Vol. 15, No. 2 (2016) 457-468
Revista Mexicana de Ingeniería Química

# STABILITY, ANTIOXIDANT ACTIVITY AND BIOAVAILABILITY OF NANO-EMULSIFIED ASTAXANTHIN

# ESTABILIDAD, ACTIVIDAD ANTIOXIDANTE Y BIODISPONIBILIDAD DE ASTAXANTINA NANO-EMULSIFICADA

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Recibido 14 de octubre de 2015; Aceptado 6 de abril de 2016

**Abstract**

Dietary carotenoids, especially xanthophylls, have attracted attention because of their characteristic biological activities, including antioxidant and anti-cancer properties. Astaxanthin (ASX) is a red-orange carotenoid pigment with, and a powerful biological antioxidant with no pro-vitamin A activity, which occurs naturally in a wide variety of living organisms. In this study, oil in water nanoemulsions of astaxanthin were prepared by high and low energy methods. The influence of oil type and surfactant concentration on the particle size was investigated. The mean diameter of the best treatment was of 40nm. The effect of nano-emulsification of ASX on its bioabsorption was evaluated in a murine model. Low ASX degradation and high antioxidant activity were determined. Absorption kinetics in rats were evaluated, and it was found that nano-emulsified astaxanthin increased carotenoid plasma concentration up to 7.5-fold when compared with a reference solution.

*Keywords*: nano-emulsion; astaxanthin; bioabsorption; plasma, bioactive compound.

**Resumen**

Los carotenoides, específicamente las xantofilas, han atraído la atención debido a su actividad biológica característica, que incluye propiedades antioxidantes y anticancerígenas. La astaxantina (ASX) es un pigmento rojo-naranja que actúa como un antioxidante biológico potente, sin que posea la actividad de la vitamina A, el cual se produce en una gran variedad de organismos vivos. En este estudio, nanoemulsiones aceite en agua de astaxantina se prepararon por métodos de alta y baja energía. Se investigó la influencia del tipo de aceite y concentración de surfactante en el tamaño de partícula. El tamaño medio de partícula del mejor tratamiento fue de 40nm. Se evaluó el efecto de la nanoemulsificación de la ASX en la bioabsorción de un modelo murino. Se obtuvo una baja degradación de ASX y una alta actividad antioxidante. Se evaluó la cinética de absorción en ratas y se encontró que la astaxantina nanoemulsificada incrementó su concentración en plasma hasta 7.5 veces más que la solución de referencia.

*Palabras clave*: nanoemulsión, astaxantina, bioabsorción, plasma, compuesto bioactivo.

## 1 Introduction

There is a growing interest within the food and beverage industries for the utilization of nano-emulsions to encapsulate and deliver lipophilic bioactive compounds, such as micronutrients, and nutraceuticals (McClements, 2007; Sanguansri *et al*., 2006; Yang *et al*., 2012). Nano-emulsions can be formulated from food-grade ingredients using simple processing operations, such as mixing, shearing, and homogenization (McClements *et al*., 2011; Carrera *et al*., 2014) and typically consist of a surfactant, oil, and water (Mason *et al*., 2006; Tadros *et al*., 2004). Oil-in-water emulsions contain surfactant-coated lipid droplets dispersed within a continuous aqueous phase.

Nano-emulsions (NE) are emulsions with very small and uniform particle size, usually in the range of 20-200 nm (Gutiérrez *et al*., 2008; Jafari *et al*., 2006; Solans *et al*., 2005), that provides them with a transparent or translucent appearance, high kinetic stability against creaming or sedimentation, and a large interfacial area (Solans *et al*., 2005;

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Flores-Miranda *et al*., 2015). NE can be easily incorporated into beverages and food gels without compromising their visual aspect (Tadros *et al*., 2004; Cavazos-Garduño *et al*., 2012). These systems are thermodynamically unstable and require a rather large energy input for their preparation. This energy input may originate from external sources of energy as high pressure homogenizers, microfluidizers, ultrasound, high shear mixers, etc; or internal sources as the chemical energy of the system (Solans *et al*., 2005). Methods that use external power are called high-energy or dispersion methods, while methods that use chemical energy stored in the system are low-energy or condensation methods.

One of the most promising potential applications of NE is the incorporation of lipophilic bioactive compounds (e.g., vitamins, nutraceuticals, and antimicrobials) into aqueous-based foods or beverages that need to remain optically transparent, such as some fortified waters, soft drinks, sauces, and dips, without compromising their attributes (Anarjan *et al*., 2012). Another potential advantage of these systems is that they often have better stability to particle aggregation and gravitational separation than conventional emulsions due to their small particle size (Tadros *et al*., 2004). The small droplet size of the lipid nano-emulsions offer solutions for availability problems of orally-administered compounds, NE can improve the solubility of water insoluble bioactive compounds, enhance permeation due to their adhesive properties, prevent enzymatic degradation in gut wall and/or liver, protect against gastrointestinal degradation and improve the control of functional ingredient release at specific locations, so as to improve their stability, bioavailability and pharmaceutical properties (Ragelle *et al*., 2012; Reyes-Munguía *et al*., 2009; Sarduní *et al*., 2005; Shrewsbury *et al*., 2009; Sozer *et al*., 2009). The digestion of lipids within gastrointestinal tract is a complex process and the uptake of any encapsulated lipophilic components have gained attention as a tool to enhance the bioavailability of bioactive components.

In this study, it was used astaxanthin as lipophilic component that can be used as ingredient to incorporate into functional product. Astaxanthin (ASX, 3,3'-dihydroxy-4,4'-diketo-$\beta$-$\beta$-carotene) is a naturally occurring carotenoid with red pigmenting properties, it is synthesized by plants and algae and distributed in marine seafood (Kidd *et al*., 2011; Miki, 1991). The presence of hydroxyl and ketone groups in each ionone ring, explains its unique chemical properties such as a higher anti-oxidant activity and a more polar configuration than other carotenoids (Miki, 1991). Studies have found that astaxanthin, have antioxidant properties which are 11-1000 times more potent than those of other carotenoids (Miki, 1991). ASX has anti-tumor (Tanaka *et al*., 1995) and anti-inflammatory activities, positive properties on blood pressure as well as a cardioprotective effect (Hussein *et al*., 2005). Astaxanthin is also available as a food supplement, but, like other carotenoids, is a very lipophilic compound with low oral bioavailability. However, this bioavailability can be enhanced in the presence of lipids (Odeberg *et al*., 2003). The purpose of this study was to evaluate the stability of astaxanthin nano-emulsion, its antioxidant activity and its absorption in a murine model.

# 2 Materials and methods

## *2.1 Materials*

ASX for feeding purposes was purchased from Future Foods, S.A. de C.V. (Mexico City) and high purity for analytical standard was obtained from Santa Cruz Biotechnology Inc. (Dallas, TX). Non-ionic surfactants Tween 40 and Span 20 were purchased from Sigma-Aldrich Co. (Mexico City), canola oil and orange essential oil (AEN) were purchased from Proteinas y Oleicos, S.A. de C.V. (Mexico City) and Frutech International (Mexico City), respectively. Other chemicals, reagent or HPLC grade, were purchased from J.T. Baker, (Xalostoc, Edo. de Mexico, Mexico).

## *2.2 Preparation of nano-emulsions*

All emulsions were prepared using canola oil or AEN and Span 20 or Tween 40 at 10 wt% at surfactant: oil ratios of 1:9, 2:8, 3:7 and 4:6.

### *2.2.1. Low-energy method*

Nano-emulsions were formulated according to the methodology described by Sarduní *et al*. (2005). Water was added to mixtures of the other two components (oil and surfactant), using a magnetic stirrer at approximately 2000 rpm (SH21-2 magnetic stirrer Homothermal). Different oil-surfactant concentrations were mixed before water addition.

#### *2.2.2. High-energy methods*

##### *2.2.2.1. Coarse emulsion preparation*

Oil in water (O/W) emulsions were formulated at different oil-surfactant concentrations and pre-mixed at 1500 rpm for 10 minutes in a Silverson model L4R homogenizer (Silverson Machines Ltd, Waterside, Chesher, Bucks., England). Coarse emulsions were then further emulsified using a High Pressure Homogenization or a probe type ultrasound. Each experiment was prepared by duplicate.

##### *2.2.2.2. High pressure homogenization*

Previously prepared coarse emulsions were passed through a high-pressure homogenizer (APV-1000 dual effect, APV, Delavan, WI) at pressure levels ranging from 100 to 500 bar and for up to 6 cycles. The best conditions were selected by the lowest particle size with a pressure of 400 bar and 5 recirculation cycles.

##### *2.2.2.3. Ultrasonication*

(Branson Ultrasonic Corporation, Danbury, 24 kHz CT, USA), set at 80 W for 15 minutes, according to the procedure reported by Seki *et al*. (2004) with some modifications. Two-thirds of the probe tip was immersed in the coarse emulsion and the sonication was turned on to the highest power and maximum amplitude. During emulsification, sonication time was adjusted through the device software. All experiments were performed in a 150 mL glass beaker containing about 100 mL of the pre-emulsion. Each experiment was run by duplicate.

### *2.3 Emulsion droplet size analysis*

Emulsion particle size distribution was determined with a Coulter LS 230 Laser Diffraction Particle Size Analyzer (Bedford Hills, NY) with a measuring range from 0.04 to 2000 $\mu$m. This instrument measures light scattered intensity angular dependence of the diluted emulsion. This test was performed by diluting an emulsion sample of 5 mL in 15 mL of distilled water. Five to seven droplets of the diluted emulsion were placed in the equipment water flow until the amount of sample required was reached, and then the equipment proceeded to automatically perform the measurement. All measurements were done by duplicate. Particle diameter distribution (PDD) was calculated using the formula used by Jafari *et al*. (2006).

$$PDD = \frac{[d(v,90) - d(v,10)]}{d(v,50)} \quad (1)$$

In this formula, d (v, 10), d (v, 50), d (v, 90) are the diameters of 10%, 50% and 90% cumulative volume, respectively. In other words, [d (v, 90) - d (v, 10)] is data range, d (v 50) is the mean diameter.

### *2.4 Preparation of astaxanthin nano-emulsions*

In order to prevent ASX light sensitivity effect, the NEs were prepared with minimal exposure to light. The oil-in-water ASX emulsion was prepared using distilled water as the continuous phase and canola oil containing astaxanthin as the disperse phase. ASX extract was first mixed with canola oil (1% w/w). Next, this solution was mixed with distilled water (90% w/w) containing 9% w/w Tween 40 as the emulsifier. The premix was homogenized by ultrasonication at 80 W for 15 minutes.

### *2.5 Determination of Astaxanthin concentration in ASX-Nanoemulsions*

Determination of ASX concentration was conducted immediately after preparation and each week during 6 months of storage at 35° C. For measurement of total concentration in ASX-NE, ASX was extracted and measured with a diode array spectrophotometer (model 845; Agilent Technologies, Waldbronn, Germany) according to the method reported of Tolasa *et al*. (2005) with some modifications. All the studies were done under red light. Briefly, 10 mL of ASX-NE was extracted 2 times with 20 mL of acetone containing 0.05% BHT with the ultra turrax for 1 min. After each extraction, samples were centrifuged at 4000 rpm for 5 min at 4°C. Acetone extracts of samples were collected in a 250 mL separatory funnel. For separation of the water-soluble compounds, 40 mL of n-hexane, 100 mL of water and 0.5 g of NaCl were added. Approximately 20-30 min after the shaking process, all the white phase was withdrawn. The upper layer was poured to a measuring cup and completed to 50 mL (raw extract). Spectrophotometric determination was performed at 470-474 nm taken from the 350-600 nm spectrum. Calculation of total astaxanthix concentration was done according to a standard curve of astaxanthin. Analyses of the samples were done in duplicate.

### *2.6 Photometric determination*

Standard curve: Approximately 3 mg astaxanthin standard and 100 mg of butylated hydroxytoluene (BHT) were placed in a 10 mL volumetric flask and dissolved in dichloromethane in an ultrasonic bath (stock solution). Then 1 mL of this stock solution was diluted in a 10 mL flask with n-hexane. Immediately after the solution was prepared, its absorbance was determined in the 350-600 nm spectrum interval. Later, concentration of solution was measured in the maximum absorbance (approximately 472 nm). Astaxanthin concentration was calculated according to the following formula:

$$C_A S X\left(\frac{\mu g}{ml} o \frac{mg}{L}\right) = Absorbance \times \frac{10000}{2100} \qquad (2)$$

where 2100 is E (1%, 1 cm)=standard absorbance of all- E-Astaxanthin solution with 1% (w/v) in spectrobath with 1 cm in hexane at 470 nm; 10000 is the scale factor.

For preparing standard curve, 0.1, 0.5, 1.0, 1.5 and 2.0 mL of each diluted astaxanthin stock solution were placed in a 10 mL flask by pipette and n-hexane:acetone:water (40:120:100) was added to the mark level. Right after the solution was prepared, its absorbance was determined. A mixture of hexane, acetone and water was used as a blank.

### *2.7 Scavenging activity of the DPPH radical*

Scavenging activity of the DPPH radical by the ASX-NE was estimated according to Duan *et al.* (2007), with some modifications: 100 $\mu$L of ASX-NE were mixed with 2.9 mL of 0.1 mM DPPH-methanol solution. After the solution was incubated for 30 min at 25 ºC in the dark, the reduction of absorbance at 515 nm was monitored using a Jenway-Genova spectrophotometer (Mod. 6305-UV/Vis, England). The control contained HPLC grade methanol instead of antioxidant solution, while blanks contained HPLC grade methanol instead of the DPPH solution. Inhibition of DPPH radicals by ASX was calculated according to the following equation:

$$\%TAA = \left(1 - \frac{Abs_{sample}}{Abs_{control}}\right) \times 100 \qquad (3)$$

where TAA is the total antioxidant activity and Abs is the absorbance. Analyses of the samples were done in triplicate.

### *2.8 pH values*

pH values were determined with a Hanna Instruments potentiometer (model 8424 Woonsocket, RI, USA), calibrated with buffer solutions of pH 4 and 7 at 25° C.

### *2.9 Redox potential*

Measurements were made using a platinum indicator electrode and a silver/silver chloride reference electrode, connected to a digital voltmeter (Hanna Instruments, model 8417, Milano, Italy) according to methodology of Manzocco *et al.* (1998). Calibration was performed against a standard redox solution (Reagecon, Shannon, Co. Clare, Ireland) having a redox potential value of 220 mV at 25° C. Electrodes were placed in a 50 mL 3-neck flask, containing 15 mL of each sample. Prior to analysis, oxygen was removed from the system by continuous nitrogen flushing for 10 min. Redox potential was monitored for at least 20 min at 25 °C, until a stable potential was reached, that was defined as the point at which changes were under 1 mV in a 1 min period. The analyses were performed by triplicate.

### *2.10 Animal study design*

Twelve weeks old male Wistar rats obtained from the Universidad Veracruzana Medical School (Xalapa, Ver.) were used. The animals were kept under standard laboratory conditions, at 25° C ±1°C, relative humidity of 55% ± 5% and light/dark periods of 12 h. The animals were housed in polypropylene cages, 6 per cage, with free access to a standard laboratory diet (Purina Rodent Chow, Puebla, Mexico) and water *ad libitum*.

The rats were randomly divided into 3 groups: control (B), astaxanthin nanoemulsion (ASX-NE) and reference (CA). 24 h before the experiment, food was withdrawn but water was offered *ad libitum*. A single dose (1 mg/kg of body weight) was administered. The dose was calculated based on the weight of the rats. Astaxanthin dosage was prepared by mixing astaxanthin with canola oil for both groups: nano-emulsion and the reference group.

#### *2.10.1 Blood sampling*

Blood samples (5 mL) were obtained pre-dose and 2, 4, 8, 12, 18, 24, 36, 48 and 72 h time after the ASX dose. All samples were collected in 5-mL EDTA tubes (Vacutainer, NJ). Samples were stored

in ice and protected from light (up to 30 min) before being centrifuged (4°C, 4,500 rpm, 10 min); plasma was separated and transferred to amber vials after centrifugation and then stored at -70° C until further analysis.

#### *2.10.2. Astaxanthin analytical method*

Analysis of ASX was performed using liquid-liquid extraction for sample preparation and Waters HPLC model 600 with a UV/visible detector. Plasma samples and control plasma were thawed at room temperature under reduced light.

Samples were examined using the methodology reported by Odeberg *et al.* (2003) with some modifications. Briefly, plasma samples were thawed at room temperature under conditions of reduced light. To extract the carotenoids 4 mL of acetone were added to 0.5 mL of plasma in 10-mL glass tubes. After vigorous mixing for 10 s, the tubes were left on a reciprocating shaker for 1 h protected from light. Thereafter, 4 mL of hexane were added and samples were mixed vigorously for 10 s before separation into two phases occurred in 1 h. The upper phase was then removed and dried with a stream of nitrogen. The dried extract was dissolved in 300 $\mu$L of methanol. The solution was syrige-microfiltered (0.22 $\mu$m; Merck Millipore, Mexico) directly into the sample vials and immediately sealed, then 25 $\mu$L of sample were injected into a C18 reverse-phase HPLC column (Symmetry, 3.5 $\mu$m, 4.6 mm x 75 mm, Waters, Milford, MA). The carotenoid was eluted using a Varian ProStar pump with a linear gradient with methanol: water: ethyl acetate, 82:8:10. The flow rate was 1 mL/min and the pressure was about 260 atm. The mobile phase was prepared fresh every time. ASX was detected at 474 nm using a Waters 2487 Dual $\lambda$ Absorbance Detector. Retention time (RT) for ASX was 2.5 min.

Quality control plasma samples and calibration standards were prepared from a stock solution of astaxanthin (99% pure, Axxora) in acetone that contained 1 mg/mL. The lower limit of quantification (LLOQ), based on peak area, was 20 ng/mL.

### *2.11 Statistical analysis*

The experiments were done in duplicate. Tukey-test was used for comparison between the two results and a one-way analysis of variance (ANOVA) was used for comparison of more than two means. A difference was considered statistically significant when $p < 0.05$.

## 3 Results and discussion

Initially, the influence of homogenization method and system composition on the formation of oil-in-water nano-emulsions was examined.

### *3.1 Emulsions produced by high-energy methods*

For this study, emulsions were formulated according to high-energy methods described previously, at different surfactant concentration (%). For high pressure homogenizing methods, emulsion particle size (EPS) is a function of emulsification pressure, temperature and product composition. In this work, experiments were conducted to examine the dependence of emulsion particle size on emulsion composition. The results are depicted in Fig. 1.

For high pressure homogenizing method, there was a steep decrease in EPS in almost all oil-in-water emulsions when surfactant concentration was increased from 6 to 9 %. This EPS is obtained as a result of the increase of homogenization pressure energy transferred into the emulsion, which reduces the size of the particles. These data are similar to those reported by Qian *et al.* (2011), who reported that by employing a high pressure homogenizer, mean particle diameters of 60-150 nm could be obtained.

Another way to decrease the emulsion particle size is by increasing the surfactant concentration in the emulsion, allowing the newly formed particles to stabilize. This is attributed mainly to sufficient surfactant concentration present to allow rapid diffusion and adsorption of surfactant to the newly formed particles; and this coincides with observations made by Kentish *et al.* (2008).

The emulsions containing canola/Tween 40 at 8 and 9% and AEN/Span 20 at 6% had EPS greater than 200 nm, suggesting an "over-processing" effect during homogenization, which is consistent with observations reported by Jafari *et al.* (2006). This phenomenon could be attributed to surfactants malfunction and an increase in Brownian motion, which increases collision probability and coalescence when energy is applied during homogenization. Under these conditions, emulsion particle size distribution is the result of the competition between two opposing processes: rupture and particle-particle coalescence.

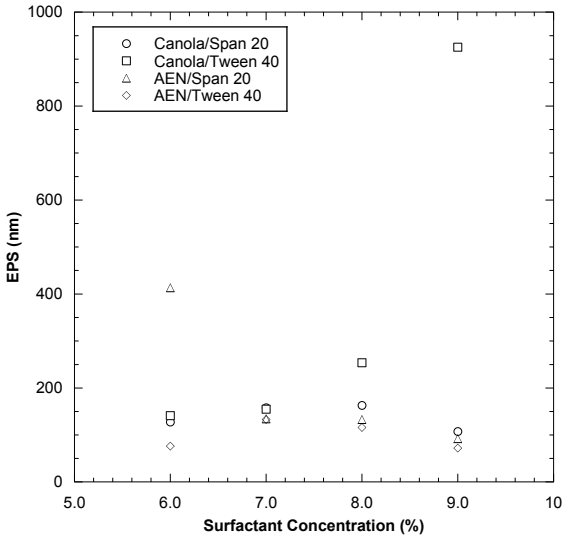


Fig 1. Emulsion Particle size obtained based on surfactant concentration (%) using high-pressure homogenization.

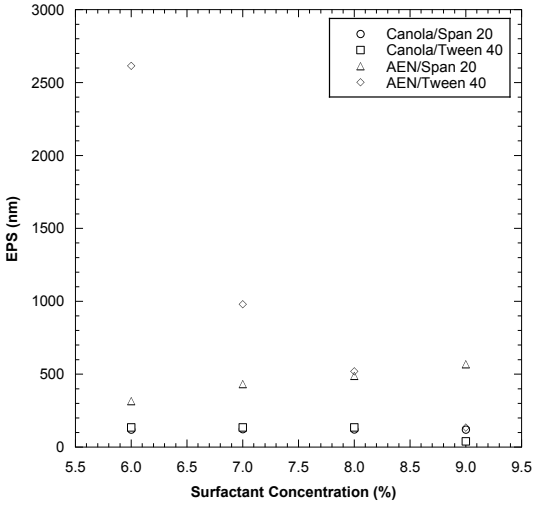


Fig 2. Emulsion Particle size (EPS) obtained based on surfactant concentration (%) using a sonication method.

The emulsification method traditionally used in the food industry is valve homogenization (McClements, 2004). This process consumes large amounts of energy, of which only a small proportion of the energy applied is effective (Tadros *et al*., 2004).

For ultrasound, EPS is a function of product composition, emulsification energy provided and time of emulsification. In this work, experiments were conducted to examine the dependence of emulsion particle size on emulsification time and emulsion composition.

Experiments were conducted to determine the effect of sonication time and after 15 minutes of sonication it was observed that EPS obtained was ca. 100 nm and translucent or bluish appearance nano-emulsions were obtained. Considering this, 15 min was chosen as the ideal time to ensure good emulsification.

The ultrasound equipment consisted of a probe, which was introduced into the pre-emulsion, and was responsible for providing the energy needed. This equipment has the advantage that the probe provides sonication energy directly to the pre-emulsion, resulting in EPS below 200 nm and near 40 nm.

Ultrasound employed for nano-emulsion formulation is well established, at least at laboratory scale (Abismaïl *et al*., 1999). However, most studies to date have focused on synthetic emulsions formulation, for the paint industry or polymeric nanoparticles preparation, which makes this method very appealing for developments in the food industry. Ultrasound emulsification occurs through two mechanisms. First, implementation of a sonic field which produces interfacial waves that become unstable over time, resulting in oil phase breakdown within the aqueous medium in the particles formed. Second, application of low frequency ultrasound causes acoustic cavitation, i.e. formation and subsequent collapse of micro-bubbles by fluctuations in the pressure of a simple sound wave.

Each of the bubbles collapse (implode on a microscopic scale), causing extreme levels of highly localized turbulence. The turbulence caused by the micro-implosions act as a very effective breaking method of new pre-particles. Studies comparing the efficiency of ultrasonic emulsification with rotor-stator dispersing, found that ultrasound to be competitive or even superior in terms of droplet size and energy efficiency (Abismaïl *et al*., 1999; Tadros *et al*., 2004).

Studies comparing the efficiency of ultrasonic emulsification with rotor-stator dispersing, found that ultrasound to be competitive or even superior in terms of droplet size and energy efficiency.

EPS can be observed for different systems under different surfactant concentrations (Fig. 2). When using ultrasound, EPS decreased favorably for systems which canola oil is used as the oil phase, resulting in EPS smaller than 200 nm and also presented a narrower particle size distribution after 15 min of sonication compared to emulsion particle size obtained with the high pressure homogenizing method described above.

On the other hand, for emulsions formulated with AEN, EPS was greater than 200 nm. This increase in EPS suggest that these systems could evidence "over-processing" and microstructural changes during homogenization by ultrasound, which is in agreement with observations reported by Jafari *et al*. (2006).

### *3.2 Emulsions produced by a low-energy method*

Emulsions were formulated according to a low-energy method described previously, at different surfactant concentrations (%). Experiments were conducted to examine the dependence of emulsion droplet size on the *low-energy method* proposed and also emulsion composition. The goal of this method was to obtain transparent or translucent emulsions with a particle size in the range of 1-200 nm using the lowest surfactant concentration possible. It was observed that using this method, transparent or translucent dispersions were not obtained; in contrast, opaque and creamy emulsions were obtained which destabilized within minutes of preparation. EPS obtained through this method was greater than 200 nm (data not shown).

Sarduní *et al*. (2005) prepared transparent or translucent nano-emulsions with particle diameters below 200 nm, using 2000 rpm and 700 rpm respectively. In this work we were unable to obtain transparent or translucent nano-emulsions using 2000 rpm. These emulsions showed a milky-like appearance, such as a traditional coarse emulsion, thus contradicting the results obtained by the same authors, perhaps this difference was due to the nature of the materials used in this study that was different to the nature of does used by Sarduní *et al*. (2005). One of these oils is a mixture of triglycerides of medium and short chain containing at least 95% of caprylic and capric acids; and the other oil has a concentration of approximately 87% ricinoleic acid, which allows both oils are good materials for nano-emulsions with particle diameters less than 100 nm.

Table 1. Emulsion particle diameter distribution obtained using high- and low- energy methods

| ***Treatment*** | | | ***Particle diameter distribution at different surfactant concentration (%)*** | | | |
|---|---|---|---|---|---|---|
| **Method** | **Oil** | **Surfactant** | **6** | **7** | **8** | **9** |
| | | Span 20 | 5.623 | 1.685 | 0.344 | 3.492 |
| | Canola | SD | ± 0.315 | ± 0.145 | ± 0.013 | ± 0.378 |
| | | Tween 40 | 0.595 | 0.755 | 5.302 | 1.982 |
| **APV** | | SD | ± 0.104 | ± 0.025 | ± 0.482 | ± 0.128 |
| | | Span 20 | 0.92 | 23.38 | 0.331 | 0.948 |
| | AEN | SD | ± 0.055 | ± 3.639 | ± 0.054 | ± 0.083 |
| | | Tween 40 | 1.693 | 1.391 | 1.577 | 1.483 |
| | | SD | ± 0.17 | ± 0.131 | ± 0.151 | ± 0.089 |
| | | Span 20 | 0.336 | 0.429 | 0.353 | 0.328 |
| | Canola | SD | ± 0.02 | ± 0.025 | ± 0.013 | ± 0.036 |
| | | Tween 40 | 1.982 | 5.302 | 0.755 | 0.595 |
| **Ultrasound** | | SD | ± 0.035 | ± 0.61 | ± 0.042 | ± 0.033 |
| | | Span 20 | 22.85 | 3.895 | 5.587 | 6.548 |
| | AEN | SD | ± 2.19 | ± 0.117 | ± 0.143 | ± 0.222 |
| | | Tween 40 | 0.336 | 0.328 | 0.429 | 0.336 |
| | | SD | ± 0.029 | ± 0.029 | ± 0.025 | ± 0.023 |
| | | Span 20 | 3.332 | 2.722 | 2.647 | 3.833 |
| | Canola | SD | ± 0.217 | ± 0.31 | ± 0.231 | ± 0.225 |
| | | Tween 40 | 0.629 | 2.006 | 5.848 | 2.066 |
| **2000 rpm** | | SD | ± 0.061 | ± 0.077 | ± 0.31 | ± 0.177 |
| | | Span 20 | 3.608 | 4.26 | 3.909 | 3.391 |
| | AEN | SD | ± 0.147 | ± 0.068 | ± 0.102 | ±0.207 |
| | | Tween 40 | 4.678 | 19.65 | 2.538 | 2.646 |
| | | SD | ± 0.891 | ± 3.339 | ± 0.201 | ± 0.294 |

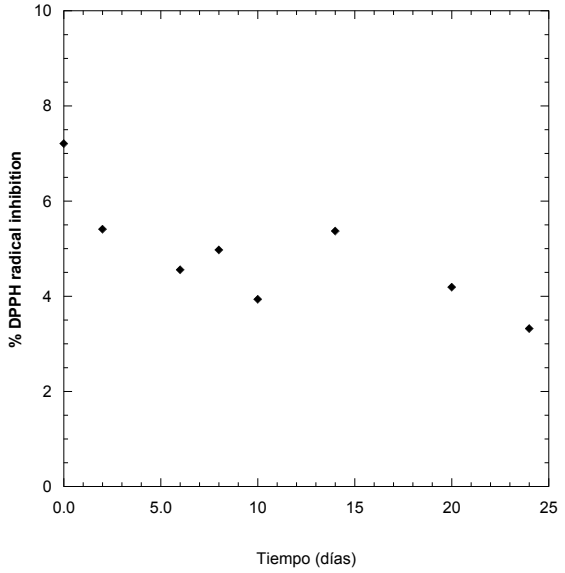


Fig 3. Changes in the chain breaking activity of nano-emulsified astaxanthin during storage.

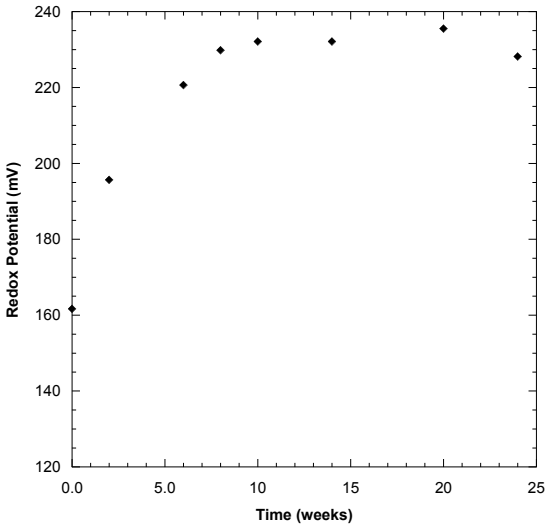


Fig 4. Changes in the redox potential of nano-emulsified astaxanthin during storage.

The oils used in this study were canola oil and orange essential oil. Canola oil is characterized by a very low level of saturated fatty acids (7%), a relatively high level of monounsaturated fatty acids (61%) and an intermediate level of polyunsaturated fatty acids, with a good balance between omega-6 and omega-3. On the other hand, AEN contains terpenes, aromatic compounds and diverse compounds derived from phenylpropane. This could be attributed to the oil composition, which influences nano-emulsion formation through the low energy method. This could suggest that efficacy of this method may depend on the nature of the materials used.

As additional information of emulsion properties the particle diameter distribution obtained for the treatments are listed in Table 1. Where it can be observed that the less particle size distribution was for ultrasound method using AEN-Span 20.

In preliminary experiments using linear regression and ANOVA with Tukey's test ($p<0.05$) there was determined that the best treatment for ultrasound and for high-pressure homogenization was for a surfactant concentration of 9% using both AEN or canola oil.

### *3.3 Breaking-chain antioxidant activity*

The Nano-emulsions were evaluated for breaking chain antioxidant activity by means of DPPH radical scavenging during storage at 35 °C. The DPPH assay has been largely used as a quick, reliable and reproducible parameter to search the *in vitro* overall antioxidant activity of pure compounds, as well as of plant extracts. Fig. 3 shows the changes in the chain breaking activity of astaxanthin nano-emulsions stored at 35° C. The results suggest a path in 2 stages: the first stage was from time zero to week 10, which showed a period of stability of the breaking chain antioxidant activity. The second stage was from week 11 to week 24 where there was a linear decrease of the chain breaking antioxidant activity. This can be attributed not only to the thermal degradation of the antioxidant (Jimenez *et al*., 2011), but also to formation of early Maillard reaction products (MRP) with pro-oxidant properties (Anese *et al*., 1999).

Another aspect to consider is the development of no reactive compounds (free radicals) produced as a result of astaxanthin auto-oxidation during emulsification, these compounds may be responsible for its low inhibition of free radicals (Anarjan *et al*., 2012).

### *3.4 Redox potential*

The antioxidant activity behavior as a thermodynamic measurement (redox potential) showed in Fig. 4 presented two stages, the first consisted from time zero to 10 weeks, with a gradual increase, reaching a value of 229 mV, in which there was a significant difference ($P > 0.05$), that suggests the probable formation of compounds capable of promoting the transfer of electrons (Jimenez *et al*., 2011); these compounds are possibly antioxidant reaction products (astaxanthin) with compounds that comprise the system. The second

stage, from 11 to 24 weeks, displayed a period of stability of the antioxidant activity behavior.

Progress of the Maillard reaction as a result of heating allows the formation of compounds with antioxidant properties. They minimize the loss of natural antioxidants and even allows improving the overall antioxidant properties of the nano-emulsions, which indicates a good antioxidant activity (Jimenez *et al*., 2011).

This data along with the chain breaking antioxidant activity data differed from the observations made by Nicoli *et al*. (2004), where they observed a good agreement between the kinetic and thermodynamic measurements, as indicated by the fact that an increase in the radical-scavenging activity corresponded to a decrease in the redox potential. The results observed in this study showed high values of redox potential, with low values of inhibition of DPPH radical, indicate quenching of singlet oxygen.

While the assessment of chain-breaking activity allows estimation of the antioxidant capacity by referring only to the most reactive compounds, the redox potential measurements give indications on the effective oxidation/reduction efficienty of all the antioxidants present, including the ?slow? ones, which cannot be detected by the traditional chain-breaking kinetic assays. These compounds play an important role in the maintenance of the chemical stability of foods during storage and probably in keeping their functional properties. On the basis of these considerations, the redox potential may be an interesting indicator of the antioxidant efficiency of food products and may be considered a better estimate of the influence of possible oxidative damage caused by processing (Reyes-Munguía *et al*., 2009).

### *3.5 pH*

pH values changed from 3.67 to 2.97 at the end of storage. This trend indicates greater antioxidant activity when pH values were significantly lower ($P > 0.05$) than the initial value measured at the beginning of storage. This is consistent with the theory that the ASX integrated trapped free radicals, which resulted in a net gain of electrons, leading to a decrease in pH.

Redox potential increased along with decreased pH values (Fig. 4), and indicates that initial ASX content was gradually lost during storage. Redox reactions such as those that occur between different natural antioxidants and oxidation products of lipids may also affect the antioxidant properties of various compounds in the nano-emulsion. These events, which mainly occur when different food matrices are mixed have almost unpredictable consequences on antioxidant properties and global stability of foods. Processing can promote or enhance these reactions (Kaur *et al*., 2001).

### *3.6 Astaxanthin content*

The astaxanthin content after 24 weeks of storage was 78.6%. However, this degradation occurred since the first week of storage. There are no statistical differences in the amount of ASX retained in the emulsion after one week of storage, showing that the nano-emulsion is stable during the time of this study. This might be attributed to carotenoid protection from nano-emulsion particle size (McClements *et al*., 2000; Sozer *et al*., 2009; Weiss *et al*., 2006). These results show a promising application of nano-emulsions for protection of lipophilic bioactive compounds during storage.

### *3.7 Absorption kinetics*

ASX plasma concentration results are depicted in Fig. 5. The results show an ASX plasma concentration 7.5-fold higher in rats fed with ASX-NE when compared to rats fed a reference solution (CA). These results are similar to reports by different authors (Ragelle *et al*., 2012; Shrewsbury *et al*., 2009). These authors reported an increase in the bioavailability of nano-emulsified bioactive compounds (2-24 fold) when compared with commercial formulas. Such bioavailability increase could be caused by small particle diameter, which provides a large interfacial area for rapid release of bioactives (Solans *et al*., 2005).

These results confirm what Anarjan *et al*. (2012) observed in their work. They evaluated the cellular absorption astaxanthin colloidal particles *in vitro* against an astaxanthin in powder, observing an increase in astaxanthin bioavailability when it was incorporated in a colloid, they also report that this increase is due to the particle diameter of the colloidal solution.

Also, this enhancement bioavailability may be attributed to the use of Tween 40 as surfactant. Odeberg *et al*. (2003) and Azeem *et al*. (2009) reported where they used surfactants such as Tween 20 and Tween 80 in nano-emulsions preparation. They observed that surfactant may serve to increase active substance permeability. The results found in our study

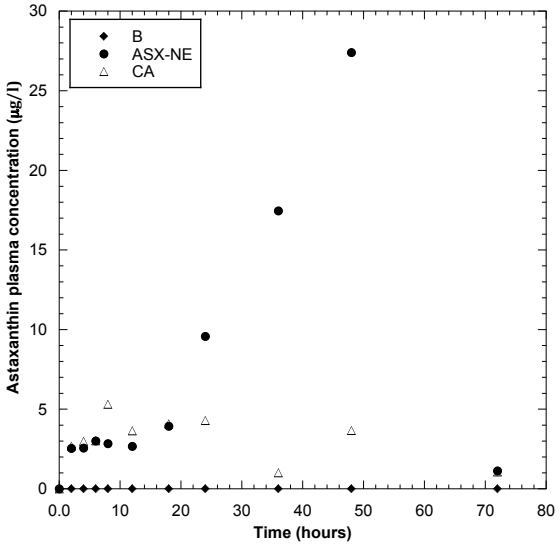


Fig 5. Plasma concentration-time profile of Astaxanthin after its oral administration at a dose of 1 mg/kg in rats fed with ASX-NE and CA.

are encouraging for further studies to improve ASX bioavailability.

# Conclusions

ASX showed good stability and high antioxidant power during storage as a nano-emulsion. We presented evidence that nano-emulsified ASX had enhanced bioabsorption when compared with a reference solution, due to the small emulsion particle size, which helps to increase the surface area of the bioactive lipophilic compound, increasing its bioavailabilty and allowing a better absorption in the organism. These data suggest encouraging applications of nano-emulsions as a vehicle to enhance bioabsorption of lipophilic bioactive compounds

This study explored the effect of methods of high and low energy on the development of nano-emulsions with different ratio of O/S, through of particle size, data particle diameter distribution and macroscopic observations, was chosen the best treatment for preparing nano-emulsions for studying astaxanthin bioavailability in a mouse model.

High energy methods proved to be a convenient strategy for nano-emulsions with monodisperse particle diameters within the range of 1-200 nm, translucent, transparent or bluish appearance. The effect of the surfactant concentration on the development of nano-emulsions stable over time was observed.

Integrating a bio-active compound (astaxanthin) at the selected treatment was observed during storage stability and high antioxidant power. Was observed an increased plasma concentration of astaxanthin when compared to a commercial astaxanthin.

The application of nanotechnology represents a great future for possible evaluation in human models and their subsequent use in foods, integrating into drinks, juices, yogurts, jellies and without affecting its appearance or texture.

## Acknowledgments

The authors wish to thank the Consejo Nacional de Ciencia y Tecnología (CONACyT) for the support received through Project 124229. We thank Margarita Virginia Saavedra Velez and Gabriel Arturo Soto Ojeda for the in vivo studies carried out at the Facultad de Ciencias Químicas of Universidad Veracruzana.

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