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OPTIMIZATION OF THE CONDITIONS FOR THE ELABORATION OF CHITOSAN NANOPARTICLES CHARGED WITH ALPHA LIPOIC ACID, ASCORBIC ACID AND ALPHA-TOCOPHEROL

OPTIMIZACIÓN DE LAS CONDICIONES PARA ELABORACIÓN DE NANOPARTÍCULAS DE QUITOSANO CARGADAS CON ÁCIDO ALFA LIPOICO, ÁCIDO ASCÓRBICO Y ALFA-TOCOFEROL

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Abstract

Advances in nano materials with antioxidant activity are considered as an emerging subject in food and pharmaceutical industries. The objective of the present work was to propose and optimize the necessary conditions for the formation of chitosan nanoparticles containing α-Lipoic acid, α-Tocopherol and ascorbic acid, as well as to determine the physicochemical and morphological properties of such particles. The determination of antioxidant activity was performed by the DPPH, FRAP and ABTS for the antioxidants mixture design in such a way that the mixture presenting the highest antioxidant activity was used in the preparation of nanoparticles that were analysed in terms of Z Potential, size and distribution, and observed in terms TEM. The design of ternary mixtures of ascorbic acid/α-Lipoic acid/α-Tocopherol showed the higher antioxidant activity with DPPH technique with the proportions: 250 mg/667 mg/183 mg respectively. Z Potential showed higher values at low pH and TPP and Tween 80 positively affected in this parameter. Size and distribution of nanoparticles, the smaller size was obtained using TPP and Tween 80 with and without the presence of the antioxidant mixture, between 52 to 260 nm.

Keywords: antioxidant activity, α-Lipoic acid, α-Tocopherol, ascorbic acid, chitosan, nanoparticles.

Resumen

Avances en nano materiales con actividad antioxidante son considerados un tema emergente en las industrias alimentarias y farmacéuticas. El objetivo del presente trabajo fue proponer y optimizar las condiciones necesarias para la formación de nanopartículas de quitosano que contengan ácido α-lipoico, α-tocoferol y ácido ascórbico, así como determinar las propiedades físico-químicas y morfológicas de estas partículas. La determinación de la actividad antioxidante fue realizada por los métodos de DPPH, FRAP y ABTS para el diseño de la mezcla de antioxidantes. La mezcla con mayor actividad antioxidante se utilizó en la preparación de nanopartículas que se analizaron en términos de potencial Z, tamaño y distribución, y se observó con TEM. El diseño de mezclas ternarias de ácido ascórbico/α-lipoico/α-tocoferol mostró una mayor actividad antioxidante con la técnica DPPH con las proporciones de 250 mg/667 mg/183 mg, respectivamente. El potencial Z mostró valores más altos a pH bajo y TPP y Tween 80 positivamente afectados en este parámetro. El tamaño y la distribución de las nanopartículas fueron homogéneos con el tamaño más pequeño utilizando TPP y Tween 80 con y sin la presencia de la mezcla antioxidante entre 52 a 260 nm.

Palabras clave: actividad antioxidante, ácido α-lipoico, α-Tocoferol, ácido ascórbico, quitosano, nanoparticulas.

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1 Introduction

Advances in nanotechnology include the design of novel materials, with improved bioavailability and nutritional value (Maynard, 2006; Mendoza and Rodríguez, 2007). In order to improve the stability of bioactive compounds during processing and storage, nanoencapsulation has been applied in the food and nutraceutical industries. The nanoencapsulation of bioactive compounds represents a viable and efficient approach to increase the physical stability of the active substances, to protect them from interactions with food ingredients and to improve their bioactivity (Acosta, 2009; Fakhreddin *et al*., 2013; Lacatusua *et al*., 2013; Domínguez-Hernández *et al*., 2016). Many of these bioactive compounds, in addition to having antioxidant activity (e.g., carotenoids, fatty acids, vitamins, phytosterols), are highly lipophilic, have poor absorption and limited bioavailability. Such drawbacks may be greatly alleviated by reducing size of the preparation in which those compounds are consumed (Barrow *et al*., 2009; Lacatusua *et al*., 2013). α-Lipoic (ALA) acid, α-tocopherol and ascorbic acid are bioactive compounds used in nanocapsules preparations.

α-Lipoic acid is an essential cofactor in mitochondrial multienzyme complexes related to energy production (Petersen *et al*., 2009). On the other hand, vitamin E or α-tocopherol is the main lipid-soluble antioxidant in the cellular antioxidant defence system and is obtained exclusively from diet (Grilo *et al*., 2014). Also, vitamin C or L-ascorbic acid is a compound derived from glucose. It presents a lactone configuration, in which the hydroxyl groups associated to the double bond function as agents with high reducing potential. This function allows it to participate in the direct reduction of oxygen (Benítez, 2006).

There is a vast scientific evidence on the outstanding antioxidant activity of α-Lipoic acid. On the other hand, its interaction with vitamin E and vitamin C, has been studied and it has been established that the combination of ALA/vitamin E and vitamin C may be beneficial to limit pathological processes in which excessive production of reactive oxygen species during the mechanisms of onset and progression of cellular damage are related, giving rise to diseases such as diabetic neuropathy, Alzheimer's disease, rheumatoid arthritis, lupus erythematosus and cardiac ischemia (Gonzalez-Pérez and Gonzalez-Castañeda, 2006, González *et al*., 2008; Yilmaz *et al*., 2013).

Special interest has been put in the development of biodegradable nanoparticles for effective administration of bioactive lipophilic components. Chitosan is important in the encapsulation of bioactive compounds due to its biocompatibility, low toxicity and biodegradability (Donsi *et al*., 2011; Harris *et al*., 2011; Fakhreddin *et al*., 2013). In recent years, due to its characteristics as colloidal protector, low viscosity at high solids content and good solubility, chitosan has been used as wall material to encapsulate bioactive substances and in the development of controlled release systems (Gharsallaoui *et al*., 2007). One of the techniques used for the preparation of chitosan-based nanoparticles is ionotropic gelation. This is based on the electrostatic interaction between the positively charged primary amino groups of chitosan, the negatively charged groups of a polyanion, such as sodium tripolyphosphate (TPP) or sodium pyrophosphate (SPP) (Racovitá *et al*., 2009), and a surfactant such as Polysorbate 80 or Poloxamer 188 (Gulati *et al*., 2013; Gulati *et al*., 2014). This technique provides a simple preparation method in an aqueous environment and avoids the use of toxic crosslinking agents which may also show effects on the physicochemical properties of the system (Agnihotri *et al*., 2004; Ibezim *et al*., 2011; Alishahi *et al*., 2011).

Although the encapsulation process depends on the nature of the bioactive substance, properties of capsules such as particle size (PS) and particle size distribution (PSD), active compound loading, particle stability, and the stability of the wall material affect the release of the active substance (Cocero *et al*., 2009). Chitosan-sodium tripolyphosphate (TPP) nanoparticles can encapsulate and release proteins, genes, hydrophilic and hydrophobic drugs, vitamins and polyphenolic compounds (Calvo *et al*., 1997; Avadi *et al*., 2009; Fan *et al*., 2012).

Several studies have shown that nanoparticles have a larger active surface area, prolonged useful life and good epithelial penetration capacity (Zhang *et al*., 2004; Deng *et al*., 2006; Liu *et al*., 2007). The objective of the present work was to propose and optimize the necessary conditions for the formation of chitosan nanoparticles containing α-Lipoic acid, α-Tocopherol and ascorbic acid, as well as to determine the physicochemical and microscopic properties of these particles.

Table 1. Effect of chemicals on the Ta1316 ADH activityDesign of ternary mixtures Simplex lattice (Design Expert v7.0) expressed in mg / L.

Run	Type	Component 1 A: Ascorbic acid	Component 2 B: α-Tocoferol	Component 3 C: α-Lipoic acid
1	Vertex	50.00	667.00	383.00
2	Vertex	50.00	50.00	1000.00
3	Central point	150.0	667.00	283.00
4	Vertex	250	50.00	800.00
5	Axial CB	100.00	204.25	795.75
6	Vertex	250.00	667.00	183.00
7	Central point	250.00	358.50	491.50
8	Central point	150.00	50.00	900.00
9	Central point	50.00	358.50	691.50
10	Vertex	250.00	50.00	800.00
11	Central point	150.00	667.00	283.00
12	Vertex	50.00	50.00	1000.00
13	Axial CB	100.00	512.75	487.25
14	Axial CB	200.00	204.25	695.75
15	Vertex	50.00	667.00	383.00
16	Vertex	250.00	667.00	183.00

2 Materials and methods

2.1 Materials

Ascorbic acid (50-81-7), α-Lipoic Acid (T5625), (±) α-Tocopherol (T3251), Chitosan (obtained from shrimp) ≥75% deacetylated (C3646), sodium penta triphosphate ($Na_5P_3O_{10}$) (238503), sodium pyrophosphate ($Na_2P_4O_7$) 2), polysorbate 80 or Tween 80 (59924) were obtained from Sigma-Aldrich (St. Louis, USA); and, poloxamer 188 (Lutrol® F68) from BASF (Mexico). Solvents used were HPLC and reagent grade.

2.2 Optimization of the antioxidant mixture using a D-optimal statistical design

The design of ternary blends was made by using the Design Expert v7.0 software (Stat Ease, Inc., Minneapolis, USA). The design used was Simplex Lattice D-Optimal for quadratic modelling which included 16 runs as shown in Table 1. D-Optimal designs are based on an optimal criterion, which guarantees that experimental points minimize the variance of the Parameters estimated for a predefined model which can be used in the study of restricted spaces where it is impossible to use the arrangement of points corresponding to designs for regular areas. In addition, the model had restrictions considering a minimum and maximum level for each compound (expressed in mg), according to the recommended daily intake and the use of these in supplements (Breithaupt-Grögler *et al*., 1999; Brigelius-Flohe and Traber, 1999; Levine *et al*., 1999; Yusuf *et al*., 2000; Naidu, 2003; Bjelakovic and Gluud, 2007; Shay *et al*., 2009). Levels were: a) 50 mg ≤ A: Ascorbic Acid ≤ 250 mg; B) 50 mg ≤ B: α-Tocopherol ≤ 667 mg; C) 183 mg ≤ C: Lipoic Acid ≤ 1000 mg.

2.3 Antioxidant activity in the design of ternary mixtures of ascorbic acid / α-lipoic acid / α-Tocopherol by means of DPPH, FRAP and ABTS techniques

According to the statistical design shown in Table 1, the antioxidant activity was considered as a response variable which was measured by three techniques. The proportion in which the maximum antioxidant activity was obtained was used for encapsulation. Methanol was used as solvent for ascorbic acid and α-lipoic acid. However, for α-tocopherol, methanol/hexane was used because α-tocopherol due its solubility and stability in this mixture during 8 weeks. (Ferrer *et al*., 1996; Müller et al., 2011; Espinosa-Velázquez *et al*., 2016).

Table 2. Determination of antioxidant activity by the FRAP method in the design of mixtures.

No. Running	COMPONENT 1 ASCORBIC ACID (mg)	COMPONENT 2 α-TOCOFEROL (mg)	COMPONENT 3 α-LIPOIC ACID (mg)	Response (μM Eq. Trolox/L)*
1	50	667	383	373.21±0.04
2	50	50	1000	122.5±0.02
3	150	667	283	475.35±0.05
4	250	50	800	348.92±0.02
5	100	204.25	795.75	239.64±0.05
6	250	667	183	538.21±0.02
7	250	358.5	491.5	491.07±0.05
8	150	50	900	223.21±0.03
9	50	358.5	691.5	254.64±0.02
10	250	50	800	335.35±0.05
11	150	667	283	527.5±0.09
12	50	50	1000	142.5±0.05
13	100	512.75	487.25	368.92±0.08
14	200	204.25	695.75	303.92±0.04
15	50	667	383	423.21±0.05
16	250	667	183	605.00±0.02

* Mean and its corresponding standard deviation

DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical activity

The method used was the proposed by Brand Williams *et al*. (1995), with some modifications (Mishra *et al*., 2012). Methanolic solutions of the designed mixtures were prepared taking aliquots and reaching a final volume of 3.0 mL. The reaction was initiated by adding a solution of DPPH (0.1 mM) in methanol with 2.0 mL of stock solution (antioxidant mixture). The reaction was maintained at 25°C for 30 min in darkness and subsequently the absorbance measured at 517 nm. Trolox was used as standard curve. The results presented for the three techniques of antioxidant activity correspond to the mixtures and are the mean of three runs performed by triplicate.

Analysis of the ferric antioxidant reducing power (FRAP)

The method used was according to Benzie and Strain (1996). For the preparation of the reagent FRAP, 2.5 mL of 2-4,6-tripyridyl triazine (TPTZ) (10 mmol/L) in 40 mmol/L of HCl, 2.5 mL (20 mmol / L) of FeCl 3 6H 2 O and 25 mL acetate Buffer (0.3 mol / L) at pH 3.6, were used. For this technique, 900 μL of the FRAP reagent were mixed with 90 μL of distilled water and 30 μL of the control mixture of antioxidants in methanol at 37°C for 30 minutes. Absorbance was read at 595 nm and Trolox reagent was used as the standard.

ABTS (2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) method

ABTS method was performed according to Erel, 2004. ABTS solutions (7mM) and potassium persulfate (2.45 mM) were prepared in 25 and 50 mL of distilled water respectively. Reagents were mixed in equal proportions and left in the dark at room temperature for 16 hours before use. After this time, the ABTS solution was diluted with methanol to obtain an absorbance of 0.700 at 734 nm. This was achieved by mixing 1.5 mL of the solution of the radical with 60 mL of methanol. After the ABTS solution was obtained under the conditions described above, the reaction was carried out using 20 μL of sample with 1980 μL of ABTS. Absorbance was evaluated at 734 nm, 6 min after reaction (Hernández-Carrillo *et al*., 2016). Trolox was used for the development of the standard curve.

2.4 *Preparation of chitosan nanoparticles loaded with ascorbic acid/α-lipoic acid/α-Tocopherol*

The nanoparticles were prepared based on the ionotropic gelation method between sodium pyrophosphate (SPP) or sodium tripolyphosphate

(TPP) and chitosan, using Tween 80 and / or Lutrol® F68 as surfactant (Calvo *et al*., 1997; Alishahi *et al*., 2011). Chitosan was dissolved in 1% (v/v) acetic acid to obtain a 0.3% (w/v) solution of chitosan for 60 minutes. The TPP or SPP was dissolved in deionized water at a concentration of 1%. 1 mL of TPP or SPP was added in 25 mL of chitosan solution with magnetic stirring at room temperature. Then, 1 mL of the surfactant (Tween 80 and/or Lutrol® F68) was added to observe the effect on the physicochemical properties of the nanoparticles. Finally, 2 mL of the mixture in which the maximum antioxidant activity was obtained were added. The mixture was stirred (600 rpm) for 30 min, then sonicated (120 volts, 1.3 Amps, 50/60 Hz) for 30 minutes. The suspension was then immediately centrifuged at 1183 g for 120 minutes. The precipitate was suspended in water, and centrifuged again for 10 minutes. The nanoparticles were suspended in deionized water for further characterization and analysis.

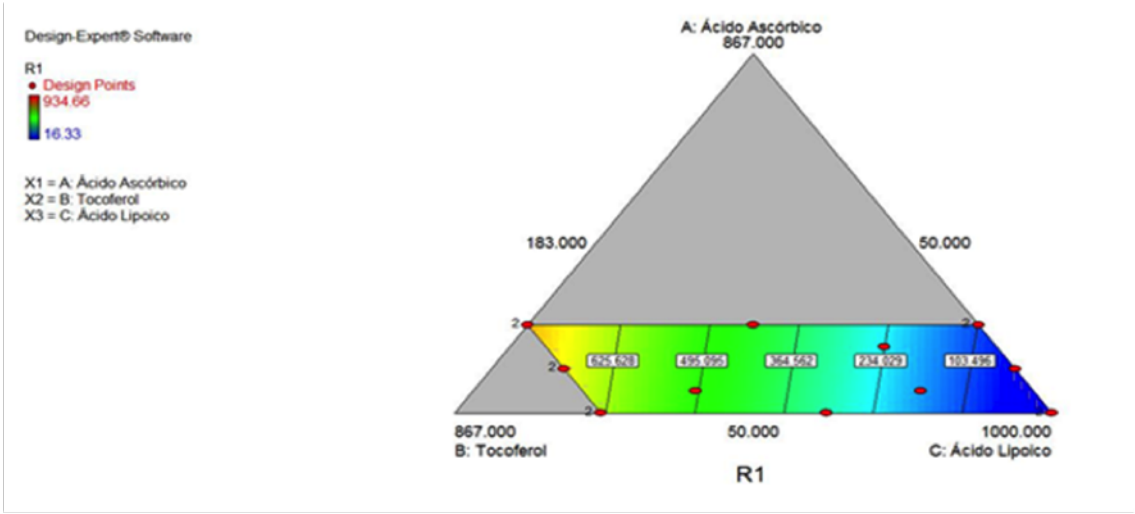

Fig. 1. Contour plot of antioxidant activity by the FRAP method. A) Ascorbic Acid B) α-Tocopherol C) α-Lipoic Acid. Orange area in the upper left corner represents the maximum antioxidant activity and, the blue area in the lower right corner, the lowest antioxidant activity.

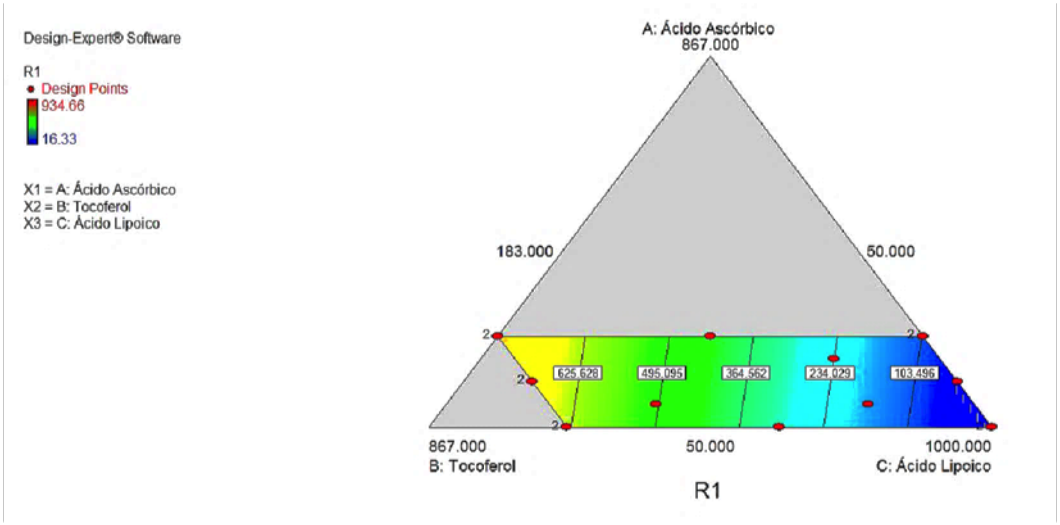

Fig. 2. Contour plot of the antioxidant activity by the ABTS method. A) Ascorbic acid B) α-Tocopherol C) α-Lipoic acid. Orange area in the upper left corner represents the maximum antioxidant activity and, the blue area in the lower right corner, the lowest antioxidant activity.

2.5 *Physicochemical characterization of nanocapsules*

Particle and size distribution

The particle and size distribution were determined by dynamic light scattering using a Zetasizer Nano ZS90 (Malvern, USA) at 25°C with a detector angle of 90 degrees. The samples were diluted in deionized water (1:200) to perform the measurements in a range of pH from 3 to 10 (Velasco-Rodríguez *et al*., 2012, Pérez-Alonso *et al*., 2015). The samples were analyzed under various conditions to study the effect of polyanion and antioxidant mixture on parameters (particle size, Z Potential, polydispersity index): 1) Chitosan/polyanion (SPP or TPP) / no antioxidant mixture 2) Chitosan / polyanion (SPP or TPP) / Mixture of antioxidants, 3) Chitosan / polyanion (SPP or TPP) / Tween 80 / without antioxidant blend, 4) Chitosan/polyanion (SPP or TPP) / Tween 80 / Mixture of antioxidants, 5) Chitosan/polyanion (SPP or TPP) / Lutrol F68 / without antioxidant blend, 6) Chitosan / polyanion (SPP or TPP) / Lutrol F68 / Antioxidant blend. Analyzes were performed in triplicate. Results were expressed as mean ± standard deviation.

Z Potential

The Z potential of the nanoparticles was determined by using a Zetasizer Nano ZS90 (Malvern, USA) at 25°C with a detector angle of 90 degrees in a pH range from 3 to 10. For this analysis, the samples were prepared in a 1:200 dilution with a 0.1 mM KCl solution, and the pH of the suspension adjusted with 0.1N NaOH and 0.1N HCl to achieve different values of pH. Z potential was measured in automatic mode (Velasco-Rodríguez *et al*., 2012).

Efficiency of encapsulation

The encapsulation efficiency (Alishahi *et al*., 2011) of the nanoparticles was analyzed by centrifugation of the suspension at 14,500 RCF per gram. The amount of ascorbic acid, α-lipoic acid and α-Tocopherol in the precipitate was determined by HPLC; 1 mL of sample was taken and, extracted with the same volume from a mixture of 2% aqueous acetic acid and absolute methanol (1:1) and filtered with a 0.20 μm Acrodisc filter prior to the analysis to remove any residual solid. Bioactive compounds in the methanol extracts were measured by using the HPLC-adapted method of Weerakody *et al*. (2008). The HPLC system (BECKMAN) consisted of a binary pump (SYSTEM GOLD 126), a degasser, with diode arrangement detector (DAD, SYSTEM GOLD 168); temperature 30°C, injection volume of 20 μL. Samples were analyzed on a Waters Nova Pack C18 reverse phase column [150 mm x 3.9 mm I.D. (4 μm), Alltech] with a mobile phase of methanol/water (65:35 v/v). The flow rate was 1 mL/min in order to remove the bioactive compounds from other components in the sample. The wavelengths at which the compounds were detected were: 266 nm for ascorbic acid, 329 nm for α-lipoic acid, 292 nm in α-tocopherol with DAD. Knowing the initial (total) concentration of each compound used to prepare the nanoparticles, the encapsulation efficiency (EE) was evaluated using the following equation:

$$E = \frac{Antioxidant_E - Antioxidant_U}{Antioxidant_U} x100\% \quad (1)$$

For the standard curve, six different concentrations were prepared for each bioactive compound dissolved in absolute methanol.

2.6 *Transmission electron microscopy (TEM) analysis*

An aliquot (5 μL) of the sample was placed in a copper grid (200 mesh) and contrasted with phosphotungstic acid (PTA). The analysis was performed after 15 minutes by TEM using a JEOl Transmission Electron Microscope (TEM), model JEM-1010 operated at 60 kV (Velasco-Rodríguez *et al*., 2012).

2.7 *Statistical analysis*

All data were expressed as mean ± standard errors. Pearsons correlation coefficient analysis was used to determine the level of significance between the dependent variables (particle size, Z potential and polydispersity index). The level of significance was set at the 5% level ($p < 0.05$). All statistical analyses were carried out with the software Design Expert and XLSTAT.

Table 3. Determination of antioxidant activity by the ABTS method in the design of mixtures.

No. Running	COMPONENT 1 ASCORBIC ACID (mg)	COMPONENT 2 α-TOCOFEROL (mg)	COMPONENT 3 α-LIPOIC ACID (mg)	Response (μM Eq. Trolox/L)*
1	50	667	383	583±0.02
2	50	50	1000	27±0.09
3	150	667	283	426.33±0.03
4	250	50	800	43±0.02
5	100	204.25	795.75	133±0.02
6	250	667	183	913±0.04
7	250	358.5	491.5	273±0.01
8	150	50	900	16.33±0.06
9	50	358.5	691.5	226.33±0.015
10	250	50	800	96.33±0.015
11	150	667	283	716.33±0.07
12	50	50	1000	106.33±0.01
13	100	512.75	487.25	546.33±0.03
14	200	204.25	695.75	276.33±0.03
15	50	667	383	689.66±0.06
16	250	667	183	934.66±0.03

* Mean and its corresponding standard deviation

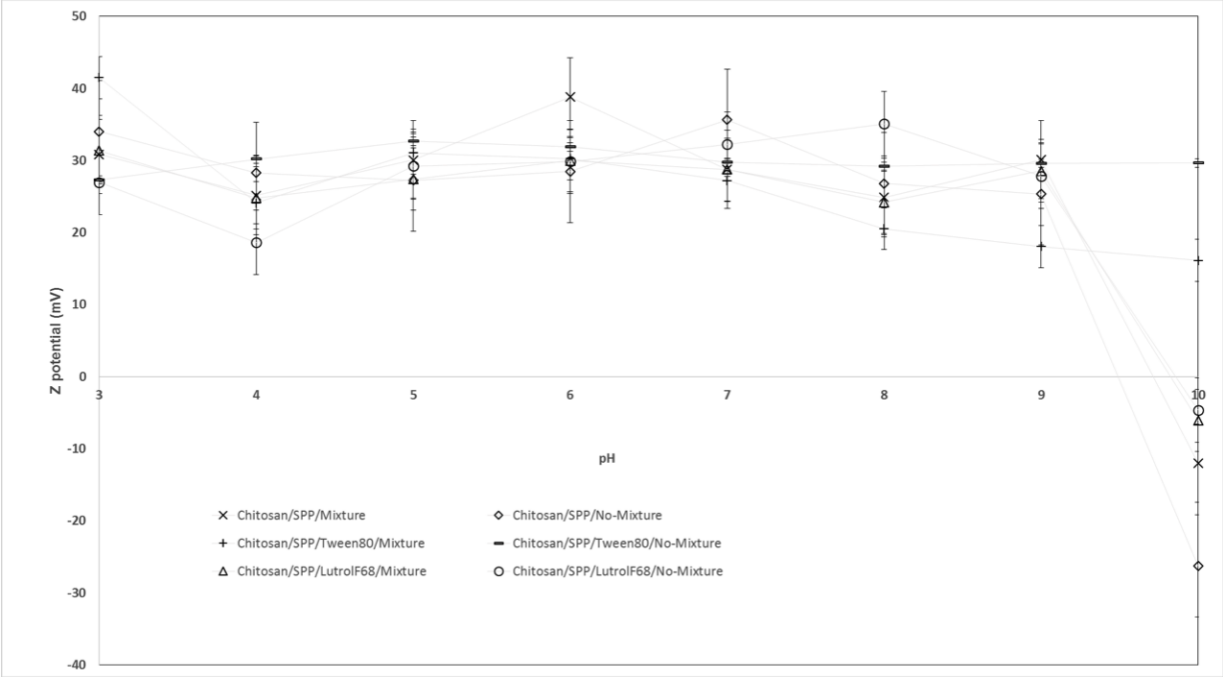

Fig. 3. Z potential at different pH for chitosan-polyanion (SPP and TPP) and chitosan-polyanion-sulfactant (Tween 80 and Lutrol F68) with antioxidant mixture (ascorbic acid /α-lipoic acid/α-tocopherol) and without antioxidant mixture.

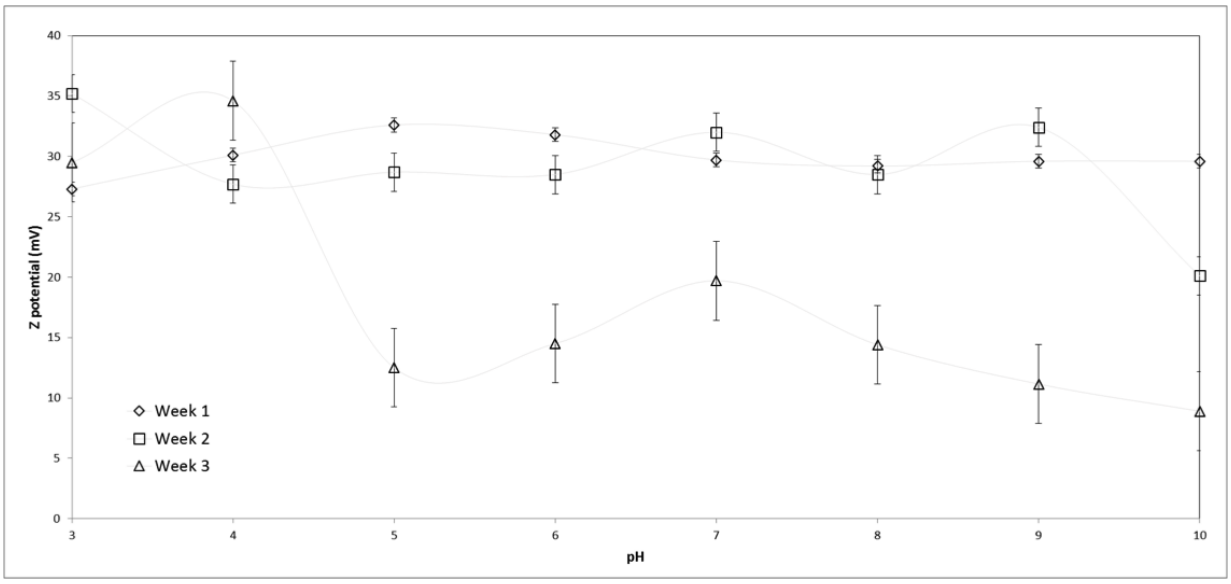

Fig. 4. Z potential kinetic for 3 weeks at different pH for chitosan-polyanion (SPP and TPP)-Tween 80 with antioxidant mixture (ascorbic acid /α-lipoic acid/α-tocopherol).

3 Results and discussion

3.1 Antioxidant activity in the design of ternary mixtures of ascorbic acid/α-lipoic acid/α-Tocopherol by means of DPPH, FRAP and ABTS techniques

DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical activity

The determination of antioxidant activity was performed by the DPPH method. However, no response was obtained from any mixture because the absorbance values were less than 0.1 in the UV-Visible spectrophotometer. Pyrzynska and Pekal (2013) reported that, steric accessibility of the DPPH radical is an important factor in the reaction, since small molecules have better access to the radical site and relatively higher antioxidant activity. Premathilaka et al. (2016), suggested that as DPPH is a stable nitrogen radical. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH, due to steric inaccessibility (Apak *et al*., 2013).

Another important factor to consider are the chemical structure and spatial geometric configuration of molecules that are important for its specific function. For example, α-tocopherol and ascorbic acid containing four hydroxyl groups react with DPPH in 5-30 min (Tripathi *et al*., 2009). Though, it is not always the case that the number of hydroxyl group is directly correlated with more antiradical efficiency. However, α-lipoic acid, with no phenolic hydroxyl group react poorly with DPPH (Mishra *et al*., 2012).

Also, Dawidowicz et al. (2012), stated that the type and amount of solvent used for the dissolution of the antioxidant compounds have a significant influence in the amount of DPPH that does not react (Gómez-Sampedro *et al*., 2016).

Analysis of the ferric antioxidant reducing power (FRAP)

The results of the antioxidant activity of the mixtures design obtained by the FRAP method expressed in μM Eq. Trolox/L, are presented in Table 2. The mixture that presented the greater reduction power of the Fe^{2+} radical was composed by ascorbic acid (250 mg), α-Tocoferol (667 mg) and α-Lipoic acid (183 mg) that had 605 ± 0.02 μM Eq. Trolox / L. For each mixture, antioxidant activity and FRAP response were analysed. A linear model was proposed since it presented a $P < 0.0001$. Also, the coefficient of linear mixing showed a high level of significance. From this analysis, an adjusted R^2 = 0.969 was obtained and the final equation (Eq. 2) was obtained in terms of the current components of the response variable, in which the three components have an additive effect on the antioxidant activity, with ascorbic acid being the component with the greatest influence on the response variable.

Table 4. Particle size, polydispersity index (PDI), isoelectric point (pI) and pH stability range for chitosan-polyanion nanoparticles (SPP and TPP) loaded with ascorbic acid / α-lipoic acid / α-Tocopherol (AOX), using Tween 80 and Lutrol F68 as Surfactant.

System	Particle Size (nm)**	Polydispersity Index (PDI)**	pI	pH at which it is stable
Chitosan/SPP/AOX	764 ± 10	0.717 ± 0.13	10	6
Chitosan/TPP/AOX	627 ± 24	0.803 ± 0.14	10	6
Chitosan/SPP/Tween 80/AOX	426 ± 61	0.634 ± 0.19	ND	8
Chitosan/TPP/Tween 80/AOX	318 ± 26	0.561 ± 0.07	ND	7
Chitosan/SPP/Lutrol F68/AOX	525 ± 69	0.494 ± 0.09	10	8
Chitosan/TPP/Lutrol F68/AOX	867 ± 20	0.700 ± 0.07	10	5

* Sodium pyrophosphate (SPP), Sodium triphosphate pentabasic (TPP), Antioxidants (AOX)

* ND = Not determined

** Mean and its corresponding standard deviation

$$\begin{aligned} Antioxidant\ activity =& 1.04347(Ascorbic\ acid) \\ &+ 0.47640(\alpha - Tocopherol) \\ &+ 0.055012(\alpha - Lipoic\ acid) \end{aligned} \quad (2)$$

ABTS Method (2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid))

In Table 3, the results of the evaluation of the antioxidant activity of the mixed design by the ABTS method (expressed in μM Trolox / L Eq.) are presented. The highest antioxidant activity determined by the ABTS method was mixture 16, composed by ascorbic acid (250 mg), α-Tocoferol (667 mg) and α-Lipoic Acid (183 mg) with a value of 934.66 ± 0.02 μM Eq. Trolox / L.

Analysis of variance showed a linear model for this experiment, P<0.0001, which indicated that the above proportions of the bioactive compounds were significant. Also, the coefficient of linear mixture showed a P<0.0001. For this model, an adjusted R^2 = 0.914 was obtained. Equation 3 was obtained in terms of the current components of the response variable, showing that ascorbic acid and α-Tocopherol presented an additive effect on antioxidant activity, being α-Tocopherol the most influential, contrary to the FRAP method. However, α-Lipoic acid had no effect on antioxidant activity.

$$\begin{aligned} Antioxidantactivity =& 0.54146(\text{Ascorbic acid}) \\ &+ 0.96657(\alpha - \text{Tocopherol}) \\ &- 0.10244(\alpha - \text{Lipoic acid}) \end{aligned} \quad (3)$$

In Figure 2, it was observed that the area for the optimal rates was delimited, thus achieving the maximum antioxidant activity. Moreover, there was an additive effect among the three components of the mixture as in the FRAP method. It should be noted that the optimal proportions of the model agreed with those obtained experimentally.

Once the optimum concentration of the compound mixture determined by the antioxidant activity was obtained (ascorbic acid (250 mg), α-tocopherol (667 mg), α-lipoic acid (183 mg)), the formation of the nanoparticles and its subsequent physicochemical characterization were carried out.

3.2 Preparation of chitosan nanoparticles loaded with ascorbic acid/α-lipoic acid/α-Tocopherol

The nanoparticles were formed immediately when the different polyanions (SPP / TPP) were mixed with the chitosan/antioxidants solution. Alishahi *et al*. (2011), mentions that nanoparticles are formed due to the molecular bonds between the phosphate groups of TPP and the amino groups of chitosan, since the ability of chitosan to rapidly gel upon contact with polyanions depends on the intermolecular and intramolecular mediated by such polyanions. Rodríguez-Hamamura *et al*. (2010), reported that the ionic gelation method depends on the molecular mass and degree of deacetylation of chitosan. The higher the molecular weight of chitosan, the greater the number of protonated amino groups in an acid solution. Thus, there was a greater number of positive charges that could interact with the negative charges of TPP, which implies a higher degree of cross-linking. In addition, the higher the degree of deacetylation of chitosan, the higher the number of amino groups to be protonated, as well as the higher ionic interaction with TPP, results in a higher gelation efficiency and greater degree of cross-linking (Zhou and Wang, 2007).

3.3 Characterization of chitosan nanoparticles / antioxidant mixture

Size and distribution of nanoparticles

The sizes of chitosan nanoparticles/antioxidant mixtures with SPP or TPP were measured with a Zetasizer Nano ZS. The effect of the polyanion and surfactant type on particle size and polydispersity index was studied, as it describes that the dispersion of the size and particle distribution, and polydispersity are characteristic parameters of nanoparticles (Müller *et al*., 2001; Du *et al*., 2009; Cerón-Montes, 2015).

The results of the particle size and distribution of nanoparticles are presented in Table 4. These values correspond to the pH where a decrease of this parameter was shown. When evaluating the ANOVA results of the linear model of the effect of five factors on particle size: pH, polyanion SPP and TPP, surfactant Tween 80 or Lutrol F68, it was found that the decrease in pH becomes an important factor in the decrease of particle size. Also, it was observed that TPP (pentasodium sodium triphosphate) gave a better result than SPP (sodium pyrophosphate), as it favoured the decrease of particle size.

Chitosan-TPP mass ratio significantly influences the characteristics of chitosan-TPP nanoparticles (Stoica *et al*., 2013). According to Hu *et al*. (2008), the ability of chitosan to quickly gel on contact with TPP relies on the formation of inter- and intramolecular crosslinking between the amino groups and the phosphate groups. This effect can be explained by the penetration of the bioactive compound molecule in chitosan network, activating hydroxyl sites and establishing physical-chemical

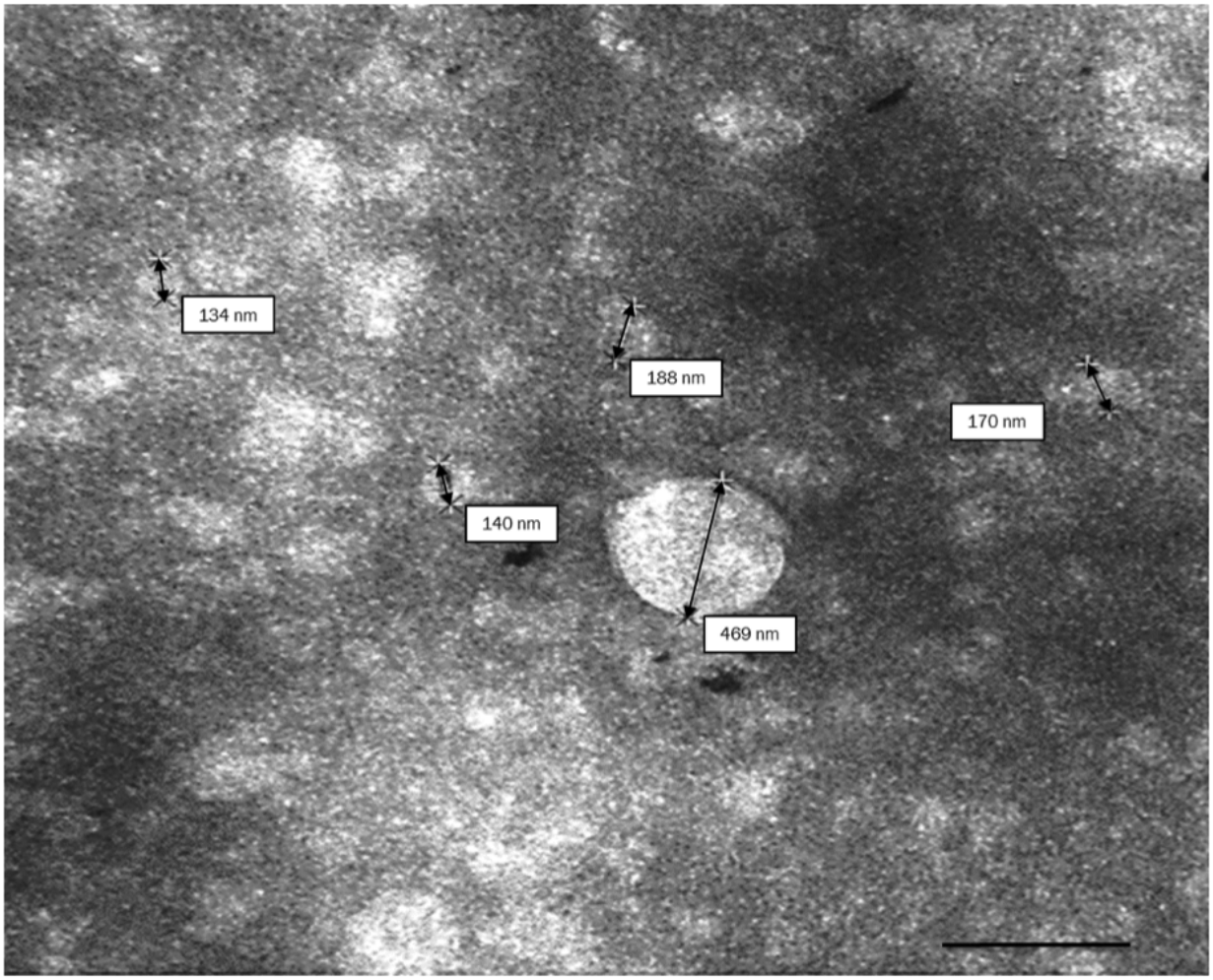

Fig. 5. TEM image of nanoparticles of chitosan/TPP/Tween 80 without antioxidant mixture. Bar = 500 nm.

electrostatic interactions and hydrogen bonds in the new system, in good agreement with literature data (Steed and Atwood, 2013).

Fan *et al*., (2012), mentioned that chitosan at low concentrations was related to formation of stable nanoparticles even at a low mass ratio of chitosan to TPP, while chitosan at higher concentration could only form stable nanoparticles at a higher mass ratio of chitosan to TPP. For example, when the concentration of TPP was fixed at 0.5 mg/mL, a chitosan concentration of 0.5 mg/mL could form stable nanoparticles at a mass ratio of 3.3:1, while a chitosan concentration of 1.0 mg/mL would form aggregates as in the present study. To explain this phenomenon, it was inferred that as chitosan concentration decreases, the intermolecular distance increases, thus leading to a decrease in intermolecular cross-linking between chitosan molecules while an increase in cross-linking density between chitosan and TPP, namely an increase in the ratio of moles of TPP to the moles of chitosan repeating units (Berger *et al*., 2004). These criteria could be used to prepare chitosan/TPP nanoparticles with smaller size, since an appropriate increase in the mass ratio induces the reduction of particle size (Jonassen *et al*., 2012).

Polydispersity index (PDI) is another factor that represents the dispersion homogeneity. The PDI for both polyanions (SPP y TPP) in the absence of surfactant was between 0.717 ± 0.13 and 0.803 ± 0.14, respectively. However, in nanoparticles with TPP and Tween 80, a decrease in PDI was obtained (0.561 ± 0.07), which indicated a relative homogenous dispersion (Fernandez-Urrusuno *et al*., 1999). Moreover, it is known that under acid conditions, there is an electrostatic repulsion between chitosan molecules due to the protonated amino groups of chitosan, meanwhile, there also exist interchain hydrogen bonding interactions between chitosan molecules (Fan *et al*., 2012).

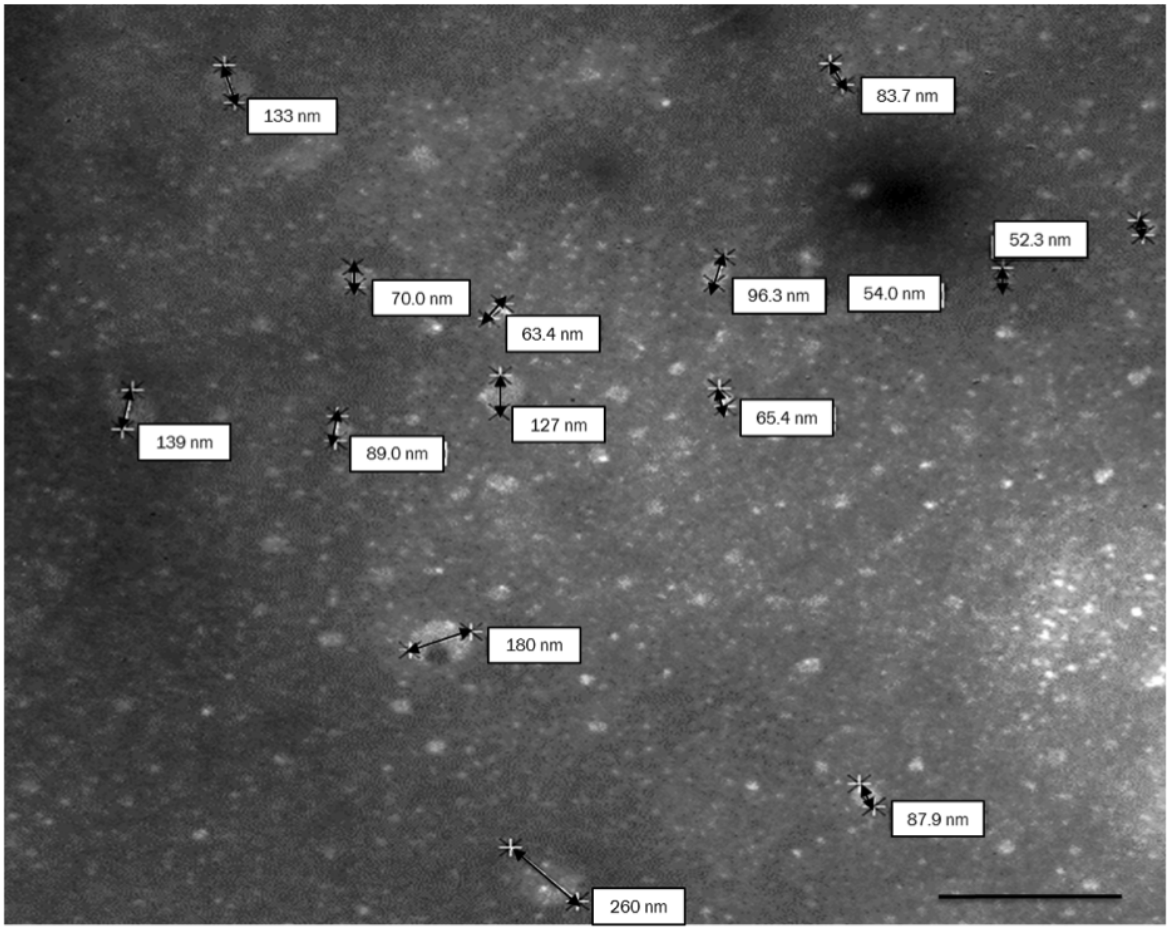

Fig. 6. TEM image of nanoparticles of chitosan/TPP/Tween 80 with antioxidant mixture. Bar = 500 nm.

Additionally, it was observed that by adding Tween 80 as surfactant, a reduction in particle size was obtained. Surfactants are used to stabilize nanoparticles by hindering their growth. The increase in the surfactant amount in colloidal dispersions may contribute to the reduction of mean particle size because of the surface active properties of surfactants (Park *et al*., 1999). Additionally, Tween 80 as a stabilizing agent is adsorbed on the surface of the nanoparticles, thereby slowing down the growth of crystal phases by reducing the surface free energy (Rajaram and Natham, 2013).

On the other hand, the effect of the pH on the formation of chitosan/TPP particles was investigated by adjusting the pH from 3.0 to 10.0. The results indicated that the critical mass ratio of chitosan to TPP for the formation of an opalescent suspension decreased with the pH. When the pH was 6.0, it was not easy to produce nanoparticles with unimodal particle size distribution, while when the pH was above 8.0, nano-particles were formed with unimodal distribution. Chitosan is a weak polyelectrolyte with a pKa around 6.5, the protonation degree of chitosan is mainly controlled by the pH of the solution. Shu and Zhu, (2002), showed that as the pH of chitosan solution increased from 4.7 to 8, the protonation degree of chitosan decreased rapidly from 100 to 0%, indicating that there is a critical pH above which chitosan starts to be deprotonated. Meanwhile, the charge number and ionic species of TPP are affected by solution pH. In original TPP solution (pH 9.7), the concentration of tripolyphosphoric ions ($P_3O_{10}^{5-}$ and $HP_3O_{10}^{4-}$) is high but the concentration of hydroxide ions is also present. The hydroxide ions or tripolyphosphoric ions in the medium, can competitively react ionically with the protonated amino groups of chitosan by deprotonation or ionic cross-linking (Mi *et al*., 1999).

Z Potential

Z Potential was mainly affected by pH (Figure 3). It was possible to observe that higher Z Potentials were obtained at pH 3 for all systems. TPP and Tween 80 positively affected this parameter. Jayakumar *et al*. (2010) reported that as chitosan is a strong basic substance since has primary amino groups with pKa = 6.3, solubilizes at low pH values. Once the conditions under which the highest Z Potentials were obtained (chitosan-polyanion (TPP)-Tween 80 in presence of ascorbic acid/α-Lipoic acid-α-tocopherol), the behaviour of this parameter with time (3 weeks) was assessed (Figure 4). Z Potential was stable for two weeks at pH values of 3-9. At pH 10, a decrement of this value was observed. These results can be explained in terms of the instability of the polyanion as from the second week.

A Z Potential value of above +25mV or below −25, ensures a high-energy barrier that stabilizes the nanosuspension (Mora-Huertas *et al*., 2010). The Z Potential of all systems was between +25mV to +35 mV, except at pH 10, where the isoelectric point was found. Gan *et al*., (2005) showed that chitosan-TPP nanoparticles exhibited a high positive surface charge across a wide pH range, and the isoelectric point of the nanoparticles was found to be at pH 9.0. Negative Z Potential values may be due to the excessive unreacted phosphate groups during the process (Shard *et al*., 2014).

Concerning the effect of polyanion, greater stability was observed when using TPP. The TPP-anions will, as a result of the crosslinking reaction, occupy some of the chitosan's positive charges (Jonassen *et al*., 2012).

Besides, these formed nanoparticles with higher Z Potential are better stabilized with the presence of surfactant (Tween 80), which is an important factor for the stability of nanosuspensions (Rajaram and Natham, 2013). Surfactant improved the stability of the system through static electricity repulsive forces, steric hindrance, and Van der Waals forces, by absorbing on to the surface of nanomaterials (Zhou *et al*., 2007; Kvitek *et al*., 2008). Tween 80 molecules acting as amphiphilic molecules deposited at the particle surface resulted in decrement of particle size. Moreover, they could shield surface charge of the antioxidants-loaded chitosan, decreasing in Z Potential (Asasutjarit *et al*., 2007).

Transmission electron microscopy (TEM) analysis

Figures 5 and 6 show the nanoparticles of chitosan/TPP/Tween80 with and without antioxidant mixture (ascorbic acid/α-Lipoic acid-α-tocopherol) respectively at pH 3.5. It is shown that the morphology is spherical for the nanoparticles with the antioxidant mixture and without it. A larger particle size was observed in the chitosan particles without the antioxidant mixture (134-469 nm), however, when the antioxidant mixture was added, size of particles decreased (52-260 nm). Also, the size of particles are smaller than those obtained by using the Zetasizer equipment. This can be explained by the fact that the diameter of the particle measured by TEM does

not consider the charged layer around the particles (Anderson *et al*., 2013; Fissan *et al*., 2014; Tuoriniemi *et al*., 2014).

Conclusions

Antioxidant activity analysis in the design of ternary mixtures of ascorbic acid/α-lipoic acid/α-Tocopherol by means of both DPPH and FRAP techniques, showed that the ascorbic acid was the compound that presented the highest antioxidant activity. Also, DPPH detected higher antioxidant activity than FRAP and ABTS. The maximum antioxidant activity was obtained in the mixture, composed of ascorbic acid/α-tocopherol/α-lipoic acid in proportions: 1:2.67:0.732, respectively. According to the size and distribution of nanoparticles, the smaller particle size was obtained using TPP as polyanion and Tween 80 as surfactant, both in the absence and in the presence of the antioxidant mixture between 52 to 260 nm. Also, a significant decrease in the polydispersity index was observed in chitosan/TPP/antioxidant mixtures using Tween 80 as a surfactant. Size of particles were smaller when using TEM than the ones from the Zetasizer. This could be explained by the fact that the diameter of the particle measured by TEM does not consider the charged layer around the particles. Z Potential showed higher values at low pH´s and TPP and Tween 80 positively affected this parameter. Z Potential values were stable for two weeks at pH values from 3-9.

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Nomenclature

EE	encapsulation efficiency
$\text{Antioxidant}_\text{E}$	antioxidant encapsulated
$\text{Antioxidant}_\text{U}$	antioxidant without encapsulation

References

Acosta E. (2009). Bioavailability of nanoparticles in nutrient and nutraceutical delivery. it Current Opinion in Colloid and Interface Science 14, 3-15.

Agnihotri, S. A., Mallikarjuna, N. N., Aminabhavi, T. M. (2004). Recent advances on chitosan-based micro and nanoparticles in drug delivery. *Journal of Controlled Release 100*, 5-28.

Alishahi, A., Mirvaghefia, A., Tehranib, M.R., Farahmanda, H., Koshioc, S., Dorkooshb, F.A., Elsabeed, M. (2011). Shelf life and delivery enhancement of vitamin C using chitosan nanoparticles. *Food Chemistry 126*, 935-940.

Anderson, W., Kozak, D., Coleman, V. A., Jämting, Å. K., Trau, M. (2013) A comparative study of submicron particle sizing platforms: accuracy, precision and resolution analysis of polydisperse particle size distributions. *Journal of Colloid and Interface Science 405*, 322-330.

Apak, R., Gorinstein, S., Böhm, V., Schaich, K. M., Özyürek, M., Güçlü, K. (2013). Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). *Pure and Applied Chemistry 85*, 957-998.

Asasutjarit C, Hirunlabh J, Khedari J, Charoenvai S, Zeghmati B., Shin U.C. (2007). Development of coir-based light weight cement board. *Construction Buildings and Materials 21*, 277-288.

Avadi, M. R., Sadeghi, A. M. M., Mohammadpour, N., Abedin, S., Atyabi, F., Dinarvand, R. (2009). Preparation and characterization of insulin nanoparticles using chitosan and Arabic gum with ionic gelation method. *Nanomedicine: Nanotechnology, Biology and Medicine 6*, 58-63.

Barrow, C. J., Nolan, C., Holub, B. J. (2007). Edible films made from tuna-fish gelatin with antioxidant extracts of two different murta ecotypes leaves (ugni molinae turcz). *Food Hydrocolloids 21*, 1133-1143.

Haider, S., Park, S.-Y., Saeed, K., Farmer, B.L. (2009). Bioequivalence of encapsulated and microencapsulated fish-oil supplementation. *Journal of Functional Foods 1*, 1-8.

Benítez, Z.D. (2006). Vitaminas y oxidorreductasas antioxidantes: defensa ante el estrés oxidativo.

Revista Cubana de Investigación Biomédica 25, 1-8.

Benzie, I.F., Strain, J.J. (1996). The Ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power". The FRAP assay. *Analytical Biochemistry 239*, 70-76.

Berger et al., J. Berger, M. Reist, J.M. Mayer, O. Felt, N.A. Peppas, R. Gurny (2004). Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. *Journal of Pharmacy and Biopharmacy 57*, 35-52.

Bjelakovic, G., Gluud, C. (2007). Vitamin Supplements. *J Natl Cancer Inst, 99*, 754-764.

Brand-Williams, W., Cuvelier, M., Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Food Science and Technology 28*, 25-30.

Breithaupt-Grögler, K., Niebch, G., Schneider, E., Erb, K., Hermann, R., Blume, H. H., Belz, G. G. (1999). Dose-proportionality of oral thioctic acid-coincidence of assessments via pooled plasma and individual data. *European Journal of Pharmaceutical Sciences 8*, 57-65.

Brigelius-Flohe, R., Traber, M. G. (1999). Vitamin E: function and metabolism. *The FASEB Journal, 13*, 1145-1155.

Calvo, P., Remunan-Lopez, C., Vila-Jato, J. L., Alonso, M. J. (1997). Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. *Journal of Applied Polymer Science 63*, 125-132.

Cerón-Montes, G.I., San Martin-Martinéz, E., Yañez-Fernández, J., Quezada-Cruz, M., Castro-Muñoz, R. (2015). Preliminary purification of anthocyanins from blue corn by adsorption and electrophoresis. *Revista Mexicana de Ingeniería Química 14*, 99-108.

Cocero, M.J., Martín, A., Mattea, F., Varona, S. (2009). Encapsulation and co-precipitation processes with supercritical fluids: Fundamentals and applications. *Journal of Supercritical Fluids 47* 546-555.

Dawidowicz, A. L., Wianowska, D., Olszowy, M. (2012). On practical problems in estimation of antioxidant activity of compounds by DPPH method (Problems in estimation of antioxidant activity). *Food Chemistry 131* 1037-1043.

Deng, Q. Y., Zhou, C. R., Luo, B. H. (2006). Preparation and characterization of chitosan nanoparticles containing lysozyme. *Pharmaceutical Biology 44*, 336-342.

Domínguez-Hernández, C.R., García-Alvarado, M.A., García-Galindo, H.S., Salgado-Cervantes, M.A., Beristáin, C.I. (2016). Stability, antioxidant activity and bioavailability of nano-emulsified astaxanthin. *Revista Mexicana de Ingeniería Química 15*, 457-468.

Donsì, F., Annunziata, M., Sessa, M., Ferrari, G. (2011). Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *Food Science and Technology 44* 1908-1914.

Du, W.L., Niu, S.S., Xu, Y.L., Xu, Z.R., Fan, C.L. (2009). Antibacterial activity of chitosan tripolyphosphate nanoparticles loaded with various metal ions. *Carbohydrate Polymers 75* 385-389.

Erel, O. (2004). A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clinical Biochemistry 37* 277-285.

Espinosa-Velázquez., R., Dorantes-Alvarez, L., Gutiérrez-López, G.F., García-Armenta, E., Sánchez-Segura, L., Perea-Flores, M.J., Ceballos-Reyes, G.M., Ortíz Moreno., A. (2016). Morpho-structural description of unripe and ripe avocado pericarp (Persea americana mill var. drymifolia) description. *Revista Mexicana de Ingeniería Química 15*, 469-480.

Fakhreddin, H.S., Zandib, M., Rezaeia, M., Farahmandghavic, F. (2013). Two-step method for encapsulation of oregano essential oil in chitosan nanoparticles: Preparation, characterization and in vitro release study. *Carbohydrate Polymers 95* 50-56.

Fan, W., Yan, W., Xu, Z., Ni, H. (2012). Formation mechanism of monodisperse, low molecular weight chitosan nanoparticles by ionic gelation technique. *Colloids and Surfaces B: Biointerfaces 90* 21-27.

Fernandez-Urrusuno, R., Calvo, P., Remunan-López, C., Vila-Jato, J.L., Alonso, M.J. (1999). Enhancement of nasal absorption of insulin using chitosan nanoparticles. *Pharmacology Research 16* 1576-1581.

Ferrer P., Alquinta C., Piquet D., Botias D., Guerrero F., Calle F., Jara M., Matallana M., Navarro E., Ortiz A., Sierra C. (1996). Procedimiento recomendado para la determinación de α-Tocoferol en suero. *Química Clínica 15* 445-449.

Fissan, H., Ristig, S., Kaminski, H., Asbach, C., Epple, M. (2014). Comparison of different characterization methods for nanoparticle dispersions before and after aerosolization. *Analytical Methods 6* 7324-7334.

Gan, Q., Wang, T., Cochrane, C., McCarron, P. (2005). Modulation of surface charge, particle size and morphological properties of chitosan-TPP nanoparticles intended for gene delivery. *Colloids and Surfaces B: Biointerfaces 44* 65-73.

Gharsallaoui A, Roudaut G, Chambin O, Voilley A., Saurel R. (2007). Applications of spray-drying in microencapsulation of food ingredients: an overview. *Food Research International 40* 1107-1121.

Gómez-Sampedro, L.J., Zapata-Montoya, J.E. (2016). Obtaining of antioxidant peptide from bovine plasma hydrolysates and effect of the degree of hydrolysis on antioxidant capacity. *Revista Mexicana de Ingeniería Química 15*, 101-109.

González-Pérez, O., Gonzalez-Castaneda, R. E. (2006). Therapeutic perspectives on the combination of α-lipoic acid and vitamin E. *Nutrition Research 26*, 1-5.

González, P.O., Moy, L.N.A., Guzmán, M.J. (2008). El alfa-tocoferol y el ácido alfa-lipoico. Una sinergia antioxidante con potencial en medicina preventiva. *Revista de Investigación Clínica 60*, 58-67.

Grilo, E. C., Costa, P. N., Gurgel, C. S. S., Beserra, A. F. D. L., Almeida, F. N. D. S., Dimenstein, R. (2014). Alpha-tocopherol and gamma-tocopherol concentration in vegetable oils. *Food Science and Technology (Campinas) 34*, 379-385.

Gulati, N., Nagaich, U., Saraf, S. A. (2013). Intranasal delivery of chitosan nanoparticles for migraine therapy. *Scientific Pharmacy 81*, 843-54.

Gulati, N., Nagaich, U., Saraf, S. (2014). Fabrication and in vitro characterization of polymeric nanoparticles for Parkinson's therapy: a novel approach. *Brazilian Journal of Pharmaceutical Sciences 50*, 869-876.

Harris, R., Lecumberri, E., Mateos-Aparicio, I., Mengíbar, M., Heras, A. (2011). Chitosan nanoparticles and microspheres for the encapsulation of natural antioxidants extracted from Ilex paraguariensis. *Carbohydrate Polymers 84*, 803-806.

Hernández-Carrillo, J.G., Valdez-Fragoso, A., Welti-Chanes, J., Mújica-Paz, H. (2015). Tracing phenolic compounds through manufacturing of edible films from orange and grapefruit peels. *Revista Mexicana de ingeniería Química 14*, 567-578.

Hu, B., Pan, C., Sun, Y., Hou, Z., Ye, H., Hu, B., Zeng, X. X. (2008). Optimization of fabrication parameters to produce chitosan-tripolyphosphate nanoparticles for delivery of tea catechins. *Journal of Agricultural Food Chemistry 56*, 7451-7458.

Ibezim, E. C., Andrade, C. T., Marcia, C., Barretto, B., Odimegwu, D. C., de Lima, F. F. (2011). Ionically cross-linked chitosan/tripolyphosphate microparticles for the controlled delivery of pyrimethamine. Ibnosina *Journal of Medicine and Biomedical Sciences, 3*.

Jayakumar, R., Menon, D., Manzoor, K., Nair, S. V., Tamura, H. (2010). Biomedical applications of chitin and chitosan based nanomaterials-A short review. *Carbohydrate Polymers 82*, 227-232.

Kvitek, L., Panacîek, A., Soukupova, J., Kolar, M., Vecîerova, R., Prucek, R., Holecova, M., Zboril, R., (2008). Effect of surfactants and polymers on stability and antibacterial activity of silver nanoparticles (NPs). *Journal of Physical Chemistry 112*, 5825-5834.

Jonassen, H., Kjøniksen, A. L., Hiorth, M. (2012). Stability of chitosan nanoparticles cross-linked with tripolyphosphate. *Biomacromolecules 13*, 3747-3756.

Lacatusua, I., Mitreaa, E., Badeaa, N., Stana, R., Opreaa, O., Meghea, A. (2013). Lipid nanoparticles based on omega-3 fatty acids as effective carriers for lutein delivery. Preparation and in vitro characterization studies. *Journal of Functional Foods 30*, 1-10.

Levine, M., Rumsey, S. C., Daruwala, R., Park, J. B., Wang, Y. (1999). Criteria and recommendations for vitamin C intake. *Jama 281*, 1415-1423.

Liu, G., Shao, L., Ge, F., Chen, J. (2007). Preparation of ultrafine chitosan particles by reverse microemulsion. *China Particuology 5*, 384-390.

Maynard, A. D. 2006. Nanotechnology: assessing the risks. *Nanotoday 1*, 22-33.

Mendoza, U.G., Rodríguez, L.J.L. (2007). La nanociencia y la nanotecnología: una revolución en curso. *Perfiles Latinoamericanos 29*, 161-186.

Mi, F. L., Shyu, S. S., Chen, C. T., Schoung, J. Y. (1999). Porous chitosan microsphere for controlling the antigen release of Newcastle disease vaccine: preparation of antigen-adsorbed microsphere and in vitro release. *Biomaterials 20*, 1603-1612.

Mishra, K., Ojha, H., Chaudhury, N.K. (2012). Estimation of antiradical properties of antioxidants using DPPH assay: A critical review and results. *Food Chemistry 130*, 1036-1043.

Mora-Huertas, C. E., Fessi, H., Elaissari, A. (2010). Polymer-based nanocapsules for drug delivery. *International Journal of Pharmaceutics 385*, 113-142.

Müller, R. H., Jacobs, C. y Kayser, O. (2001). Nanosuspensions as particulate drug formulations in therapy rationale for development and what we can expect for the future. *Advanced Drug Delivery Reviews, 47*, 3-19.

Müller L., Fröhlich K., Böhm V. (2011). Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (aTEAC), DPPH assay and peroxyl radical scavenging assay. *Food Chemistry 129*, 139-148.

Naidu, K. A. (2003). Vitamin C in human health and disease is still a mystery? An overview. *Nutrition Journal 2*, 1.

Park, C. B., Miller, R. D., Xia, J. (1999). Multichannel analysis of surface waves. *Geophysics 64*, 800-808.

Pérez-Alonso, C., Campos-Montiel, R.G., Morales-Luna, E., Reyes-Munguía, A., Aguirre-Álvarez, G., Pimentel-González D.J. (2015). Stabilization of phenolic compounds from Opuntia oligacantha forst by microencapsulation with agave sap (aguamiel). *Revista Mexicana de Ingeniería Química 14*, 579-588.

Petersen, S.K., Moreau, R., Smith, E., Smith, A. y Hagen, M. (2009). Alpha-lipoic acid as a dietary supplement: Molecular mechanisms and therapeutic potential. *Biochimica et Biophysica Acta 1790*, 1149-1160.

Premathilaka, R., Wickramasinghe, I., Hewage, S. (2016). In vitro antioxidant activity of leaf extracts in three medicinal plants; *Costus specious* (Koen.) Smith, *Coccinia grandis* (L.) J. Voigt and Wattakaka Volubilis. *European Journal of Academic Essays 3*, 141-146.

Pyrzynska, K. Pękal, A. (2013). Application of free radical diphenylpicrylhydrazyl (DPPH) to estimate the antioxidant capacity of food samples. *Analytical Methods 5*, 4288-4295.

Racoviţă, S., Vasiliu, S., Popa, M., Luca, C. (2009). Polysaccharides based on micro-and nanoparticles obtained by ionic gelation and their applications as drug delivery systems. *Revue Roumaine de Chimie 54*, 709-718.

Rajaram, S., Natham, R. (2013). Design and characterization of ascorbic acid stabilized rifampicin nanoparticles for oral delivery. *International Journal of Biological & Pharmaceutical Research 4*, 993-999.

Rodríguez Hamamura, N., Valderrama Negrón, A., Alarcón Cavero, H., López Milla, A. (2010). Preparación de partículas de quitosano reticuladas con tripolifosfato y modificadas con polietilenglicol. *Revista de la Sociedad Química del Perú 76*, 336-354.

Steed, J. W., Atwood, J. L. (2013). *Supramolecular Chemistry*. John Wiley and Sons.

Shay, K. P., Moreau, R. F., Smith, E. J., Smith, A. R., Hagen, T. M. (2009). Alpha-lipoic acid as a dietary supplement: molecular mechanisms and therapeutic potential. *Biochimica et Biophysica Acta (BBA)-General Subjects 1790*, 1149-1160.

Shu, X. Z., Zhu, K. J. (2002). Controlled drug release properties of ionically cross-linked chitosan beads: the influence of anion structure. International Journal of Pharmaceutics 233, 217-225.

Stoica, R. Somoghi, R., Ion, R. (2013). Preparation of chitosan-tripolyphosphate nanoparticles for the encapsulation of polyphenols extracted from rose hips, digest. *Journal of Nanomaterials and Biostructures 8*, 955-963.

Tripathi, A., Ren, Y., Jeffrey, P. D., Hughson, F. M. (2009). Structural characterization of Tip20p and Dsl1p, subunits of the Dsl1p vesicle tethering complex. *Nature Structural and Molecular Biology 16*, 114-123.

Tuoriniemi, J., Johnsson, A. C. J., Holmberg, J. P., Gustafsson, S., Gallego-Urrea, J. A., Olsson, E., Pettersson, J.B.C., Hassellöv, M. (2014). Intermethod comparison of the particle size distributions of colloidal silica nanoparticles. *Science and Technology of Advanced Materials 15*, 1-10.

Velasco-Rodríguez, V., Cornejo-Mazón, M., Flores-Flores, J. O., Gutiérrez-López, G. F., Hernández-Sánchez, H. (2012). Preparación y propiedades de nanopartículas de quitosano conteniendo ácido alfa lipoico. *Revista Mexicana de Ingeniería Química 11*, 155-161.

Weerakody, R., Fagan, P., Kosaraju, S. (2008). Chitosan microspheres for encapsulation of α-lipoic acid. *International Journal of Pharmaceutics 357*, 213-218.

Yilmaz, O., Ersan, Y., Ozsahin, A. D., Ozturk, A. I., Ozkan, Y. (2013). Consequences of the combined α-tocopherol, ascorbic acid and α-lipoic acid on the glutathione, cholesterol and fatty acid composition in muscle and liver of diabetic rats. *Iranian Journal of Basic Medical Sciences 16*, 165-172.

Yusuf, S., Dagenais, G., Pogue, J., Bosch, J., Sleight, P. (2000). Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *The New England journal of medicine 342*, 154-160.

Zhang, H., Oh, M., Allen, C., Kumacheva, E. (2004). Monodisperse chitosan nanoparticles for mucosal drug delivery. *Biomacromolecules 5*, 2461-2468.

Zhou, W. y Wang, Z. L. (Eds.). (2007). *Scanning microscopy for nanotechnology: techniques and applications*. Springer science and business media.