella zofingiensis ATCC 30412	Heterotrophic, fed-batch	3.2	Sun *et al.* 2008[16]
Chlorococcum sp.	Mixotrophic, batch	2.1	Liu and Lee 2000[17]
Chlorococcum sp.	Mixotrophic, batch	0.7	Ma and Chen 2001[18]
Haematococcus pluvialis CCAP34/7	Photoautotrophic, batch	2.7	Harker *et al.* 1996[19]
Haematococcus pluvialis SAG19-a	Photoautotrophic, batch	2.0	Brinda *et al.* 2004[20]
Haematococcus pluvialis NIES-144	Photoautotrophic, batch	2.4	Zang *et al.* 2016[21]
Haematococcus pluvialis JNU35	Mixotrophic, batch	5.6	Wang *et al.* 2019[22]
Haematococcus pluvialis NIES-144	Photoautotrophic, batch	7.7	Kang *et al.* 2005[23]

Table 2: Antioxidant capacity (TEAC) of some common carotenes.

Compound	TEAC (mM)	Source
Astaxanthin	0.03 ± 0.03	Miller *et al.* 1996[32]
β-carotene	1.61 ± 0.07	Van der Berg *et al.* 1999[33]
Lycopene	2.31 ± 0.07	Van der Berg *et al.* 1999[33]
Zeaxanthin	1.70 ± 0.05	Van der Berg *et al.* 1999[33]
Lutein	1.40 ± 0.04	Miller *et al.* 1996[32]
β-criptoxanthin	1.65 ± 0.02	Van der Berg *et al.* 1999[33]

Astaxanthin molecular inclusion complexes with cyclodextrins

Molecular inclusion is an encapsulation technique that takes place at the molecular level and involves the entrapment of a guest molecule inside the cavity of a host molecule through physico-chemical forces, such as hydrogen bonds, van der Waals forces or hydrophobic interactions.[63] These complexes are formed through a reaction that takes place only in the presence of water.[64] The most common "host" molecules are Cyclodextrins (CD), which are composed of a hydrophilic outer part and an internal hydrophobic part. The cyclodextrins form inclusion complexes that contain cavities which can be occupied by molecules of low solubility in the aqueous media.[64] Inclusion complexes may exhibit improved chemical or biological properties relative to the host molecule. Inclusion can improve aqueous biological properties, aqueous solubility, dissolution and bioavailability.[65] Inclusion can also increase the physico-chemical stability of medications[65] and improve their shelf life;[65] modify the medication administration site and dosing time profile;[66] reduce or eliminate unpleasant taste and smell;[66] prevent interactions between medications or medications with excipients;[67] and convert liquid medications into microcrystalline or amorphous powders.[63] Dong *et al.* (2014) carried out a study of the inclusion complex of astaxanthin with hydroxypropyl-β-cyclodextrin, Figure 8, performing an optimization with the response surface methodology, UV spectroscopic profiles, FT-IR, thermogravimetric analysis (TG) and differential thermal analysis (DTA), properties of water solubility, storage stability, as well as an evaluation of the activity of uptake of 1,1-diphenyl-2-picrilhydrazyl radical (DPPH·) and reducing power.[68] This study showed that it is possible to form complexes at a 500:1 mass ratio hydroxypropyl-β-cyclodextrin/astaxanthin, with an inclusion percentage of 30.4 ± 1.3%. Due to the formation of the inclusion complex, the decomposition temperature of astaxanthin increases by approximately 50°C. The reducing power and DPPH· Radical scavenging activities of the inclusion complexes were much higher than those of pure astaxanthin and the inclusion complexes showed good solubility in water with a concentration of up to 50 mg/mL. Astaxanthin in the inclusion complex with hydroxypropyl-β-cyclodextrin is quite stable with respect to light and temperature.

Astaxanthin nanoemulsions

Nanoemulsions are emulsions with a droplet size of the order of 100 nm. A typical nano-emulsion contains oil, water and an emulsifier. The addition of an emulsifier is essential for the formation of nanoprobes, since it reduces the interfacial tension between the oil and water phases of the emulsion. The emulsifier also plays an important role in the stabilization of nano-emulsions through repulsive electrostatic interactions and steric hindrance.[69] The emulsifier used is a surfactant and the preparation of nano-emulsions can be carried out through either high energy or low energy methods.[70] Among the high energy methods are high pressure homogenization (HPH)[71] and ultrasound.[72] Low energy methods exploit the specific properties of the system to produce small drops without significant energy consumption. Among these methods are spontaneous emulsification,[73] phase inversion temperature (PIT)[74] and the emulsion inversion point (EIP).[75,76] Nano-emulsions give the product characteristic properties that can be important in the fields of personal care, cosmetics and health sciences. This is because they reduce the adverse effect of gravity on the emulsion with respect to systems with larger particle sizes and, therefore, reduce sediment formation, decrease flocculation or coalescence and make the delivery of active ingredients effective and homogeneous.

Nano-emulsions containing astaxanthin as a bioactive principle may be useful for developing anti-aging skin products. For example, Kim *et al.*[77] produced oil nano-emulsions in water with astaxanthin through high pressure homogenization (HPH).[78] In that study they optimized the influence of emulsification conditions, which include the type of emulsifier, concentration, passage time, astaxanthin concentration and co-antioxidants. In the study they used two emulsifying agents, glyceryl/citrate/lactate/linoleate/oleate and hydrogenated lecithin. The results showed that astaxanthin was stable in the nano-emulsion over a one-month test cycle.

Self-emulsifying drug delivery systems (SEDDS) are homogeneous, isotropic, thermodynamically stable dispersions formed by oil, surfactant and co-surfactant / co-solvent. These form nano-emulsions with low energy demand. Self-emulsification is generated with the help of gentle *in vivo* agitation provided by gastrointestinal motility.[78] SEDDS reduce the energy associated with a solid-liquid phase transition and prevent the dissolution process after oral intake. When the SEDDS drug is solubilized in submicron drops of oil, it offers a large contact area.[79] In addition, fine drops of oil quickly transfer from the stomach and promote a wide distribution of the drug throughout the gastrointestinal tract, thus minimizing irritation. Recently, Mao *et al*.[80] developed a solid self-emulsification system to improve the bioavailability of astaxanthin in an oral administration formulation.[80] Initially, the system was in a liquid state and became solid when mixed with a solid absorbent vehicle, promoting the encapsulation of astaxanthin. The substances used as vehicles were silicon dioxide and calcium acid phosphate. Both vehicles allowed a sustained release of astaxanthin. The authors found that encapsulated astaxanthin does not lose its antioxidant activity. The *in vitro* digestion rate was slower in the calcium acid phosphate formulation than in the silicon oxide.

CONCLUSION

Astaxanthin is a powerful antioxidant agent that has a protective effect against reactive oxygen species. Likewise, astaxanthin formulations based on structured systems in liquid or solid phase, such as nano-emulsions, micro- and nano-encapsulated forms, can lead to the stabilization of astaxanthin during intake. This behavior has been observed in *in vitro* digestion systems. Studies have shown there is no single method that meets all the transport and protection needs of astaxanthin. The formulation method to use will depend on the application.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare and are responsible for the content and writing of the manuscript.

ABBREVIATIONS

ADP: Adenosine diphosphate; **APP:** Amyloid precursor protein; **APP/PS1:** Double transgenic mice; **APOP:** Advanced protein oxidation product; **ARE:** Antioxidant response element; **ARPE-19:** Retinal pigment epitelial cell line; **BMI:** Body mass index; **CAT:** Catalase; **CD:** Cyclodextrins; **COX-2:** Cyclooxygenase-2; **C16:0:** Palmitic acid; **C16:1ω7:** Palmitoleic acid; **C18:1ω9:** Oleic acid; **C18:2ω6:** Linoleic acid; **C18:3ω6:** ɣ-linolenic acid; **C18:4ω3:** Stearidonic acid; **DCFCH-DA:** Dichloro-dihydrofuorescein diacetate; **DHA:** Docosahexanoic acid; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **DTA:** Differential termal analysis; **ED_{50}:** 50% effective dose; **EIP:** Emulsion inversion point; **ESR:** Electron spin resonance; **FT-IR:** Fourier transform infrared; **GRAS:** Generally recognized as safe; **GSH:** Glutathione; **HeLa:** Human cervical cancer cells; **HepG2:** Human liver cancer cell line; **HUVEC:** Human umbilical vein endotelial cells; **HPH:** High pressure homogenization; **iNOS:** Nitric oxide synthase; **LOOH:** Lipid hydroperoxides; **MDA:** Malondialdehyde; **NF-Kb:** Nuclear translocation factor-kappa B; **NO:** Nitric oxide; **Nrf2:** Transcription factor; **$^1O_2$:** Strongest singlet oxygen extinction activity; **O/W:** Oil in water emulsions; **PCPLC:** Poly(ethylene oxide)-4methoxycinnamoylphthaloyl-chitosan; **PIT:** Phase inversión temperature; **PI3K/Akt:** Phosphatidylino-sitol-3-kinase/protein kinase B; **PON1:** Paraoxonase 1; **PS1:** Presenilin-1-protein; **RoGFP1:** Basic (constitutively fluorescent) green fluorescent protein; **ROS:** Reactive oxygen species; **RPE:** Retinal pigment epitelial; **SEDDS:** Self-emulsifying drug delivery systems; **SOD:** Superoxide dismutase; **TAC:** Total antioxidant capacity; **TEAC:** Trolox equivalents of antioxidant capacity; **TG:** Thermogravimetric analysis; **Q_{10}:** Coenzyme Q10.

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