The Effect of Various Antioxidants on the Degradation of O/W Microemulsions Containing Esterified Astaxanthins from *Haematococcus pluvialis*

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Abstract: Esterified astaxanthins are used as functional nutraceuticals and pigments in many food products. Unfortunately, the utilization is currently limited due to their chemical instability and poor water-solubility. In this study, esterified astaxanthins were quantified and purified from *Haematococcus pluvialis* using a novel and precise approach. By HPLC-(+)APCI-MS/MS, twenty esterified astaxanthin molecular species were identified, of which classified into eight monoester forms (approximately 85%, w/w) and twelve diester forms (approximately 15%, w/w), depending on the number of fatty acids that bind thereto. The MS data showed that the predominant fatty acids in astaxanthin esters of *H. pluvialis* are usually a long chain fatty acid with 16~18 carbon atoms, such as C18:1, C18:2, C18:3, C18:4, C16:0, C16:1. The purity of the esterified astaxanthins was determined to be $96.8 \pm 1.2\%$ after purification. A well water-dispersible microemulsion was fabricated using high purity esterified astaxanthins, ethyl butyrate, Tween 80 and ethanol; and that emulsion exhibited a mean particle radius around 60 nm. The chemical degradation of esterified astaxanthins was monitored under accelerated stress storage conditions. After storage for 20 days, the results indicated that the degradation of esterified astaxanthins was effectively slowed by the addition of antioxidants to the microemulsions. By investigating the dependence of the chemical degradation of the esterified astaxanthins in O/W microemulsions on the concentration of the additives, it was concluded that the effectiveness of the additives at inhibiting the degradation of the esterified astaxanthins decreased in the following order: EDTA > ascorbic acid > vitamin E acetate. The utilization of antioxidants in combination was less effective than using them individually. These results provide information for designing effective delivery systems, thereby delaying the chemical degradation of esterified astaxanthins in foods, beverages and other commercial products during long-term storage.

Key words: esterified astaxanthins, microemulsion, stability, antioxidants, degradation

1 INTRODUCTION

Astaxanthin is one of the most important and economically valuable ketocarotenoid pigments that is naturally synthesized in certain plants, algae and bacteria. This pigment is accumulated in aquatic animals, including salmon, trout, red seabream, shrimp, lobster and fish eggs and in birds such as flamingoes and quails through their place in natural food chains^{1,2)}. Because astaxanthin exhibits various effects relating to the prevention of some human health disorders, such as cancer, cardiovascular disease, cataracts and macular degeneration, great attention has been paid to this class of natural pigments³⁾. Therefore, astaxanthin has been used in cosmetics, pharmaceuticals,

and functional food additives.

Haematococcus pluvialis is a common green algae and is recognized as the best source of material for the manufacture of natural astaxanthin⁴⁾. Three forms of astaxanthin have been reported in *H. pluvialis*: free astaxanthin, astaxanthin monoesters and astaxanthin diesters (Fig. 1)⁴⁾, of which astaxanthin monoesters predominate^{5,6)}. Furthermore, previous studies had been shown that there were no difference between esterified form carotenoid and free form carotenoid on the intestinal absorption⁷⁾. Currently, two methods can be used to quantify astaxanthin in *H. pluvialis*: UV-visible spectroscopy (UV-Vis) and high performance liquid chromatography (HPLC)⁸⁻¹⁰⁾. However, it

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Accepted January 14, 2015 (received for review November 20, 2014)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
<http://www.jstage.jst.go.jp/browse/jos/> <http://mc.manuscriptcentral.com/jjocs>

Fig. 1 Structures of astaxanthin, astaxanthin monoesters, and astaxanthin diesters. R, saturated or unsaturated alkyl chains.

is difficult to quantify esterified astaxanthins in *H. pluvialis* due to the complex molecular composition. Therefore, it is necessary to establish a precise and reliable method to quantify astaxanthin esters in *H. pluvialis*.

The term “esterified astaxanthins” refers to a group of related fat-soluble molecules that contain conjugated polyunsaturated hydrocarbon chains, exposing them to autoxidation^{11, 12}. Due to the low chemical stability and poor water solubility of these molecules, a number of challenges are associated with incorporating esterified astaxanthins into commercial products. One of the main challenges associated with the use of esterified astaxanthins is their high susceptibility to chemical degradation. A number of factors have previously been shown to promote the oxidation of astaxanthin esters, including light¹³, heat¹⁴, highly acidic environments¹⁵, singlet oxygen¹⁶, free radicals¹⁷, and transition metals¹⁸. The beneficial health properties of esterified astaxanthins can be lost if they undergo chemical degradation in foods and other products. Another factor limiting the application of esterified astaxanthins is that these molecules are highly lipophilic and exhibit very low water solubility; thus, their direct dispersal into aqueous media is difficult¹⁹. Esterified astaxanthins must either be dispersed into a lipid phase or incorporated into a suitable delivery system. Therefore, an efficient delivery system for esterified astaxanthins is required that can effectively increase their water solubility and stability without adversely affecting product quality (e.g., beneficial health properties, appearance, and taste).

Previous studies have shown that various approaches can be used to increase the chemical stability of lipid emulsions; these include the addition of antioxidants, metal ion chelation, interfacial engineering, particle size control, and environmental regulation^{20, 21}. Furthermore, interest is increasing in replacing synthetic antioxidants with natural or nature-identical antioxidants in food and beverage prod-

ucts²². Ascorbic acid and vitamin E acetate are conventional antioxidants. Ethylenediaminetetra-acetic acid (EDTA) is a strong chelating agent that has previously been shown to be highly effective at inhibiting lipid and lycopene oxidation in oil-in-water (O/W) emulsions^{11, 20}. EDTA and ascorbic acid are water-soluble antioxidants, whereas vitamin E acetate is oil-soluble; thus, the physical location of these additives within the microemulsions will be different.

The objective of this study was to gain a better understanding of three typical antioxidants (water-soluble, oil-soluble and chelating) in terms of their ability to inhibit the chemical degradation of esterified astaxanthins in enriched (O/W) microemulsions. The effectiveness of the antioxidants was measured in isolation and in combination. By understanding the mechanisms of these additives, we expect that the results of this study will support the application of esterified astaxanthins in foods, beverages, pharmaceuticals and cosmetics.

2 MATERIALS AND METHODS

2.1 Materials

Haematococcus pluvialis dry powder (2.0 wt% of total astaxanthin, as determined by HPLC) was purchased from Yunnan Alphy Biotech Co., Ltd. Tween 80, EDTA, ascorbic acid, vitamin E acetate, HPLC-grade methanol (Merck, Germany) and methyl tertiary butyl ether (MTBE) were purchased from Burdick & Jackson (Muskegon, USA). Astaxanthin (97.3%) was purchased from Dr. Ehrenstorfer Co., Ltd. (Germany). All other chemicals used were of analytical grade. Ultrapure water was used to prepare all solutions and emulsions.

2.2 Preparation of high-purity esterified astaxanthins

Briefly, 50 g of *H. pluvialis* dry powder was mixed with 150 mL of ethyl acetate, shaken for 5 min under nitrogen in the dark, and then centrifuged for 10 min at 2,800 rpm. The supernatant containing the carotenoid mixture (mainly comprising esterified astaxanthins) and the cellular residue was repeatedly extracted and centrifuged until colorless. The combined supernatants were then dried in a rotary evaporator at 30°C to obtain a crude extract. The crude extract was then purified using a silica gel column and gradient elution with *n*-hexane/acetone (100/0→96/4→92/9→88/12, v/v); low polarity impurities, astaxanthin diesters, mixtures of astaxanthin diesters and astaxanthin monoesters, and astaxanthin monoesters were eluted, in that order. The fractions containing astaxanthin diesters and astaxanthin monoesters were then combined and rotary evaporated to dryness at 25°C, yielding highly purified esterified astaxanthins.

2.3 Molecular species analysis of esterified astaxanthins

The molecular species and average molecular weight of the esterified astaxanthins was determined using positive-ion high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (HPLC-(+)APCI-MS/MS). Crude extract (10 mg) and 1 mg of high-purity esterified astaxanthins were dissolved in 100 mL of methanol/MTBE (1/1, v/v), respectively. The sample was then analyzed using an Agilent 1260 series HPLC system (Agilent, Waldbronn, Germany) equipped with a G1314A pump, an automatic injector and a G1315B diode array detector (DAD); the system was connected with a workstation computer. Reversed-phase separation was performed on a YMC carotenoid C30 column (4.6 mm × 250 mm, 5 µm), which was maintained in a column oven at 35°C. The mobile phase consisted of methanol (A) and TBME (B). The following gradient was used (min/% B): 0/0; 16/0; 22/22; 42/22; 50/40; 60/40; 65/80; 70/0. A flow rate of 1.0 mL/min was used, and the injection volume was 10 µL. The column was monitored at 476 nm, and analyses were performed in duplicate. To analyze the astaxanthin molecular species, a C30-HPLC module equipped with DAD was coupled to an Agilent 6410B spectrometer (Agilent, Waldbronn, Germany). Detection was performed using APCI in the positive ionization mode. The voltage of the corona needle was set to 4 kV. Nitrogen was used as the drying gas, and the carrier gas was used at a flow rate of 5 L/min; the nebulizer was set to 45 psi, and the dry gas temperature was maintained at 350°C. Mass spectra were obtained in the mass range (m/z) of 200–2,000. MS^2 was detected in the product ion scan and precursor ion scan modes. The fragmentor voltage and collision energy were set to 60 V and 15 eV, respectively.

2.4 Purity analysis of the highly purified esterified astaxanthins

To quantify the astaxanthin and esterified astaxanthins in *H. pluvialis*, the molecular species of astaxanthin were analyzed using HPLC-APCI-MS/MS; the average molecular weight was then calculated using the following formula:

$$M_{(\text{average})} = \sum (M_i * i\%)$$

In the formula, $M_{(\text{average})}$ represents the average molecular weight of total astaxanthins in *H. pluvialis*; M_i represents the molecular weight of astaxanthin molecular species i in *H. pluvialis*; $i\%$ is the content of astaxanthin molecular species i relative to total astaxanthins in *H. pluvialis*, as calculated based on the relative peak area of astaxanthin molecular species i . Finally, the content of esterified astaxanthins was calculated using the following formula:

$$\text{esterified astaxanthins \%} = (M_{(\text{average})} * n) / m$$

In the formula, n represents the amount of total astaxanthins in *H. pluvialis*, as determined from a calibration

curve created using free astaxanthin as a reference material; m represents the weight of the sample.

2.5 Preparation of esterified astaxanthin O/W microemulsions

An oil phase was prepared by dispersing 3% (w/v) of purified esterified astaxanthins in ethyl butyrate with mild heating (<5 min, 40°C); the dispersion was then stirred at ambient temperature for approximately 1 h to ensure full dissolution. The samples were flushed with nitrogen during this process to inhibit degradation of the esterified astaxanthins. Oil-in-water microemulsions were prepared by homogenizing 10 mL of oil phase (3% esterified astaxanthins in ethyl butyrate, w/v) with 90 mL of aqueous phase (pH 7.0, 15% Tween 80, 7% ethanol in buffer solution, v/v) at ambient temperature (25°C). The ratio of oil phase to aqueous phase was kept constant at 1:9 (v/v). An emulsion pre-mixture was prepared for 2 min using a high-speed blender (Biospec Products Inc., Bartlesville, OK), which was then passed through a high-pressure microfluidizer (Model 101, Microfluidics, Newton, MA) three times at 9,000 psi; organic solvents were then removed by reduced pressure distillation at 40°C. The final concentration of esterified astaxanthins in emulsion was 0.36% (w/v) detected by HPLC-APCI-MS/MS. The freshly prepared emulsions were divided into aliquots and sealed in glass tubes covered by aluminum foil.

2.6 Measurement of particle size and zeta potential

The particle size distribution, mean particle radius (r , Z-average), and zeta potentials of the diluted emulsions were measured using a commercial dynamic light-scattering instrument (Nano-ZS, Malvern Instruments, Worcestershire, UK). Samples were diluted (1:100, v/v) with buffer solution before analysis to avoid multiple scattering effects. The buffers used for dilution had the same pH and ionic composition as the samples being analyzed.

2.7 Measurement of the degradation of esterified astaxanthins in O/W microemulsions

The chemical degradation of the esterified astaxanthins in O/W microemulsions during storage was measured using two approaches: UV-visible spectroscopy and colorimetry²². All measurements were repeated three times.

The UV-Vis absorbance ($\lambda = 485$ nm) and spectra of the esterified astaxanthin O/W microemulsions were measured using a UV-Vis spectrophotometer (Shimadzu, Japan). Samples were diluted (1:100, v/v) with dimethyl sulfoxide before analysis to avoid multiple scattering effects. A dimethyl sulfoxide solution was used as a blank. A microemulsion without esterified astaxanthins was also analyzed as a control. The degradation of esterified astaxanthins was monitored based on UV-Vis absorbance ($\lambda = 485$ nm).

The tristimulus color coordinates (L^* , a^* , b^*) of the mi-

croemulsions were measured using a hand-held colorimeter (Color Munki, X-Rite, Grand Rapids, MI). The L^* value is a measure of lightness (higher values indicate lighter colors); the a^* value is a measure of redness/greenness (positive values indicate red colors, negative values indicate green colors); the b^* value is a measure of yellowness/blueness (positive values indicate yellow colors, negative values indicate blue colors). Microemulsions were placed into a transparent flat-faced cuvette, the measuring device of the colorimeter was pressed against the cuvette surface, and the color was recorded. The total color difference (ΔE^*) was then calculated using the following formula:

$$\Delta E^* = [(L_t^* - L_0^*)^2 + (a_t^* - a_0^*)^2 + (b_t^* - b_0^*)^2]^{1/2}$$

Here, L_t^* , a_t^* , and b_t^* are the measured color coordinates of the microemulsions at storage time t , and L_0^* , a_0^* , and b_0^* are the initial color coordinates of the microemulsions.

2.8 The effect of antioxidants on microemulsion stability

The physical and chemical stabilities of esterified astaxanthin O/W microemulsions were tested by adding different types and amounts of antioxidants. Water-soluble antioxidants, EDTA (0 to 0.5%, w/v) and ascorbic acid (0 to 0.5%, w/v) were dissolved in the aqueous phase of emulsion samples (pH 7.0). Oil-soluble antioxidants and vitamin E acetate (0 to 0.5%, w/v) were dissolved in the oil phase before homogenization. The microemulsion samples were transferred to glass tubes under an air atmosphere and stored under fluorescent illumination at 37°C for 20 days.

2.9 Statistical analysis

All experiments were performed in triplicate, and means and standard deviations were calculated. Significant differ-

ences ($p < 0.05$) between selected treatments were established using a Two-Sample t -Test Assuming Unequal Variances (Excel 2007, Microsoft).

3 RESULTS AND DISCUSSION

3.1 Separation and Quantification of Esterified Astaxanthins

The esterified astaxanthins were first purified and quantified, because their composition and purity strongly affect the preparation of microemulsions²³. High-purity esterified astaxanthins were obtained using silica gel column chromatography as described above. The esterified astaxanthin contents before and after purification are presented in Fig. 2 and Table 1. Peak 1 (m/z 597.4 and m/z 579.4) represented protonated quasimolecular ions $[M+H]^+$ and $[MH-18]^+$ of astaxanthin, as determined based on contrast- and positive-ion-mode mass spectra, and peak 1 was not present after purification.

The MS data used to identify astaxanthin and esterified astaxanthins are summarized in Table 1. Twenty esterified astaxanthin molecular species were identified, of which classified into eight monoester forms (approximately 85%, w/w) and twelve diester forms (approximately 15%, w/w), depending on the number of fatty acids that bind thereto. The results showed that the predominant fatty acids in astaxanthin esters of *H. pluvialis* are usually a long chain fatty acid (especially oleic acid) with 16 or more carbon atoms, such as C18:1, C18:2, C18:3, C18:4, C16:0, C16:1. These results are compatible with previous researches in which the fatty acids consisted of esterified astaxanthins in *H. pluvialis* were analyzed⁴⁻⁶. The purity of total astaxan-

Fig. 2 HPLC chromatogram (DAD, 476 nm) of ingredients in *H. pluvialis* extracts. The identification corresponding to the numbers representing the peaks of HPLC spectrum were shown in Table 1, and the number with different letters represents the isomers of astaxanthin ester. A: crude extract; B: purified esterified astaxanthins.

Table 1 Mass spectroscopy data and the relative contents of astaxanthin and esterified astaxanthins in the *H. pluvialis* extract.

No.	<i>m/z</i>			Product ion	Identification	Content (%)	
	[M+H] ⁺	[M+H-FA ₁] ⁺	[M+H-FA ₂] ⁺			Crude extract	Purified esterified astaxanthins
1	597.4			579.4	Astaxanthin	3.6 ± 0.3	—
2	855.6	579.4		837.5	ME-C18:4	4.4 ± 0.5	3.1 ± 0.4
3	857.6	579.4		839.8	ME-C18:3	18.1 ± 0.8	20.5 ± 0.3
	857.8	579.4		579.4	ME-C18:3		
4	859.6	579.3		841.7	ME-C18:2	24.7 ± 0.5	25.7 ± 0.6
5	861.8	579.3		843.8	ME-C18:1	17.3 ± 0.4	19.1 ± 0.4
	861.8	579.3		579.3	ME-C18:1		
6	835.7	579.3		579.3	ME-C16:0	16.7 ± 0.5	16.9 ± 0.3
7	863.8	579.4		579.4	ME-C18:0	0.4 ± 0.1	0.1 ± 0.1
8	1,117.4	855.8		855.8	DE-C18:4/C18:2	0.8 ± 0.2	0.4 ± 0.1
9	1,119.9	841.7		841.7	DE-C18:3/C18:2	2.3 ± 0.3	2.2 ± 0.2
10	1,122.0	841.7		841.7	DE-C18:2/C18:2	2.7 ± 0.2	3.0 ± 0.2
11	1,124.0	843.7	841.5	841.5	DE-C18:2/C18:1	2.1 ± 0.3	2.0 ± 0.1
11	1,124.9	843.8	843.8	841.8	DE-C18:1/C18:1	0.1 ± 0.1	0.1 ± 0.1
12	1,127.0	845.8	843.8	845.8	DE-C18:1/C18:0	0.6 ± 0.2	0.5 ± 0.2
12	1,096.0	839.7		839.7	DE-C16:0/C18:3	1.7 ± 0.2	1.8 ± 0.2
13	1,097.9	841.6	817.6	841.6	DE-C18:2/C16:0	2.1 ± 0.2	2.3 ± 0.3
13	1,097.6	843.2		843.2	DE-C16:1/C18:1	0.1 ± 0.2	0.1 ± 0.1
14	1,100.0	843.8	817.4	843.8	DE-C16:0/C18:1	1.7 ± 0.2	1.9 ± 0.1
14	1,103.0	817.6		817.6	DE-C18:0/C16:0	0.1 ± 0.1	0.1 ± 0.1
15	1,073.7	817.3	817.3	817.3	DE-C16:0/C16:0	0.5 ± 0.2	0.5 ± 0.1
Mr						883.2	895.4

ME: astaxanthin monoester; DE: astaxanthin diester. i.e. “ME-C18:4” means “Astaxanthin-C18:4”; “DE-C18:4/C18:2” means “Astaxanthin-C18:4 and C18:2”.

The purity of crude extract and purified esterified astaxanthins were 10.4 ± 0.8% and 96.8 ± 1.2%, respectively.

thin was 10.4 ± 0.8% in the crude extract and 96.8 ± 1.2% in the purified extract. Overall, the results indicate that HPLC-(+)APCI-MS/MS was an effective method for the quantification and purification of astaxanthin and esterified astaxanthins obtained from *H. pluvialis*, and this technique can be used for the quantification and purification of esterified astaxanthins obtained from other biological samples (e.g., Antarctic krill, shrimp, and crab), while analyzing the molecular species of esterified astaxanthins.

3.2 Formation and Physical Stability of Microemulsions

Microemulsions were prepared by homogenizing 10% (v/v) oil phase (3% high purity esterified astaxanthins in ethyl acetate, w/v) with 90% (v/v) aqueous phase (15% Tween 80, 7% ethanol in buffer solution, v/v). The mean particle radius (approximately 60 nm) was obtained immediately

after homogenization (Fig. 3(A)). The microemulsions were translucent, indicating that well-dispersed aqueous microemulsions were formed ($r < 100$ nm). For application, it is important that microemulsions remain physically stable during storage. Thus, we monitored changes in the mean particle radius and transparency over time during storage of the microemulsions under fluorescent illumination at 37°C for 20 days (Table 2, Fig. 3). The mean particle radius increased to a small extent during storage, and the rates of particle growth of the microemulsions with no antioxidant, EDTA, ascorbic acid, and vitamin E acetate were 15.81 ± 2.13%, 10.54 ± 0.94%, 8.07 ± 1.36%, 5.38 ± 0.71%, respectively. The mean particle radii were all less than 100 nm, and the microemulsions remained transparent throughout the experimental period (Fig. 3(B)), indicating that the microemulsions were relatively stable against

Fig. 3 Astaxanthin ester-enriched O/W microemulsions stored under fluorescent illumination at 37°C for 20 days. A: Particle size distribution, B: An image of emulsions containing various antioxidants after storage. a: no antioxidants; b: EDTA (0.05%, w/v); c: ascorbic acid (0.05%, w/v); d: vitamin E acetate (0.05%, w/v).

Table 2 Mean particle radii of esterified astaxanthins O/W microemulsions containing various antioxidants.

Antioxidant	Mean Particle Radius (nm)		
	0 Day	20 Days	Increase (%)
Control (none antioxidants)	61.21 ± 1.31	70.89 ± 0.85	15.81 ± 2.13
EDTA (0.05%, w/v)	58.49 ± 1.05	64.65 ± 0.74	10.54 ± 0.94
Ascorbic acid (0.05%, w/v)	59.36 ± 0.82	64.14 ± 0.58	8.07 ± 1.36
Vitamin E acetate (0.05%, w/v)	61.86 ± 1.14	65.20 ± 0.97	5.38 ± 0.71

droplet growth. Nevertheless, we did observe and note a noticeable change in the particle size distribution of the emulsions after storage. Initially, the microemulsions exhibited a monomodal distribution, and most particles exhibited radii of less than 400 nm. Most of the particles remained relatively small after storage ($r < 400$ nm), but some evidence showed the existence of a new population of larger particles ($r > 1,000$ nm), which was formed after 20 days of storage. These larger particles were observed in the control sample and in all of the samples containing antioxidants. Based on previous researches on the stability of oil-in-water emulsions^{20, 23–25}, we hypothesize that these large particles are flocs. Because the zeta potentials of the microemulsions were determined to be approximately only -2 mV using a Nano Zetasizer, no great electrostatic repulsion would have developed among the droplets. Furthermore, the emulsifier molecule (Tween 80) that was adsorbed on the surfaces of the lipid droplets in the microemulsions has been shown to undergo conformational changes when stored under accelerated stress storage conditions (fluorescent illumination, 37°C); these changes might lead to the exposure of non-polar groups, thereby increasing the hydrophobic attraction between lipid droplets. While net attractive forces are stronger than net repulsive forces, the droplets will tend to flocculate. In addition, the microemulsion remained transparent throughout the experimental period, and we did not observe any

visible evidence of gravitational separation (creaming or sedimentation) in the samples during storage (Fig. 3(B)); thus, most of the droplets did not flocculate and remained small.

Overall, we found that the addition of various antioxidants to the microemulsions did not apparently affect their initial particle size or long-term stability (Table 2). A possible explanation for this phenomenon is that the antioxidants were primarily present either in the water phase (EDTA or ascorbic acid) or in the oil phase (vitamin E acetate). Consequently, the antioxidants were not particularly surface-active and did not compete with Tween 80 at the oil-water interface. Moreover, it should be noted that accelerated stress storage conditions (fluorescent illumination, 37°C) were used in this study. However, in practice, microemulsions would usually be stored in the dark at lower temperatures and would be expected to be more stable against droplet aggregation than under the research conditions used here.

3.3 The Effect of Antioxidants on Esterified Astaxanthins Degradation

In this section, we examined the effect of various antioxidants on the chemical degradation of esterified astaxanthins in O/W microemulsions using both UV-visible spectroscopy and colorimetry.

The Effect of Antioxidants on Spectral Absorption.

Fig. 4 The UV/VIS absorption spectra of esterified astaxanthin O/W microemulsions containing various antioxidants during storage under fluorescent illumination at 37°C for 0, 4, 9, 12, 15 and 20 days. (a): control (no antioxidants); (b): EDTA (0.05%, w/v); (c): ascorbic acid (0.05%, w/v); (d): vitamin E acetate (0.05%, w/v).

Because a good correlation was found between the spectrum absorption ratio and astaxanthin content^{4, 5, 9}, UV-Vis absorbance measurements and spectra of esterified astaxanthin O/W microemulsions containing various antioxidants were recorded during storage (Fig. 4). The tested antioxidants exhibited an approximately linear decrease in the absorbance at 485 nm during storage. However, the extent of the absorbance decrease varied; in particular, the absorbance of the microemulsion without antioxidants decreased by 93.02% after storage for 20 days. The absorbances of the microemulsions containing EDTA, ascorbic acid and vitamin E acetate were reduced by 20.95%, 38.89% and 48.57%, respectively. Figure 4 shows that the height of the absorption peak at 485 nm measured for the microemulsions gradually decreased and a new absorption peak appeared near 405 nm during storage. The absorption peak at 485 nm was eventually lost in microemulsions without antioxidants. These absorbance changes might be due to the oxidation of esterified astaxanthins, and a series of auto-oxidation products were generated during storage²⁶. Therefore, it can be concluded that antioxidants effectively inhibited the chemical degradation of esterified astaxan-

thins. In addition, the rate of degradation of esterified astaxanthins measured in this study was slower than that obtained in previous reports on astaxanthin²⁷. In that report, a reduction of 99% in concentration of astaxanthin was shown after storage for 6 days under fluorescent illumination at 25°C. However, in our systems, the absorbance of the esterified astaxanthin microemulsion without antioxidants decreased by approximately 35% after storage for 9 days under fluorescent illumination at 37°C. This result can be attributed to the fact that esterified astaxanthins are more stable than free astaxanthin. The reason for this phenomenon might be presumed that astaxanthin has a highly unsaturated structure, which lead to molecule is extremely susceptible to chemical degradation, and the esterification of OH groups of astaxanthin with fatty acids might stabilize the compound against chemical degradation. In addition, the type of emulsifier used might also affect the rate of degradation of esterified astaxanthins. These assumptions will be examined in future studies.

The Effect of Antioxidants on Color Fading. Esterified astaxanthins represent a series of carotenoids that exhibit an intense red color; however, this coloration fades in re-

Fig. 5 Color changes in esterified astaxanthin-enriched oil-in-water microemulsions containing various antioxidants (no antioxidants ●; EDTA, 0.05%, w/v, △; ascorbic acid, 0.05%, w/v, ○; vitamin E acetate, 0.05%, w/v, □) during storage under fluorescent illumination at 37°C for 20 days. (a) lightness; (b) redness; (c) yellowness; (d) total color difference.

sponse to chemical degradation²⁸). Therefore, the chemical stability of the esterified astaxanthins in O/W microemulsions was monitored by measuring color fading. Changes in the tristimulus color coordinates (L^* , a^* , b^*) of the microemulsions during storage under fluorescent illumination at 37°C are presented in Fig. 5(a, b and c). In the absence of antioxidant, the microemulsions exhibited an appreciable increase in lightness (L^*) and a decrease in their redness ($+a^*$) and yellowness ($+b^*$) during storage. The increased microemulsion lightness can be attributed to the fact that the L^* value of emulsions usually increases as their color intensity decreases (smaller $|a^*|$ and $|b^*|$ values); therefore, more light was reflected from the surface of the emulsions²⁹). The changes in lightness, redness, and yellowness during storage were much less pronounced in the presence of antioxidants than in their absence. The effects of antioxidants on the total color difference (ΔE^*) of the microemulsions are presented in Fig. 5(d). The ΔE^* values of the microemulsions changed markedly after 4 days as follows: 73.91 (no antioxidant), 1.06 (EDTA), 2.31 (ascorbic acid) and 8.76 (vitamin E acetate) after storage for 20 days. The ΔE^* value is a convenient means of quantifying the overall color changes in microemulsions during storage²²). Therefore, the above results showed that the tested antioxidants

remarkably delayed the color fading of the esterified astaxanthin microemulsions.

Moreover, the effect of increasing the additive concentration on the chemical degradation of the esterified astaxanthins was investigated by measuring changes in total color loss (ΔE^*) during storage. The rate of color loss was calculated from the slope of ΔE^* versus storage time based on linear regression analysis. The rate of color degradation decreased as increasing amounts of antioxidants were added to the microemulsions (Fig. 6), thus demonstrating the effectiveness of the three antioxidants tested. The most dramatic change in color degradation occurred at antioxidant concentrations of less than 0.001% (w/v), indicating that antioxidants clearly affected color degradation, even at low concentrations. When the concentration of antioxidant was equal to or greater than 0.05% (w/v), the rate of color degradation did exhibit further changes. EDTA was much more effective at inhibiting color degradation than the other additives at a given concentration.

EDTA primarily chelates transition metal ions that normally catalyze lipid oxidation^{21, 30}). Decker *et al.* reported that chelating agents, when used as antioxidants, can inhibit metal-catalyzed reactions by a variety of mechanisms, including the prevention of metal redox cycling, the

Fig. 6 Color degradation rates of esterified astaxanthins in O/W microemulsions containing various concentrations of antioxidants (EDTA Δ , ascorbic acid $\circ$, and vitamin E acetate $\square$) during storage under fluorescent illumination at 37°C for 20 days.

occupation of metal coordination sites, the formation of insoluble metal complexes, and the steric hindrance of interactions between metals and lipid substrates³¹. It is known that transition metal contaminants can promote lipid oxidation in emulsions only at low levels^{11, 32}, and these contaminants can come from a variety of sources (e.g., raw materials, oils, emulsifiers, water, and contact with containers). Several recent studies have shown that the addition of EDTA is highly effective at retarding the chemical degradation of unsaturated lipids, such as lycopene and unsaturated fatty acids^{32–34}, in emulsions. Based on these findings and the fact that EDTA effectively inhibits the degradation of esterified astaxanthins, we speculated that the samples might have contained low concentrations of transition metals, such as iron (Fe^{2+}) or copper (Cu^{2+}), which promote the chemical degradation of esterified astaxanthins in microemulsions. Therefore, the addition of a transition metal chelator to microemulsions that contain esterified astaxanthins appears to be highly effective at improving their stability against chemical degradation.

Ascorbic acid and vitamin E acetate are vitamins that might act as antioxidants in foods through a variety of mechanisms, such as oxygen scavenging and free radical scavenging³⁵. However, ascorbic acid and vitamin E acetate are much less effective than EDTA at chelating transition metals and inhibiting color degradation (Fig. 6). Under certain conditions, ascorbic acid can act as a pro-oxidant and convert ferric ion to the more reactive ferrous ions, which can decompose lipid hydroperoxides into free radicals³³. However, no pro-oxidant activity of ascorbic acid was observed in this study, possibly because the form of

Fig. 7 Relative color degradation rates for esterified astaxanthin-enriched oil-in-water microemulsions containing various antioxidants during storage under fluorescent illumination at 37°C for 20 days: no antioxidant (control); water-soluble antioxidant (0.05% EDTA, w/v); oil-soluble antioxidant (0.05% vitamin E acetate, w/v); water-soluble and oil-soluble antioxidants (0.05% EDTA + 0.05% vitamin E acetate, w/v). Different letters indicate data that significantly differ ($p < 0.05$).

iron that promotes the degradation of esterified astaxanthins is primarily the oxidized (ferric) form of iron, and ascorbic acid, which reduces ferric to ferrous ions, would reduce the concentration of the active pro-oxidant³⁶. In this study, vitamin E acetate is not a highly effective antioxidant, probably because the esterification of tocopherol with acetic acid reduces its chemical reactivity. These reasons might explain, in part, why the effectiveness of the additives at inhibiting color fading decreased in the following order: EDTA > ascorbic acid > vitamin E acetate.

3.4 The Effect of Antioxidant Combinations on Esterified Astaxanthin Degradation

To demonstrate whether combinations of antioxidants were more effective at protecting esterified astaxanthins from degradation in microemulsions than single antioxidants, we tested systems containing a mixture of a water-soluble antioxidant (EDTA) and an oil-soluble antioxidant (vitamin E acetate). The esterified astaxanthin degradation rates were calculated from plots of total color difference against time for microemulsions stored under fluorescent illumination at 37°C for 20 days. The relative color degradation rates for microemulsions containing no antioxidants (control), water-soluble antioxidant only (0.05% EDTA, w/v), oil-soluble antioxidant only (0.05% vitamin E acetate, w/v), and an antioxidants combination (0.05% EDTA + 0.05% vitamin E acetate, w/v) (Fig. 7) were 100%, 1.4%, 11.9% and 6.7%, respectively (the relative degradation rate is calculated as the degradation rate for the sample divided by the degradation rate for the control).

The microemulsions containing antioxidants exhibited slower color fading than the control sample, and the effectiveness of the antioxidants at inhibiting the degradation of the esterified astaxanthins decreased in the following order: EDTA > EDTA + vitamin E acetate > vitamin E acetate. This experimental result conflicts with the previous reports that combinations of antioxidants were usually more effective than individual antioxidants at inhibiting oxidation³⁷⁾. In that study, ascorbic acid has been postulated to regenerate oxidized vitamin E, increasing the ability of vitamin E acetate to inhibit lipid oxidation. However, in our system, the mixture of antioxidants was less effective than the EDTA alone at retarding esterified astaxanthins degradation. We concluded that the phenomenon might be due to the vitamin E acetate may have an antagonistic effect on the degradation reaction³⁸⁾.

4 CONCLUSIONS

Overall, the well-dispersed oil-in-water microemulsions containing esterified astaxanthins were prone to color fading during storage under fluorescent illumination at 37°C, and this is attributed to chemical degradation of the esterified astaxanthins. Moreover, this degradation was effectively retarded by antioxidants, especially EDTA. The results of our study will prove important for application for the rational design of microemulsion based delivery systems to protect and delivery esterified astaxanthins in foods, beverages and other commercial products. Currently, we are attempting to identify the effect of various emulsifiers and methods of microencapsulation on the degradation of esterified astaxanthins in food-grade O/W emulsions. And the stability of esterified astaxanthin microemulsions in different model food systems engineering will be examined in our future studies.

ACKNOWLEDGEMENTS

Financial support for this study was provided by National Key Technology Research and Development Program of the Ministry of Science and Technology of China (No. 2012BAD33B07), National Natural Science Foundation of China (No. U1406402), and Program for Changjiang Scholars and Innovative Research Team in University (IRT1188).

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