Microencapsulation of astaxanthin in a chitosan matrix

I. Higuera-Ciapara*, L. Felix-Valenzuela, F.M. Goycoolea, W. Argüelles-Monal

Laboratory of Supercritical Extraction, Centro de Investigación en Alimentación y Desarrollo A.C., Carretera a la Victoria, Km 0.6 A.P. 1735, Hermosillo, Sonora 83000, Mexico

Abstract

Synthetic astaxanthin was microencapsulated in a chitosan matrix cross-linked with glutaraldehyde by using the method of multiple emulsion/solvent evaporation. A powdered product was obtained containing microcapsules with a diameter of 5–50 μm. The stability of the pigment in the microcapsules was studied under storage at 25, 35 and 45 °C for 8 weeks by measuring isomerization and loss of concentration of pigment. Pigments were extracted from the microcapsules with a mixture of dichloromethane/methanol (50:50) and the astaxanthin was analyzed by HPLC with a diode array detector (480 nm). Results showed that the microencapsulated pigment did not suffer isomerization nor chemical degradation under the investigated storage conditions.

Keywords: Astaxanthin; Chitosan; Microcapsules; Emulsion

1. Introduction

Astaxanthin (3,3′-dihydroxy-β-β′-carotene-4-4′-dione) is the main ketocarotenoid responsible for the red–orange color in salmonids and crustacean (Gentles & Haard, 1991; Shahidi & Synowiecki, 1991). The most important commercial application of astaxanthin, obtained either from synthetic origin or from natural sources, e.g. microalgae, yeast, or crustacean by-products, is in the aquaculture industry where it is used in the formulation of feeds for farmed salmon to provide the typical muscle color, which is widely accepted by consumers throughout the world. Astaxanthin has several key biological functions in fish. It serves as a precursor of vitamin A, it is associated with reproduction and embryo development and also with protecting cells against oxidative damage (Parajo, Santos, & Vazquez, 1996; Putnam, 1991). In human nutrition, astaxanthin has been gaining widespread popularity as a dietary supplement due to its powerful antioxidant properties. Currently, several astaxanthin products derived from microalgae are available in the marketplace, and being promoted as anticancer and anti-inflammatory agents as well as immunostimulants.

As most carotenoids, astaxanthin is a highly unsaturated molecule and thus, can easily be degraded by thermal or oxidative processes during the manufacture and storage of foods. This can cause the loss of their nutritive and biological desirable properties as well as the production of undesirable flavor or aroma compounds. Generally, carotenoids are found in nature as all-*trans* molecules in which all the double bonds are in the *trans* configuration (Urich, 1994). It is also well known that high temperature and light conditions may promote the isomerization to the *cis* forms. The *cis* isomers of the provitamin A carotenoids have less activity than their corresponding all-*trans* carotenoids (Sweeney & Marsh, 1973).

Due to their intrinsic high instability, these compounds are not usually handled in their crystalline form but rather as stabilized emulsions or microcapsules. The microencapsulation process is generally performed by forming a polymeric matrix or coating layer around a particular compound in order to protect its biological activity from environmental factors and enhance its physicochemical stability. Among the most commonly used matrixes are those comprised by hydrolyzed starch and gelatin. Methods employed are based on emulsion preparation between the carotenoid and the encapsulating matrix, followed by spray

* Corresponding author. Tel.: +52-662-2892400; fax: +52-662-2800055.

E-mail addresses: higuera@cascabel.ciad.mx (I. Higuera-Ciapara); lfelix@cascabel.ciad.mx (L. Felix-Valenzuela).

drying of the emulsion. The main disadvantage of these methods is that carotenoid losses occur during the process and the product obtained does not present a good stability.

The use of chitosan has not been reported for carotenoid microencapsulation. Chitosan is a copolymer formed by units of 2-deoxy-*N*-acetyl-D-glucosamine and 2-deoxy-D-glucosamine linked by β-1,4 glycosidic bonds. Chitosan is obtained from deacetylation of chitin (poly-*N*-acetyl-2-amino-2-deoxy-D-glucopyranose), which in turn is obtained from crustacean shells. Thus, crustacean by-products are an inexpensive source of chitosan.

Due to the nature of its chemical configuration, the chitosan chain has a rather rigid configuration and this property has been successfully used for the preparation of films, gels and spheres which are currently used in the biomedical, cosmetic and food industries. One of their main applications is in the production of microcapsules with delayed release of drugs in which the chitosan chains are cross-linked with dialdehydes like glutaraldehyde or tricarboxilic acids such as citric acid. Previous studies performed in our laboratory showed that chitosan can be utilized for astaxanthin microencapsulation (Félix, 1999; Higuera, Félix, Goycoolea, & Arguelles, 2002). The information on microencapsulation and stability of astaxanthin is very scarce; thus, the objective of this study was to microencapsulate it in a chitosan matrix cross-linked with glutaraldehyde and to study its stability under different storage temperatures.

2. Materials and methods

Astaxanthin was obtained from Sigma Chemical Co. (St Louis, MO). Chitosan was provided by the laboratory of biopolymers at CIAD and was a batch previously prepared from chitin obtained from shrimp shells (*Penaeus* spp.) following the protocol outlined by Lizardi (1998). The molecular mass (Mv) of chitosan was around 180 000 Da and the acetylation degree 14.7%. HPLC grade analytical reagents used were methanol (Sigma Chemical Co., St Louis, MO), dichloromethane (J.T. Baker, Phillipsburg, NJ), acetonitrile and water (Aldrich, Milwaukee, WI).

2.1. Microencapsulation of astaxanthin

Microencapsulation of astaxanthin was carried out through the multiple emulsion/solvent evaporation technique by Genta, Giunchedi, Pavanneto, Conti, Perugini and Conte (1997) with slight modifications consisting of the following. A primary emulsion (o/w) was prepared as a first step with an astaxanthin solution dissolved in dichloromethane (0.01 g/ml) (dispersed phase) and a 3% chitosan solution in a mixture of acetic acid (2% v/v)/methanol (4:1), with Tween 80 (1.6% w/v) as an emulsifier (continuous phase). The emulsion was prepared at room temperature and 9500 rpm using an Ultraturrax Janke & Kunkel T25 homogenizer. Later on, a multiple emulsion (o/w/o) was prepared adding the previously described emulsion in mineral oil (1:9 v/v ratio), with Span 20 (2% w/v) as an emulsifier. The multiple emulsion was prepared in a laboratory reactor (Janke & Kunkel Ika Laborrtechnick Model LR-A1000, Germany) at 50 °C and 100 rpm. The homogenizer was attached to the reactor vessel in order to achieve a stirring speed of 8000 rpm to produce the proper dispersion of the two phases. Once the multiple emulsion was formed, glutaraldehyde was added (50% aqueous solution) at a rate of 1 ml for each 10 ml of chitosan solution utilized in the primary emulsion. The cross-linking reaction was allowed to proceed at 50 °C during 2 h. The aqueous solvents were vacuum evaporated using a rotating evaporator (Buchi RE 121) at 60 °C during 7 h. The microcapsule suspension thus obtained was then centrifuged at 1590*g* for 5 min. The mineral oil was decanted and the microcapsules were washed three times with small volumes of petroleum ether and vacuum-dried at 40 °C during 24 h. Three batches of microcapsules were produced and later were mixed in order to perform the astaxanthin stability test during storage under controlled conditions. The size range of the microcapsules was measured through observation in a microscope (Carl Zeiss AxioLab Model) by randomly selecting 10 fields and measuring the largest and smallest diameter microcapsules. Microcapsule moisture was also measured at 80 °C under vacuum for 24 h.

2.2. Sample storage

Samples weighing approximately 0.35 g were placed in 10 ml open amber vials, which were then placed in desiccators containing a $MgCl_2{\cdot}6H_2O$ saturated solution prepared according to Budavari (1989), to afford a relative humidity of approximately 33%. Each desiccator contained also a vial with commercial powdered astaxanthin as a control. The desiccators were sealed with parafilm, covered with aluminum foil and stored at 25, 35 and 45 °C, respectively. The storage temperatures were recorded inside of the stove where the desiccators containing the samples were placed. Duplicate samples of the three treatments were taken weekly and analyzed by HPLC during 8 weeks.

2.3. Microcapsule extraction for HPLC analysis

In order to measure the astaxanthin content of the microcapsules during their storage, 50 mg of sample were weighed in an amber vial with a screw top. Five milliliter of a dichloromethane/methanol (HPLC grade) (50:50) mixture was added. The vial was closed tightly and placed under agitation at 400 rpm during 30 min. The mixture was centrifuged at 2800*g* for 5 min using a Beckman GS-6R centrifuge at 20 °C. The extract was decanted. The residue was subjected to three further extractions and pooled with the previous extract for one more centrifugation step under the same conditions described above. The extracts were

filtered (0.22 μm) and stored at −18 °C for HPLC analysis. In the case of the astaxanthin control samples, a solution (~0.3 mg/ml) was prepared using a 50:50 dichloromethane/methanol solution. This was then filtered through 0.22 μm and stored at −18 °C for its HPLC analysis.

2.4. HPLC astaxanthin extract analysis

HPLC analysis was performed in a Waters Liquid Chromatograph, equipped with a G9760F pump, a HP diode array detector system (Model 1100) and a reverse phase column [Beckman Ultrasphere C18 (5 μm) measuring $250 \times 4.6\ mm^2$]. Twenty microliter of samples were eluted at room temperature with a mobile isocratic phase consisting of 85% methanol, 5% dichloromethane, 5% acetonitrile and 5% water. Absorption spectra were obtained in the 250–700 nm interval and detection was performed at 480 nm (Yuan & Chen, 1998). The chromatogram peaks were identified and quantified using an external astaxanthin standard. The standard was dissolved in a dichloromethane/methanol (HPLC grade) mixture (25:75) and filtered (0.22 μm) through a porous membrane. From this stock solution, dilutions were prepared using methanol to prepare the calibration curve. Results were processed using and HPLC Chemstation Spectral SW module and astaxanthin concentration results in the microcapsules were subjected to an analysis of variance and mean comparison according to Tukey–Kramer (NCSS 1997).

3. Results and discussion

3.1. Microcapsule preparation

Chitosan is currently used to prepare microcapsules used for the slow release of pharmaceutical compounds (Majeti & Kumar, 2000). Two of the most commonly used techniques for microencapsulation of hydrophobic and hydrophilic compounds are spray drying and emulsification/evaporation of solvent (Genta et al., 1997). This last technique is usually combined with chemical cross-linking of chitosan and was used in this study for the astaxanthin microencapsulation. In this technique, Genta et al. (1997) recommend the use of a 1.6% (w/v) solution of chitosan in order to obtain a stable emulsion. In this study, the concentration used was 3% so that the polymeric membrane would be thicker and stronger to avoid its rupture and pigment diffusion toward the oily phase when preparing the multiple emulsion. At this concentration, the chitosan solution was very difficult to handle due to its high viscosity but spherical microcapsules were obtained (Fig. 1). These microcapsules had a non-homogeneous size and a diameter of 5–50 μm. The yield of the process (i.e. amount of microencapsulated astaxanthin/utilized astaxanthin) was about 92% (Félix, 1999). The moisture content of the powder obtained was 11.25%.

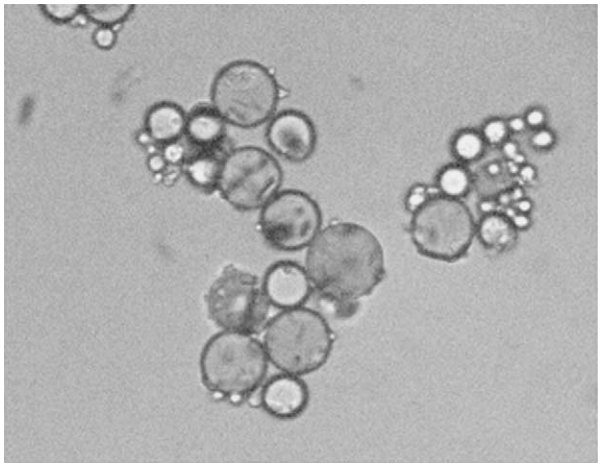

Fig. 1. Microphotograph (100 ×) of astaxanthin microcapsules in a chitosan matrix.

3.2. Microcapsule extraction

Before selecting the proper solvent system for pigment extraction, several trials were made using various solvents or their mixtures at several ratios: acetone, methanol, dichloromethane/acetone (50:50) and dichloromethane/methanol (25:75 and 50:50). The system that afforded the best carotenoid extraction was the dichloromethane/methanol (50:50). However, such a system did not result efficient as it extracted only 77% of the total microencapsulated astaxanthin. The extraction yield (%) was obtained by dividing the quantity of astaxanthin from microcapsules according to the HPLC analysis by the theoretical concentration obtained from the amount of astaxanthin used in the microencapsulation process, considering that the efficiency of the microencapsulation method was 92%. Four consecutive extractions were performed and even though the fourth extract was colorless, the residue had a dark orange color, indicative that pigment removal was not complete. In spite of this, a dichloromethane/methanol (50:50) mixture was preferred for pigment extraction from the samples under the conditions previously mentioned in the methodology section. It has been shown that dichloromethane/methanol mixtures are efficient carotenoid extraction solvents (Yuan & Chen, 1998). It is likely that the chitosan matrix hindered total extraction of the pigment.

3.3. HPLC astaxanthin analysis

The HPLC technique allows the separation of astaxanthin isomers; thus, chromatograms of the extracts from the microcapsules before and after storage were compared in order to find out if the pigment was destabilized due to isomer formation during the stability experiment. Retention times for astaxanthin peaks (Figs. 2 and 3) both from extracts and external standard varied from 6.4 to 7.2 min in the analysis carried out during storage. This was due to

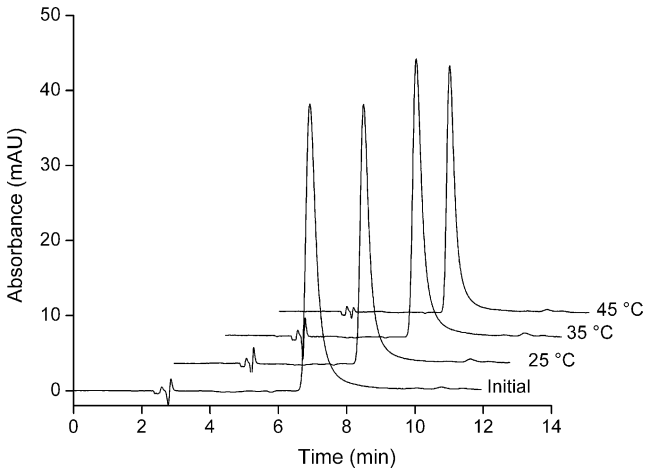

Fig. 2. Representative HPLC profile of the extract of microcapsules, initially and after 8 weeks storage. Chromatographic conditions are described in the text.

the fact that analysis was performed at room temperature (~25 °C) without control of column temperature. However, the variation coefficients between the areas of sample duplicates in all cases were less than 5%. The presence of only one peak in the chromatogram and in the tri-dimensional spectra of both the initial extract and the microcapsules extracts stored at 25, 35 and 45 °C, suggests that the pigment did not experience isomerization at any of the above temperatures during the 8 weeks of storage. Therefore, it can be argued that the matrix formed during the microencapsulation process protects the pigment from thermal and oxidative (air was not eliminated from the vials containing the samples) degradation. However, the astaxanthin control showed the same behavior as the microcapsules extracts. Fig. 3 shows that only the astaxanthin *trans* peak appeared after 6.6 min in the experimental temperatures after 8 weeks of storage. In a similar study, Wagner and Warthesen (1995) studied the stability of carrot carotenes microencapsulated in hydrolyzed starch by spray drying. These authors found that the carotenes in the spray-dried carrot powder did not experience isomerization at 45 °C with or without exposure to light. Contrary to this report, Chen and Tang (1998) found that all-*trans* α- and β-carotene from spray-dried carrot pulp powder microencapsulated in a gelatin/sacharose matrix stored in darkness at 4, 25 and 45 °C did isomerize to 9 *cis*, 13 *cis* and 15 *cis* α- and β-carotene, respectively. According to this, it is clear that the stability of microencapsulated carotenoids depends upon the process conditions used, as they determine the properties of the final product obtained. In our study it is likely that the manufacturing conditions were not aggressive enough to destabilize the pigment since neither the control nor the microcapsules extracts presented isomers.

3.4. Storage stability evaluation

Astaxanthin stability was evaluated on the basis of retained pigment quantity during microcapsules storage at 25, 35 and 45 °C. For this purpose, weekly quantification by HPLC of the pigment concentration of the microcapsules extracts was performed. As can be seen in Fig. 4, the astaxanthin concentration varied between 18 and 21 mg/g in the three treatments. However, the behavior of the samples stored at 25 °C which had a concentration of about 20–21 mg during the first 6 weeks, was significantly different ($p < 0.05$) to that of samples stored at 45 °C which had a pigment concentration around 18 mg/g. As far as the time factor was concerned, no significant differences were observed ($p > 0.05$) from the initial astaxanthin concentration (20.68 mg/g) and the end of the experiment (18.63, 18.08 and 17.7 mg/g at 25, 35 and 45 °C, respectively). As can be observed in Fig. 4, none of the treatments showed a marked decreasing tendency in astaxanthin concentration in the microcapsules during

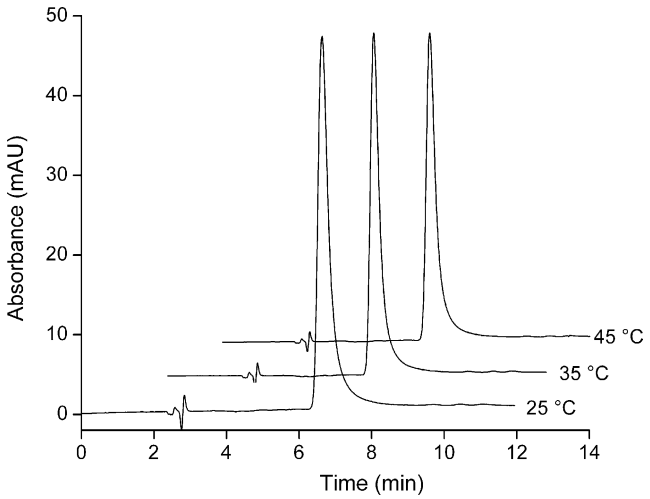

Fig. 3. Representative HPLC profile of pure astaxanthin after 8 weeks storage. Chromatographic conditions are described in the text.

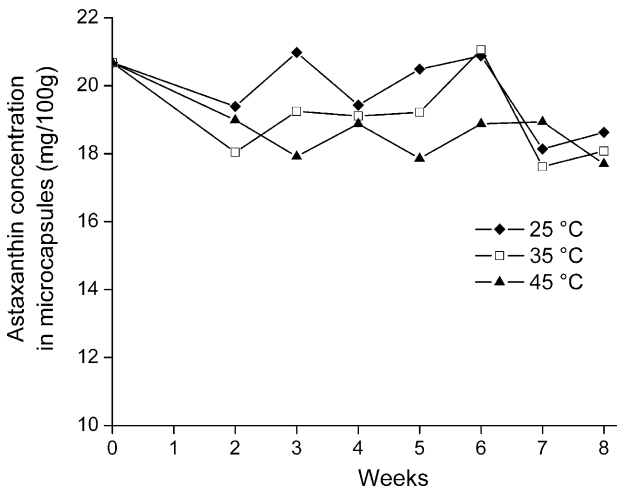

Fig. 4. Evolution of astaxanthin concentration in stored microcapsules at 25, 35 and 45 °C (relativity humidity of 33%).

storage. This would have indicated pigment degradation. Rather, some fluctuations due to storage time were observed. While results from the HPLC technique were in general highly reproducible, when the final extract concentration was calculated (mg astaxanthin/mg microcapsules) some results showed substantial variability. Approximately 28% of the data showed a coefficient of variation between 8.5 and 10%. This is probably due to variation during the extraction method since these were performed simultaneously for all treatments. It is also necessary to perform further work to improve the technique of astaxanthin extraction from the microcapsules.

On the other hand, there are no published reports on the stability of pure astaxanthin microencapsulated in chitosan. In some studies performed with carotenes in different matrixes it has been suggested that degradation takes place mainly due to auto-oxidation (Desobry, Netto, & Labuza, 1997; Wagner & Warthesen, 1995). The auto-oxidative products do not present color properties, as they do not absorb at the same wavelengths as carotenes (Wagner & Warthesen, 1995). In our study, according to Fig. 4, it is suggested that no pigment loss due to oxidation occurred under the treatments performed.

4. Conclusions

Under the conditions of this study, the microencapsulated astaxanthin was maintained in stable conditions as measured by isomerization and pigment degradation. The extraction method utilized did not prove be very effective for total pigment extraction from the microcapsules. Thus, more studies are needed in this regard. This is the first reported study of astaxanthin microencapsulation in a chitosan matrix so it is important to highlight that the conditions for microcapsules preparation can be considered as a preliminary step for future process development. It is also deemed necessary to perform further studies on astaxanthin stability under different storage conditions to generate further scientific information on this important subject.

References

Budavari, S. (1989). *The Merck index*, USA: Merck & Co. Inc.

Chen, B. H., & Tang, Y. C. (1998). Processing and stability of carotenoid powder from carrot pulp waste. *Journal of Agricultural and Food Chemistry*, *46*, 2312–2318.

Desobry, S. A., Netto, F. M., & Labuza, T. P. (1997). Comparison of spray-drying, drum-drying and freeze-drying for β-carotene encapsulation and preservation. *Journal of Food Science*, *62*, 1158–1162.

Félix, V. L (1999). Extracción supercrítica de astaxantina de biodesechos de jaiba (*Callinectes sapidus*) con CO_2/etanol y su microencapsulado con quitosano. *Tesis de maestría*, Centro de Investigación en Alimentación y Desarrollo, A.C. Hermosillo, Sonora, México.

Genta, I., Giunchedi, P., Pavanneto, F., Conti, B., Perugini, P., & Conte, U. (1997). Preparation of chitosan microparticulate drug delivery systems. In R. A. A. Muzzarelli, & M. G. Peter (Eds.), *Chitin handbook* (pp. 391–396). Italy: European Chitin Society.

Gentles, A., & Haard, N. F. (1991). Pigmentation of rainbow trout with enzyme treated and spray dried *Phaffia rhodozyma*. *The Progressive Fish Culturist*, *53*, 1–6.

Higuera, C. I., Félix, V. L., Goycoolea, V. F. M., & Argüelles, M. W. M. (2002). *International Patent Application* PCT/MX02/00085. Método de preparacion de microcápsulas de astaxantina en quitosano y producto obtenido. Septembee. pp. 11.

Lizardi, M. J (1998). Implementación de tecnologías alternativas para la obtención de polisacáridos funcionales a partir de biodesechos pesqueros quitinosos. *Tesis de Maestría*, Centro de Investigación en Alimentación y Desarrollo, A.C. Hermosillo, Sonora, México.

Majeti, N. V., & Kumar, R. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, *46*, 1–27.

Parajo, J. C., Santos, V., & Vazquez, M. (1996). Producción biotecnológica de astaxantina por *Phaffia rodozyma*. *Alimentación, Equipos y Tecnología, Ene/feb*, 153–160.

Putnam, M. (1991). A review of the nature, function, variability and supply of pigments in salmonid fish. In N. de Pauw, & J. Joyce (Eds.), *Aquaculture and the environment* (pp. 245–263). Gent. Bélgica: European Aquaculture Society.

Shahidi, F., & Synowiecki, J. (1991). Isolation and characterization of nutrients and value-added products from snow crab (*Chinoecetes opilio*) and shrimp (*Pandalus borealis*) processing discards. *Journal of Agricultural and Food Chemistry*, *39*, 1527–1532.

Sweeney, J. P., & Marsh, A. C. (1973). Liver storage of vitamin A in rats fed carotene stereoisomers. *Journal of Nutrition*, *103*, 20–25.

Urich, K. (1994). *Comparative animal biochemistry*, Germany: Springer.

Wagner, L. A., & Warthesen, J. J. (1995). Stability of spray-dried encapsulated carrot carotenes. *Journal of Food Science*, *5*, 1048–1053.

Yuan, J. P., & Chen, F. (1998). Chromatographic separation and purification of *trans* astaxanthin from the extracts of *Haematococcus pluvialis*. *Journal of Agricultural and Food Chemistry*, *46*, 3371–3375.