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The Bioavailability of Astaxanthin Is Dependent on Both the Source and the Isomeric Variants of the Molecule

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Abstract

Astaxanthin is a marine carotenoid that has a number of potential health benefits, including a very strong antioxidant potential. Present in the flesh of salmonids and shellfish, its natural sources currently on the market for food supplements come from the algae *Haematococcus pluvialis* and krill. However other natural sources can be found and may be of interest. Cellular uptake studies were performed on Caco-2/TC7 colonic cells. The cells were cultured on a semi-permeable membrane to create a polarized and functional epithelium, representative of the intestinal barrier. Four sources of astaxanthin were selected and compared; synthetic, natural extracts from bacteria, algae or yeast. Astaxanthin was incorporated at a concentration of 5 μ M into mixed micelles and applied to cultured cells and concentration of astaxanthin measured by HPLC in both apical and basolateral compartments. Small variations in bioavailability were observed at 3 hours. After 6 hours, only the algae source of astaxanthin was still present in the apical compartment as the esterified form. Structure-activity relationships are further discussed. Animal experiments using yeast and algae sources in different types of matrices confirm the role of source and formulation in the bioavailability potential of astaxanthin.

Keywords: *astaxanthin, bioavailability, Caco-2 cells, carotenoids, lipid metabolism*

INTRODUCTION

Astaxanthin belongs to the family of carotenoids and has powerful antioxidant properties. Increasing interest has been targeted toward this molecule and its biological benefits in recent decades. Several studies compared the antioxidant activity of astaxanthin with other carotenoids. One study found that astaxanthin neutralized twice as efficiently singlet oxygen as beta-carotene (and almost 80 times more effective than vitamin E) in chemical solution (Mascio et al., 1991). Lycopene by comparison was 30% more efficient than astaxanthin. Similar results were observed by researchers working on in vitro system of human blood cells treated with various carotenoids and then exposed to singlet oxygen. Again, lycopene has proved the most effective, followed by zeaxanthin and beta-carotene (Tinkler et al., 1994). Astaxanthin also neutralizes free radicals. A study shows that it is 50 times more effective in preventing peroxidation of fatty acids than beta-carotene or zeaxanthin (Terao, 1989). In most aquatic animals in which it can be found, astaxanthin has several essential biological functions, including protection against the oxidation of polyunsaturated essential fatty acids protect against the effects of UV light, immune response modulation, pigmentation, communication, reproductive behaviour and the improvement of reproduction (Lorenz & Cysewski, 2000).

Astaxanthin cannot be synthesized by most animals and must be acquired from the diet. Although mammals and most fish are unable to convert other dietary carotenoids to astaxanthin, crustaceans (such as shrimp) and some fish species (koi) have a limited ability to convert closely related dietary carotenoids into astaxanthin, although most astaxanthin recovered comes from their diet. Mammals do not have the capacity to synthesize astaxanthin and unlike beta-carotene astaxanthin has no pro-vitamin A activity in these animals.

Astaxanthin is naturally present in the human diet with seafood such as krill, shrimp, lobster, cod, mackerel, salmon or other coloured fish. In wild salmon, concentrations of

astaxanthin can reach 40 mg/kg while farmed salmon can only reach up to 8 mg/kg (Ambati et al., 2014). The daily intake of 2-4 mg astaxanthin, recommended for physiological effects, thus corresponds to the absorption of 100 g of wild salmon or 500 g of farmed salmon. The main non-animal natural sources of astaxanthin are the microalgae *Haematococcus pluvialis* and yeast *Phaffia rhodozyma*. Only algae astaxanthin is currently marketed as dietary supplement for humans; *Phaffia rhodozyma* yeast extracts on the other hand are currently only used for animal feed (fish, eggs). The synthetic source is also widely used in animal feed, while production by the bacterium *Paracoccus carotinifaciens* is more anecdotal. These four sources present specific geometric and optical isomers detailed in Tab. 1. The astaxanthin profile being identical in fish flesh to that present in their diet (Storebakken et al., 1985), the different forms used in aquaculture therefore find themselves indirectly in the human diet.

The various stages of transportation, digestion, absorption and transport in the plasma of dietary carotenoids were examined in mammals (Furr & Clark, 1997). In plasma, the non-polar carotenoids such as beta-carotene, alpha-carotene or lycopene are usually transported by very low density lipoproteins (VLDL) and low density lipoproteins (LDL) and the polar carotenoids such as zeaxanthin, lutein or astaxanthin are more likely to be transported by LDL and high density lipoprotein (HDL). Similarly, a limited number of clinical studies have investigated the bioavailability of astaxanthin from algae (Mercke-Odeberg et al., 2003; Okada, Ishikura, & Maoka, 2009) or a synthetic esterified form (Coral-Hinostroza et al., 2004; Østerlie, Bjerkeng, & Liaaen-Jensen, 2000) but none so far on fermentative sources such as yeast or bacteria.

To our knowledge, no report comparing the different sources of astaxanthin has been published. This study therefore aims to compare on a cellular epithelial transport model four sources of astaxanthin used in animal feed, some of which are used or intended for human consumption.

Tab. 1. Sources of astaxanthin (AX) and their structural variations

Source	AX (ppm)	Optical isomers (%)			Geometrical isomers (%)		Derivation (%)		
		3R,3'R	3R,3'S	3S,3'S	All-trans	Cis	Free	Mono-ester	Diester
farmed salmon	2.5-8	25	50	25					
wild salmon	5-30	12-17	2-6	78-85					
krill	120	9-55	7-21	38-70	75	25	4-5	31-35	61-64
<i>Phaffia rhodozyma</i>	5000-10000	98	-	2	70-90	10-30	100	-	-
<i>Paracoccus carotinifaciens</i>	20000	-	-	100	95	5	100	-	-
<i>Haematococcus pluvialis</i>	10000-40000	4	8	88	70	30	5	85	15
Synthetic	100000	25	50	25	65-75	25-35	100	-	-

MATERIALS AND METHODS

All reagents were purchased from Sigma Aldrich (France), except for the different astaxanthin extracts which were generously donated by Ajinomoto Foods Europe, Algatechnologies Ltd and ACS Dobfar spa for the yeast, algae and bacterial sources, respectively. We followed an existing protocol (Mimoun-Benarroch et al., 2011) to mimic the intestinal absorption of lipophilic molecules solubilized in the form of bile salt micelles. This

formulation, based on the composition of postprandial duodenal contents in humans (Armand et al., 1996), appears to be the closest to physiological conditions. In brief, cholesterol (0.1 mM), phosphatidyl choline (0.5 mM), lyso-phosphatidyl choline (1.5 mM), monoolein (0.03 mM), sodium oleate (0.5 mM) and astaxanthin (5 μ M) were mixed in a methanol:chloroform solution (2/1; v/v) and evaporated under a stream of nitrogen. Sodium taurocholate (5 mM) was then diluted in half a volume of culture medium (DMEM) without serum and without phenol red and added to the dried lipid residue and vigorously mixed by sonication at 25 W for 3 min. When the medium is translucent, the second volume of culture medium is added and the solution stirred overnight to allow the micelles to stabilize. The solution is filtered (0.2 μ m) before treatment to retain only the uniform size of micelles.

Media and cell culture solutions were purchased from Life Technologies (France). Caco2/TC7 cells were a generous gift from Dr Monique Rousset (Université Pierre et Marie Curie-Paris 6, UMR S872, Les Cordeliers, Paris). They were cultured in DMEM Glutamax medium, 4.5 g/L glucose, 1 % antibiotics, 1 % nonessential amino acids, 20 % inactivated foetal calf serum, in an incubator at 37 °C and 10 % CO₂. To mimic the enterocyte transport, the cells were seeded at a density of 0.25×10^6 cells in inserts containing a semi permeable PET membrane (23.1 mm in diameter; 1 μ m porosity) placed in 6-well plates (Becton Dickinson). The use of inserts allows differential access to the two poles of the cell, the apical compartment representing the intestinal lumen and basolateral compartment representing the internal circulation. In brief, cells were cultured with complete medium (20% serum) for 7 days after seeding until it formed a compact cell monolayer, as validated by transepithelial electrical resistance (TEER, Millipore). Cells were kept in a serum-free medium on the apical side and complete medium on the basolateral side for a further two weeks to induce differentiation and structural configuration similar to physiological conditions. The cells were used after 21 days of culture, that is to say when they are contiguous and polarized. On D20, the culture media were replaced with identical media without phenol red. On D21, the apical medium was replaced with 500 μ l of test medium described in section 2.1, while the basolateral medium was replaced with 1.5 mL complete medium. The treatment was stopped after 3 or 6 hours of incubation by collection of the different media (apical, basolateral). Finally the cellular layer was washed with PBS before being scraped from the insert and collected in 500 μ l PBS. Samples were kept frozen (-80 °C) until analysis.

The extraction protocol is adapted from Mercke-Odeberg et al (2003). Briefly, 6 volumes of hexane and 6 volumes of acetone were added, the sample vortexed for 1 min before centrifugation for 5 min at 2500 rpm, room temperature. The organic phase was transferred to a new tube and evaporated under nitrogen flux. The astaxanthin pellet was recovered in 200 μ L acetone for HPLC analysis. In the case of tests on the algae source of astaxanthin, the extraction residue was taken up in 1.5 ml of acetone supplemented with 1.5 mL of cholesterol esterase solution (Sigma) in 50 mM Tris HCl pH 7 (4 U / ml) and incubated for 2 h at 37 °C, then 0.5 g of sodium decahydrate and 1 mL of petrol ether were added to the solution before centrifugation 3 min at 3500 rpm. The organic phase was transferred to a new tube containing 1 g anhydrous sodium sulfate. This step was repeated and the supernatant evaporated under a nitrogen stream. The pellet was recovered in 1.5 mL of acetone, dissolved by ultrasound for 30 sec and filtered (0.45 μ m) prior to HPLC analysis. The HPLC system (Surveyor PDA ThermoFisher Scientific) was connected to a PDA detector selected to 470 nm. YMC column 30 (25 cm x 4.6 mm id; particles 5 microns; pores 100 Å) was preceded by a guard column with the same characteristics. This system was kept at a constant temperature of 30 °C during elution (constant flow 1 ml/min). After 0.5 min of isocratic condition using 4% solvent A (ddH₂O), 15% of solvent B (MTBE) and 81% solvent C (Methanol), carotenoids were eluted over 50 min with a

linear gradient of 15 to 90% of solvent B, solvent A remaining constant throughout the elution time. The column was re-equilibrated for 5 min between each analysis. Quantification was performed using a standard curve of all-trans astaxanthin (CaroteNature, Switzerland). The integration and analysis of astaxanthin peaks were performed using the ChromQuest software.

All raw data were the product of at least two independent experiments. Statistical tests were performed using SPSS v17.0 for Windows (SPSS Inc). Because of the relatively small number and heterogeneity of replicates ($n=5$ to 13, depending on the treatment), all statistical analyses were by default non parametric. Kruskal-Wallis tests were performed to compare the global sets of data and, when significant, post-hoc analyses were done using Dunn's pair to pair comparison.

RESULTS AND DISCUSSION

Absorption data

The absorption of astaxanthin present in the four sources was time-dependent with a median value for the unabsorbed fraction of 41.2%, 53.8%, 48.5%, 35.6% after 3 hours of treatment and 15.6%, 44.2%, 2.6%, 6.7% after 6 hours of treatment for the yeast, algae, bacterium and synthetic sources, respectively (Fig. 1). Although a lower rate of absorption is observed at 3 hours between the esterified and free forms, the difference is only significant between algae and synthetic astaxanthin ($p<0.05$). At 6 hours, however, the difference is statistically highly significant against all three non-esterified sources ($p<0.001$ for all three forms).

As a control molecule, beta-carotene had an unabsorbed fraction of 31.9% at 3 hours and was fully absorbed after 6 hours. This increased rate of absorption is not significant at 3 hours compared to astaxanthin but is significant at 6 hours against yeast ($p<0.01$) and algae ($p<0.001$) astaxanthin but not the two other sources. Therefore even if the transport mechanism is via passive transfer, the rate of transfer is different.

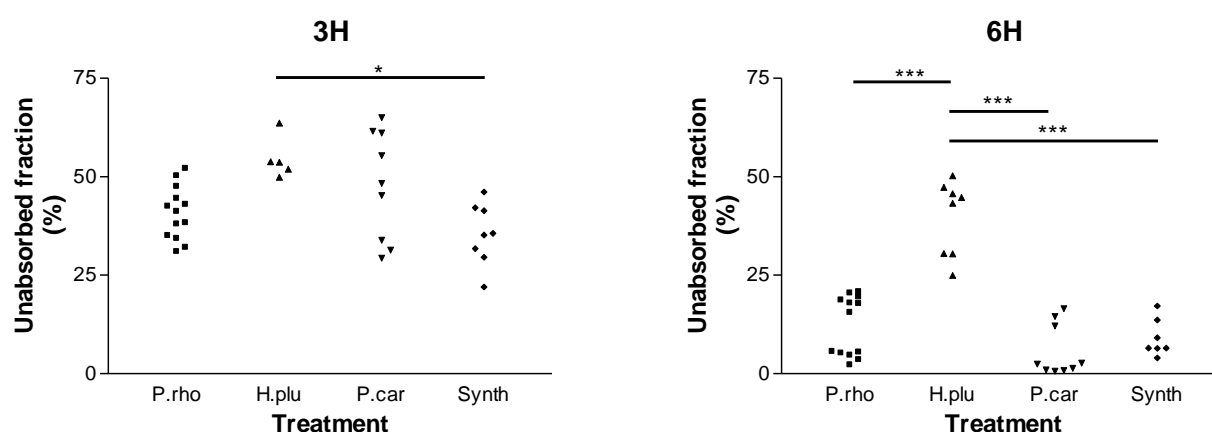


Fig. 1. Unabsorbed fractions of astaxanthin isoforms after 3 and 6 hours incubation

Caco2/TC7 cells were culture for 21 days on transwells prior to treatment. Astaxanthin ($5\mu\text{M}$) were added on the apical medium (DMEM serum free) and the apical, basal (DMED 10% serum) and cell fractions were collected after 3 or 6 hours. Carotenoids were quantified and expressed as percentage of unabsorbed molecule (apical). All datapoints are presented on the scatterplot for yeast, algae, bacterium and synthetic sources, in order. Dunn's pair-to-pair comparisons were performed for statistical significance. * $p<0.05$; *** $p<0.0005$

Over a third of astaxanthin remained unabsorbed (44.2%) after 6 hours for the algae source (Fig. 1). As the percentage of unabsorbed astaxanthin is not greatly improved between

3 and 6 hours of treatment (non-significant), the chromatograms confirm that only esterified astaxanthin remains in the apical medium while only free form is present in the cells (data not shown). This suggests a role for hydrolysis of astaxanthin esters leading to delayed absorption of the molecule.

Transfer to the basolateral side was very poor for astaxanthin (up to 2%, whatever the source) while it was fast and complete for beta-carotene (Tab.2). This difference is highly significant ($p < 0.001$) against all sources of astaxanthin at both 3 and 6 hours of treatment. From the different sources of astaxanthin, there is no molecule detected in the basolateral compartment with algae and the transfer through the basolateral membrane seems to be time-dependent for yeast and synthetic astaxanthin, although very slow, but not for the bacterial astaxanthin. The question remains as to why the astaxanthin accumulates in the cells rather than being exported to the basolateral medium in a similar manner to beta-carotene. This may suggest the implication of an active transport system.

Tab.2. Absorption of astaxanthin and beta-carotene by Caco2/TC7 cells

Source	3h				6h			
	% Absorbed (BL+C)		of which in BL		% Absorbed (BL+C)		of which in BL	
P.rho	59.22 (6.75)	<i>b</i>	1.01 (0.50)	<i>b</i>	87.83 (7.54)	<i>b</i>	2.20 (1.69)	<i>b</i>
P.car	51.94 (13.83)	<i>ab</i>	1.19 (2.08)	<i>b</i>	94.08 (6.58)	<i>c</i>	0.75 (1.58)	<i>a</i>
H.plu	45.26 (5.29)	<i>a</i>	0.00 (0.00)	<i>a</i>	60.20 (9.50)	<i>a</i>	0.00 (0.00)	<i>a</i>
Synth	64.38 (7.78)	<i>b</i>	1.00 (0.95)	<i>b</i>	90.78 (4.70)	<i>bc</i>	2.54 (1.70)	<i>b</i>
B-car	60.32 (15.67)	<i>b</i>	55.49 (13.91)	<i>c</i>	100.00 (0.00)	<i>d</i>	95.87 (6.52)	<i>c</i>

Caco2/TC7 cells were culture for 21 days on transwells prior to treatment. Astaxanthin or beta-carotene ($5\mu\text{M}$) were added on the apical medium (DMEM serum free) and the apical, basal (DMED 10% serum) and cell fractions were collected after 3 or 6 hours. Carotenoids were quantified and expressed as percentage of absorbed molecule (cell + basolateral). Data are expressed as mean and standard deviation and different letters in columns indicate a significant difference between the treatments (Kruskall-Wallis followed by Dunn's pair-to-pair comparisons).

There are very few investigations on the bioavailability of astaxanthin reported. The main reason may be that records of astaxanthin used as a bioactive molecule in human nutrition are no more than a couple of decades old. As a consequence, studies of this carotenoid are relatively sparse. In addition, investigations on the bioavailability represent only a very small part of the available publications, which are mostly on physiological benefits. It may be as well due to the highly competitive market leading to preclinical research remaining confidential. However it is possible to compare our results with those published on other carotenoids.

O'Sullivan et al. (2004) showed that astaxanthin was better accumulated into Caco-2 cells over a 24-hours period compared to beta-carotene, which is in contradiction with our observations. However cells were cultured as monolayer rather than on a transwell system and the delivery matrix was Tween40/80 rather than biliary micelles. In addition, cells were treated with a mixture of carotenoids rather than individual compound, which may impact the absorption rate of individual molecules. A similar result with higher cellular uptake and secretion to the basolateral side was observed on a transwell culture set-up when comparing

astaxanthin to beta-carotene presented on the apical side in Tween40 with chylomicron-stimulating molecules for 16 hours (O'Sullivan, Ryan & O'Brien, 2007). Sy et al. (2012) did not find any carotenoid into the basolateral medium after a 3 hours incubation in the apical side with natural or synthetic micelles. This is consistent with our observations for astaxanthin (less than 2% in the basolateral fraction) but not beta-carotene. All experimental conditions were similar between the two set-ups. Our data also showed a higher transfer rate to the cellular fraction at 3 hours (around 50%) compared to the authors (10%) but all agree on a similar uptake rate for the two carotenoids at 3 hours. The higher uptake rate should not be due to adsorbed carotenoids as the washing step was performed in our experiments as well.

The rate of absorption measured was increased for the free form compared to esterified astaxanthin from algae. This is consistent with the data reported by Lyons et al. (2002), where cellular incorporation from synthetic astaxanthin was nearly twice higher than algae astaxanthin. Esterification is a common feature of lipid-soluble molecules. Carboxyl ester lipase, also known as cholesterol esterase is produced in the pancreatic juice for an activity at the brush border (Ikeda et al., 2003) and is the main actor of hydrolysis of carotenoid esters in the intestinal lumen before absorption of the free form by the enterocytes. However, in a similar experimental setup, fucoxanthin was hydrolysed when exposed to Caco-2 cells producing the free form fucoxanthinol similar to that produced by hydrolysis with pancreatic lipase (Sugawara et al., 2002). This activity of endogenous lipase or carboxylesterase activity at the apical membrane or secreted into the apical medium close to the brush border is consistent with previous reports (Annaert et al., 1997; Spalinger et al., 1998). Similarly, absorption of zeaxanthin, another xanthophyll is facilitated by prior hydrolysis of its esters (Chitchumroonchokchai & Failla, 2006). Therefore the partial absorption of astaxanthin and detection of the free form only in the cellular fraction observed herein may be due to the endogenous hydrolytic activity of the cells. Further investigations need to be performed as well to assess the esterase activity in cells exposed to free and ester forms of astaxanthin. In addition, it may be interesting to do a pre-digestion with pancreatic juice. The uptake and role of esterified astaxanthin on mucosal absorption patterns remain unclear at this time. Sugawara et al, 2009, observed some esterification of astaxanthin by Caco-2 cells after 24 hours treatment. It remained very partial with up to 2% of the astaxanthin present in the basolateral compartment being esterified compared to up to 10% for peridininol esters. Although we did not detect any astaxanthin esters in the basolateral medium, our treatment time was much shorter at 3 and 6 hours and the process may be time-dependent (Sy et al., 2012).

Animal studies (data not shown) have been performed in order to better understand the impact of the source and the food matrix in absorption parameters. A study on hamsters showed that powder matrix greatly reduced the plasma recovery of astaxanthin from the yeast *Phaffia rhodozyma* and delays the peak of plasma concentration by up to 4 hours. This is in agreement with the clinical study from Mercke-Odeberg et al (2003) showing an increased bioavailability of astaxanthin from the algae *Haematococcus pluvialis* in lipid compared to powder matrix. Another study in rats compared the two main sources (algae and yeast) prepared in the same lipid carrier (safflower oil). Data showed that this specific carrier was not suitable to dissolve the free form (yeast) into a solution but rather into a fine suspension of astaxanthin crystals. Hence, the bioavailability was much lower for the yeast compared to algae due to the exchange area that was much lower (Ajinomoto Foods Europe, personal data).

These studies were thus contradictory with the *in vitro* data regarding the rate of absorption between yeast and algae astaxanthin. However two reasons can be suggested for this observation. The first is in the formulation for the *in vivo* studies, leading to lower solubility of the free form thus lower available concentration of the molecules. The second may be related

to hydrolysis of astaxanthin esters on the intestinal area, possibly due to stimulation of the enzyme through the presence of the ester, which was not observed *in vitro*.

Mechanisms of action

The expression of several lipid carriers was measured by RT-qPCR after 3 and 6 hours of treatment (data not shown). The maximum expression was observed at 6 hours, when the absorption was mostly complete. The activation therefore is not necessarily directly related to an active transport of the molecules. Incubation with carotenoid-free micelles, a wide range of genes were modulated, indicating that most carriers were modulated primarily by bile acids, in agreement with the physiological mechanisms of absorption of dietary lipids. When comparing astaxanthin treatments against micelles, very few modulations were significant. Astaxanthin from the yeast *Phaffia rhodozyma* stimulated the expression of a range of genes at 6 hours, including Fabp2 ($p < 0.05$), Scarb1 ($p < 0.05$) and Scp2 ($p < 0.01$). Astaxanthin from algae *Haematococcus pluvialis* stimulated expression of Scarb1 ($p < 0.05$) and Scp2 ($p < 0.05$) at 6 hours. Synthetic astaxanthin, finally, stimulated the expression of Scp2 ($p < 0.05$) at 6 hours, while bacterial astaxanthin did not seem to significantly affect any gene expression. All this may suggest a structure-dependent activation of the lipid carriers involved in the uptake or at least stimulated by astaxanthin isomers. In the case of Scp2, a possibility may be the role of the geometrical isomer cis-astaxanthin which is virtually absent from the bacterial extracts and present in up to 30% in the other forms (Tab. 1).

During et al. (2005) in a cell study using the Caco-2 cell line showed that the bioavailability of carotenoids was partially inhibited by the use of an antagonist of cholesterol transport. Apolar carotenoids seemed more sensitive to this inhibition compared to polar carotenoids such as zeaxanthin or lutein. An inhibitor of the SR-B1 transporter also had a partial effect on carotenoids uptake, suggesting a role in active transport of beta-carotene. The results of Reboul et al. (2005), suggesting that lutein is at least partially carried by an active mechanism involving the SR-B1 receptor. Further work reviewed in 2013 point to the role of SR-B1 carrier protein for a range of carotenes and xanthophylls (Reboul, 2013). The corresponding gene, Scarb1 was modulated by both beta-carotene and some sources of astaxanthin in our study. The inhibition of the expression of AbcA1 is however inconsistent with the putative role of the carrier protein in the excretion of carotenoids on the basolateral membrane of the enterocyte (Reboul, 2013), nor is it consistent with the high transfer rate of beta-carotene to the basolateral medium as observed herein. Our tests have not to date included the use of inhibitors of the lipid carriers highlighted in our results or suggested in publications. Additional tests will be needed to refine these observations and to better understand variations between the different sources of astaxanthin.

The RIVAGE study (Lairon et al., 2009; Vincent et al., 2002) showed a relationship between a polymorphism of the gene Scarb1 (SR-B1 transporter) and cardiovascular markers like blood sugar, insulin and cholesterol levels, while Fabp2 polymorphism had lower physiological repercussions but mainly on triglycerides. Variations and stimulation of lipid metabolism genes could have a functional impact beyond the active transport of carotenoid. As the genetic expression was at the highest once absorption was completed, the transporters may be involved in facilitated uptake of other lipids rather than astaxanthin itself. With this in mind, most forms of astaxanthin seem to particularly target the intestinal sterol carrier protein (SCP2) and modulate bile acid excretion into the lumen and formation of lipid carriers such as micelles for general lipid uptake as well as intracellular cholesterol transport (Shen, Howles, & Tso, 2001). The wider effect of the yeast astaxanthin may suggest a further role in stimulating circulating HDL-cholesterol (SR-B1) or the intestinal recognition protein and intracellular utilization of long chain fatty acids and their esters (FABP2).

CONCLUSION

We have observed through this series of experiments variation in the bioavailability and gene expression of lipid carriers after treatment for 3 to 6 hours of Caco-2/TC7 cells with astaxanthin in mixed micelles. Our main conclusion relates to the delay in uptake due to the esterification of astaxanthin, the algae source being partially absorbed when the free form in the yeast or other extracts is nearly completely absorbed after 6 hours of treatment. We found very few differences between geometric and optical isomers of astaxanthin, but further structural study would be needed to confirm those observations. Further investigations are required as the esterified form from algae is currently the only non-dietary source of astaxanthin present for human nutrition and while their physiological effects seem to be more and more dissected, the first contact with the host at the intestinal mucosal barrier remains a black box.

Our second conclusion is based on the expression of genes related to lipid or cholesterol transport, some of which are also known to be involved in the transport of carotenoids, but the timing seems off between the observed uptake of the molecule and genetic expression. The implications are perhaps more complex and the RIVAGE study suggests possible physiological consequences of modulating these carriers.

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