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Design and determination of double emulsions ($W_1/O/W_2$) for the trapping of antioxidant compounds sensitive to thermal processes

Diseño y estabilización de emulsiones dobles ($W_1/O/W_2$) para el atrapamiento de compuestos antioxidantes sensibles a procesos térmicos

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Abstract

Currently, there are several advances in the development of functional foods, since consumers are increasingly aware of the close relationship between food and the health of the body. Thus, this research proposes the encapsulation of bioactive compounds, extracted from the xoconostle fruit using micro emulsions for its protection at high temperatures in the jam making process. For the extraction, three treatments were proposed, with different means of extraction (EX); EX1: water, EX2: ethanol and EX3: ethanol-water mixture (1:1). Statistical analysis revealed that the extraction medium did not have an effect on antioxidant capacity. The emulsion was made with the EX1 extract, forming a multiple water-in-oil-in-water emulsion (W1/O/W2), using three biopolymers whey protein concentrate (WPC), whey protein isolate (WPI) and gum arabic (GA) in concentrations of 6.0%, 7.5%, and 9.0%, in which physical stability and stability to creaming were determined. The 7.5% WPI emulsion presented greater stability. The antioxidant capacity tests in the jam showed that the emulsion is efficient to preserve the antioxidant capacity, observing an increase in this in EXC/EM, compared to the EX and EXS/EM treatment.

Xoconostle, Aguamiel, Antioxidants

Resumen

Actualmente, existen diversos avances en desarrollo de alimentos funcionales, ya que los consumidores son cada vez más conscientes de la estrecha relación entre alimentación y salud del organismo. Es así, que la presente investigación, propone la encapsulación de compuestos bioactivos, extraídos del fruto xoconostle utilizando microemulsiones para su protección a las altas temperaturas del proceso de elaboración de mermelada. Para la extracción, se plantearon tres tratamientos, con diferentes medios de extracción (EX); EX1: agua, EX2: etanol y EX3: mezcla etanol-agua (1:1). El análisis estadístico reveló que el medio de extracción no tiene un efecto en la capacidad antioxidante. La emulsión se realizó con el extracto EX1, formando una emulsión múltiple agua-en-aceite-en-agua (W1/O/W2), empleando tres biopolímeros concentrado de proteína de suero (WPC), aislado de proteína de suero (WPI) y goma arábiga (GA) en concentraciones de 6.0%, 7.5 %, y 9.0%, en las cuales se determinó la estabilidad física y la estabilidad al cremado. La emulsión de WPI 7.5 %, presentó mayor estabilidad. Las pruebas de capacidad antioxidante en la mermelada mostraron que la emulsión es eficiente para conservar la capacidad antioxidante observando un incremento de esta en EXC/EM, comparado con el tratamiento EX y EXS/EM.

Xoconostle, Aguamiel, Antioxidantes

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Introduction

The world's population is considering important changes in their eating habits, due to the influence that scientific progress has had in demonstrating the effects of functional foods or bioactive compounds on certain functions of the organism, obtaining as a benefit the well-being and health of the consumer. Therefore, a functional food can be designed according to two aspects, one where the presence of compounds that can be harmful to health can be limited, or increasing the proportion of those that have a positive influence on the consumer (Jiménez-Colmenero, 2013), due to this it is necessary to incorporate new technologies for the optimisation of the extraction processes of bioactive components, to strategies that involve their protection during their inclusion in different food matrices, to increase their stability and bioavailability.

However, several investigations have focused on the use of agro-industrial by-products of plant origin as a source of bioactive compounds, especially those with antioxidant activity, so that extracts are produced with quantities that have this property (Castromonte et al., 2020). Among these antioxidant components are polyphenols, which are a group of secondary metabolites in plants that have gained commercial importance; polyphenols are compounds that differ in their structure, which determines their biological functions and which, when incorporated into foods, confer functional properties that provide practical applications (Ríos-Aguirre and Gil-Garzón, 2021). However, it is important to take into account that these compounds, being outside their source of origin, are susceptible to environmental factors such as light, oxygen and pH, causing their degradation and therefore a decrease or loss of their functionality, so encapsulation becomes more relevant, since it is a micro-packaging technology (Castromonte et al., 2020), which involves the coating or entrapment of a pure material or a mixture inside another material forming a capsule of between 5 to 300 microns in diameter (Esquivel-González, Ochoa Martínez and Rutiaga-Quiñones, 2015).

The main function of these capsules is to protect the material both from adverse environmental conditions and during its passage through the gastrointestinal tract, thereby promoting a controlled release of the bioactive component, increasing its shelf life and bioavailability (Castromonte, Wacyk, Valenzuela, 2020). Therefore, food production continuously seeks strategies for the integration of these compounds without losing their integrity and therefore without compromising the sensory quality of food products (Buyukkestelli and Sedef Nehir, 2021).

Water-in-oil-in-water (W1/O/W2) double emulsions are one of the carrier systems used for encapsulation, protection, and delivery of hydrophilic and hydrophobic active components. Double emulsions are complex multiphase systems in which small water droplets (inner water phase W1) are trapped inside larger oil droplets (W1/O), which are subsequently dispersed in a continuous water phase (outer water phase W2). Recently, double emulsions have been studied in the release of hydrophilic bioactives in the food and pharmaceutical industries. Several parameters have been shown to affect the droplet stability of double emulsions, e.g. the phase mass fraction and the type of emulsifier. However, double emulsions are thermodynamically and kinetically unstable systems with a strong tendency towards coalescence, flocculation and creaminess due to the presence of two interfaces with opposite curves in a single structure. The proximity of these interfaces also induces diffusion from one interface to the opposite interface. Subsequently, this destabilises the structure to form a single emulsion. Furthermore, the stability of double emulsions after heat treatment can be important for improving the safety and shelf life of many food products containing active components in the food industry (Jo and van der Schaaf, 2022).

Therefore, the present research proposes the design of multiple emulsions for the protection of antioxidant compounds extracted from xoconostle fruit for incorporation into a jam.

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Materials and methods

Conditioning of the raw material

The xoconostle was harvested from the plot of Mr. Silvestre Jiménez Montiel, located in the municipality of Tlanalapa, State of Hidalgo, Mexico, whose coordinates are Latitude 19°48'41.33 "N, Longitude 98°35'28.65 "W, at an altitude of 2460 m above sea level. The fruits were selected according to their optimum ripeness, working with fruits in which pink colour was predominant (Pimienta-Barrios et al., 2008), in addition to verifying that there were no perforations or presence of pests that could cause any alteration in the fruit.

The total weight of the xoconostles was obtained and then they were washed and disinfected with a solution of sodium hypochlorite in water (5 ppm). Subsequently, the epidermis, mesocarp and pulp or seeds (endocarp) were separated. Each fraction was weighed to calculate the yield. Finally, they were vacuum packed and stored frozen, protected from light until further analysis.

Extraction of bioactive compounds from xoconostle pulp

Anthocyanins were extracted from the pulp with seeds. The process consisted of an initial grinding for three minutes in an industrial blender (Oster brand), followed by filtering through an organza cloth to remove larger particles. The treatments were established according to the experimental design shown in Table 1. Three treatments were proposed, in which the extraction medium was changed: 1) distilled water (EX1), 2) ethanol (EX2) and 3) a 1:1 mixture of ethanol and water (EX3), these three treatments with and without the application of temperature and a maceration time of 2 hours (Romero-López, 2015).

Treatment	Extraction medium	Temperature (°C)	Time (hours)
EX_1	Water	25	2
EX_2	Ethanol	45	2
EX_3	Water-ethanol	25	2

Table 1 Experimental design for the extraction of xoconostle pigments with different solvents

After the extraction time, the samples were centrifuged at 6000 rpm for 20 minutes (Thermo Scientific Medialite 6 PL Centrifuge), the supernatant was decanted and the antioxidant activity was determined by the ABTS method to corroborate that there was no decrease in this parameter. The extracts were kept refrigerated (4°C), protected from light until further use and analysis.

Emulsion design

The methodology proposed by Rodríguez-Hueso et al. (2014) was applied, forming a multiple water-in-oil-in-water (W1/O/W2) emulsion in two stages. In the first stage, a W1/O primary emulsion was formed, with a 70:30 ratio, 70 % aqueous phase (xoconostle pulp extract) and 30 % oil phase with canola oil (Capullo®, Unilever de México, S.A. de C.V., Tultitlán Edo. de México, Mexico) at a total emulsifier concentration of 8 % w/w. The hydrophilic emulsifier used was Panodam SDK (monoglyceride and diglyceride esters of diacetyl tartaric acid) and the hydrophobic emulsifier was Grindsted PGRR 90 (fatty acid esters of polyglycerol and polyricinoleate), both from Danisco México, S. A. de C. V. The emulsification process was carried out with a Wiggen-Hauser D-500 Disperser homogeniser at 10,000 rpm for five minutes.

For the second stage, 30 mL of the primary emulsion W1/O was reemulsified in 70 mL of aqueous medium, testing different biopolymers; Whey Protein Concentrate (WPC), Whey Protein Isolate (WPI) and Gum Arabic (GA) at concentrations of 6. 0%, 7.5 % and 9.0% as external aqueous phase (W2) in a Wiggen-Hauser D-500 Disperser homogeniser at 4500 rpm for five minutes, producing double emulsions (W1/O/W2).

Physical stability of the emulsion

Particle size analysis of the W1/O/W2 double emulsions was carried out under an optical microscope (VELAB model VE L5MCD digital) and applying Pixemetre image analyser software (version 5.1) to measure the length of the average diameter of the oil globules of the double emulsion.

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The diameter of 30 randomly selected globules was determined to determine the measurement of the diameters of the primary and double emulsion, immediately after their realisation and subsequently over a 14-day time. Taking into account what is reported by Nava, (2019) the droplet size was determined, as it depends on the strength with which the emulsion is made and is responsible for the water solubility of the ingredients.

Stability of the emulsion upon creaming

Determined by observing a creaming layer at the top and the presence of a clear water phase at the bottom of the tube containing the double emulsion, the creaming index (CI) was calculated by applying the following formula:

$$CI(\%) = \frac{hW}{hT}\ x\ 100 \qquad (1)$$

Where hW is the height of the clear water phase at the bottom and hT is the total height of the double emulsion. The accumulation of the phase with a higher proportion of oil at the top is similar to the formation of cream, but with a less clear phase at the bottom (Yesiltas et al., 2021).

Antioxidant activity analysis

ABTS-+2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) method.

A solution of ABTS-+ radical cations at a concentration of 7 mM (ABTS-++) was prepared and activated with 2.45 mM potassium persulphate for 12 h under dark conditions at room temperature prior to analysis. The ABTS-++ radical is diluted in potassium phosphate buffered saline (PBS) to adjust the absorbance to 0.700 ± 0.020 at a wavelength of 734 nm. To measure the antioxidant capacity, 10 µL of sample was mixed with 990 µL of radical solution and the absorbance at 734 nm was recorded on a UV-Vis Digital spectrophotometer (FIAXA 325-1000 nm-722G), readings were taken at time zero and 6 min after sample addition to determine the percentage inhibition of antioxidant activity (Re et al., 1999; Guzmán-Maldonado, 2010).

Antioxidant activity assay. DPPH method (1,1-diphenyl-2-picrylhydrazine)
A solution was prepared from 7.4 mg of DPPH (SIGMA-ALDRICH) by gauging to 100 mL with absolute ethanol and shaken until complete homogenisation. For the standard calibration curve, concentrations of 300, 200, 100, 100, 50 and 0 mmol/L Trolox were used as standard solution. From each dilution or sample, 200 µL were taken, 1000 µL of DPPH were added and finally left to stand for one hour until subsequent reading at 520 nm, using a UV-Vis Digital spectrophotometer (FIAXA 325-1000 nm-722G) (Llica, 2008).

Determination of total phenols

It was determined using Folin-Ciocalteu reagent (SIGMA ALDRICH), at 10% (solution A), sodium carbonate at 7. 5 % (solution B), and for the standard curve 100 mg of gallic acid in 10 mL of deionised water, from which concentrations of 0, 10, 50, 100, 100, 300, 500, 700, 900 and 1000 mg/mL were prepared, of each dilution 100 µL were mixed with 500 µL of solution A and 400 µL of solution B, after 30 minutes of rest the absorbance at 760 nm was obtained. This procedure was applied for each sample analysed (Ordoñez et al, 2020).

Jam production

According to the NMX-131-1982 standard; and according to Paltrinieri, (1997); Jiménez and Bonlla, (2012); Makanjuola, (2019) the characteristics that a jam should have are 64 -65° Brix, pH of 3.4, acidity of 0.4282 g of citric acid and 6 % of pectin. Table 2 shows the proposed formulations. Adjustments were made to the amount of pectin, so that the minimum amount necessary for gel formation could be determined. Subsequently, the xoconostle jam was made by substituting the sugar with mead honey (MA) and adding the bioactive compounds extracted from the xoconostle pulp; table 3 shows the different treatments. The antioxidant activity and total phenol content were determined for each jam produced.

*Formulation	Xoconostle (g)	MA (g)	Pectin (g)
1	145.79	125	1.25
2	145.79	125	2.50
3	145.79	125	3.75
*The amount of final product was 225 g.			

Table 2 Treatments to determine the amount of pectin suitable for gel formation

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Statistical analysis

All determinations were performed in triplicate, reporting the mean and standard deviation. Additionally, a one-way analysis of variance was used, followed by a Tukey mean comparison test with a 95 % confidence level, the analysis was performed using the statistical programme Sigma Plot 12.0 (Systat Software, Inc. SigmaPlot for Windows).

Jam	Xoconostles (g)	MA (g)	Pectin (g)	Pulp extract (mL)
1	140.0	225	7.3	20
2	140.0	225	7.3	20*

Table 3 Formulations for jam production with pulp extract

*Microencapsulated pulp extract.

Results

Extraction of bioactive compounds

For the selection of the extraction process, the percentage inhibition of the ABTS-+ radical was determined for each treatment. These measurements revealed that the antioxidant capacity is not affected by the extraction method, since the statistical analysis revealed that there are no significant differences between the treatments (EX1: 1.78, EX2: 1.51, EX3: 1.89 μmol ET /g of extract), so it was decided to use the EX1 treatment, since this refers to the use of water as the extraction medium and no temperature. The results are related to those obtained by Hernández-Fuentes et al., (2015) in xoconostle pulp, who indicate values of 1.10 to 1.22 μmol ET /g. Sánchez-González, (2016) recommends extraction with aqueous ethanol solutions from 20 to 50% to achieve complete extraction. In this case, as there were no significant differences, the treatment with water without solvents was applied due to the fact that the product in which the extract will be used is intended for a foodstuff. Soto and Rosales, (2016), report greater extraction efficiency using 80% ethanol in aqueous solution, as it favours obtaining phenolic compounds and total antioxidant activity; however, these extracts are for medicinal use, Bustos (2012) mentions that the extraction process used depends directly on the type of compound and the food matrix from which the extraction is carried out.

This author reports that the best extraction of vitamin C, flavonoids and anthocyanins from apple peel is achieved with boiling water, even compared to ethanolic or methanolic solutions.

Physical stability of the emulsion

Different treatments of double emulsions were carried out, using different W2 aqueous phases formed with three biopolymers (WPC, WPI and GA) and with a concentration variation of 6.0, 7.5 and 9.0 %, respectively. In the emulsions formed by WPC at a concentration of 6.0 and 7.5 %, the same trend was presented, increasing the droplet size of the double emulsion from 7.92 ± 0.68 and 9.07 ± 1.28 μm at day 1 to 20.09 ± 1. 88 and 20.97 ± 2.23 μm at day 14, respectively, being the most stable compared to the 9.0 % concentration that presented a diameter increase of 23.85 ± 2.76 at day 1 and 33.57 ± 2.58 μm at day 14 (Figure 1a). This behaviour may be due to the fact that the stabilisation of the oil-water interfaces with a higher percentage of biopolymer is presenting a drastic increase in size indicating an instability system in which the emulsion droplets are growing. The same behaviour is observed in the WPI emulsions (Figure 1b), where the double emulsions similarly do not show a drastic change in size; stabilised with WPI6.0% and WPI 7.5% from 11.41 ± 2.97 at day 1 and 17.95 ± 1.72 μm at day 1 to 23.09 ± 2.84 and 20.21 ± 3.99 μm at day 14, respectively, not so for the emulsion stabilised with WPI9.0%. In the GA emulsions at 6.0% concentrations a peculiar behaviour was presented (Figure 3c), from a large double emulsion droplet size of 48.46 ± 2.57 μm there was a decrease to 28.75 ± 1.89 μm. This same behaviour was observed for the 7.5% concentration. However, at the 9.0% concentration the opposite occurs as there is a drastic increase in emulsion size from 15.09 ± 2.05 at day 1 to 51.56 ± 2.49 μm at day 14 (Figure 1c), indicating that an instability system is occurring possibly due to saturation of the biopolymer.

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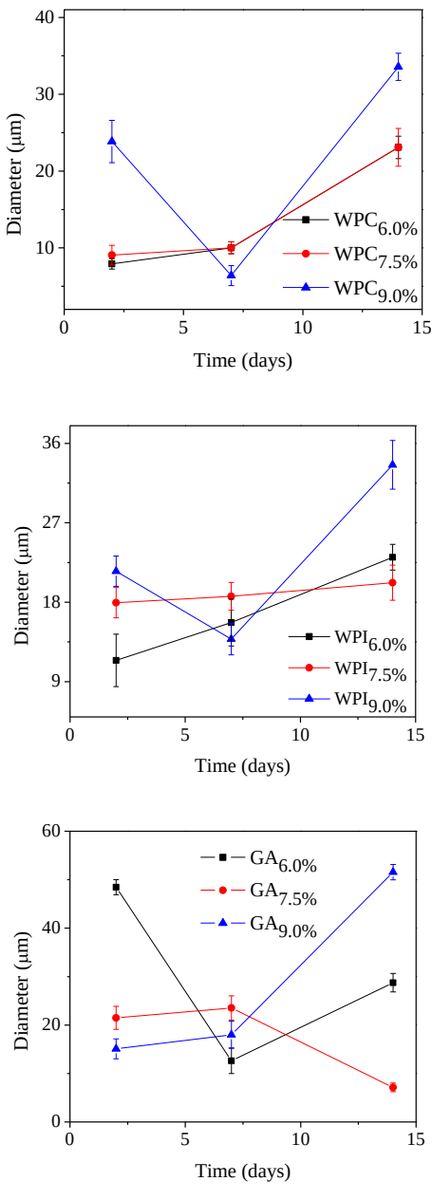

Figure 1 Change in diameter of the double emulsions. Where (a); WPC for concentrations of 6.0%, 7.5% and 9.0%; (b) WPI for concentrations of 6.0%, 7.5% and 9.0%; and (c) GA for concentrations of 6.0%, 7.5% and 9.0%

Derived from the above results, it was decided to make only the comparison study of the double emulsions stabilised with the three biopolymers at the concentrations of 6.0 and 7.5% as can be seen in figures 2a and 2b. In the double emulsions stabilised with 6.0%, it can be seen in figure 2a, the change in diameter of the double emulsions according to the biopolymer used, presenting a smaller diameter and better stability over time in the double emulsion stabilised with WPC 6.0%. As for the 7.5% concentration, the WPI biopolymer is the one that presents better stability compared to the 6.0% concentration over time, because its increase is not significant (Figure 2b).

According to McIntyre et al., (2018), the formation of a stable emulsion will depend on the ability of the proteins or biopolymers to migrate to the oil-water interface and reduce the interfacial tension, and for this to be achieved, the proteins must be sufficiently soluble. Therefore, the main form of instability in the emulsions formed is coalescence that arises due to the inability of the biopolymers to adequately stabilise the oil surface area generated during homogenisation. The biopolymers are absorbed at the oil-water interface, where they decrease the free energy of the emulsion system allowing stabilisation. However, only a proportion of the biopolymer is actually at the interface; an unadsorbed fraction is present in the aqueous phase as an aggregate. Higher levels of biopolymers not adsorbed in the aqueous phase may result in the biopolymer concentration not being sufficient to allow adequate coverage at the oil-water interface. If such a case occurs, biopolymer aggregates could be shared between adjacent emulsion droplets, resulting in flocculation and, consequently, a marked increase in droplet size (McIntyre et al., 2018). This theory agrees with the results reported in the present work, as the droplet size of the emulsions was larger when a higher concentration of biopolymers (9.0%) was used.

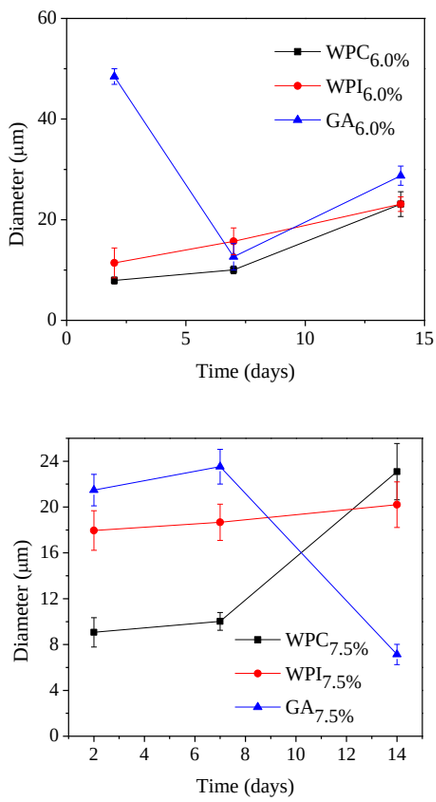

Figure 2 Change in diameter of the double emulsions (a) concentration 6% and (b) concentration 7.5%

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Emulsion stability at creaming

The creaming index (CI) was applied to further evaluate the emulsion stability for different storage periods (1, 7 and 14 days). One of the main indications of physical instability of the emulsions was creaming (CI). Creaming describes the upward movement of oil droplets due to gravitational separation that leaves a clear water phase at the bottom (Yesiltas et al., 2021). Lipid droplets in emulsions stabilised by different biopolymers and in their different concentrations tend to move upwards in the upper layer, while water-soluble substances may move downwards due to gravity (Chen et al., 2020). A lower CI value corresponds to a smaller emulsion separation, representing a more stable system with less separation. These results can be seen in Table 4. All double emulsion treatments tested showed an increase in CI as storage time increased. The double emulsions stabilised in the external aqueous phase with GA 6.0% at day 1 showed the lowest CI value, but after 14 days of storage it increased drastically. The 7.5% biopolymer concentration, stabilised with 7.5% WPI, had the lowest creaming rate after 14 days of storage, from 25.0 ± 2.23% at day 1 to 29.0 ± 0.23% at day 14. In contrast, the 9.0% concentration of biopolymers showed lower CI at day 1 but with a large increase at day 14 of storage. Therefore, in figure 2 it can be observed that, with increasing concentration of the biopolymers, the lowest CI values occurred at the shortest storage time (1 day), but as the storage time increased, the value increased. Chen et al., (2020), report stability of nano-emulsions and evaluated the creaming index only for a storage time of 48 hours, which indicates that the systems evaluated in the present work are more stable over time.

Analysis of antioxidant activity and phenols in marmalade

In addition to the treatments shown in table 3, a control sample was made for the production of jam, which consisted of jam to which no xoconostle pulp extract (Ms/E) was added. For each treatment, the percentage of ABTS-+ radical inhibition, DPPH and total phenol concentration were determined (Table 5).

Treatment	CI (%)		
	1 day	7 days	14 days
$WPC_{6.0\%}$	38.0± 0.23	48.0 ± 1.32	51.0 ± 1.99
$WPI_{6.0\%}$	25.0 ± 1.45	38.5 ± 2.78	41.0 ± 2.01
$GA_{6.0\%}$	13.0± 2.31	56.5 ± 1.45	57.0 ± 1.99
$WPC_{7.5\%}$	42.5 ± 1.83	54.0 ± 3.01	56.0 ± 2.99
$WPI_{7.5\%}$	25.0 ± 2.23	26.5 ± 2.41	29.0 ± 0.23
$GA_{7.5\%}$	15.0 ± 1.56	62.5 ± 1.12	65.0 ± 1.25
$WPC_{9.0\%}$	25.0 ± 2.78	51.0 ± 0.89	57.5 ± 2.03
$WPI_{9.0\%}$	15.0 ± 0.89	49.0 ± 0.99	50.5 ± 1.78
$GA_{9.0\%}$	15.0 ± 0.28	60.0 ± 3.09	75.0 ± 0.78

Table 4 Creaming index of double emulsions stabilised in the external aqueous phase (W_1) with different biopolymers and concentrations

The results show that the antioxidant capacity of the jam increases with the addition of pulp extract (table 5). Additionally, when comparing the percentage of inhibition of the ABTS radical in the extract (10.2 %) with that of Ms/E (10.7 %), an increase in this inhibition was observed, which may be due to the addition of the fruit pulp. In general, the highest percentage of inhibition, as well as of total phenols is presented in EXC/EM, revealing that the encapsulation process is efficient in the conservation of the functional properties of the extracted bioactive compounds by protecting them from the high temperatures that occur in the elaboration of the jam.

Treatment	ABTS-+ (% inhibition)	DPPH (mg TE/100mL)	Total phenols (mg/100g)
EX	$10.2±4.2^a$	$2.05±0.03^a$	$2.3±3.6^a$
$M_{s/E}$	$19.7±2.8^b$	$2.21±0.72^a$	$4.55±1.7^b$
$EX_{S/EM}$	$29.1±5.8^c$	$2.83±0.7^a$	$5.3±5.0^b$
$EX_{C/EM}$	$36.1±1.6^d$	$3.11±0.1^b$	$12.3±3.6^c$

Table 5 Results of antioxidant activity of xoconostle extract applied to a food system (jam)

Average of three determinations ± Standard deviation. Where: EX: Xoconostle extract, Ms/E: Jam without extract, EXS/EM: Extract without encapsulation applied in jam and EXC/EM: Encapsulated extract applied in jam. a, b, c different letters in superscripts indicate significant differences ($P<0.05$).

The results obtained are in agreement with other research. Šaponjac et al., (2016) and Davidov et al., (2012) mention that the incorporation of encapsulated bitter cherry pomace and grape seed extracts respectively in biscuits positively influences their functional characteristics and even the shelf life of the product.

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This research, together with the present report, shows that the incorporation of encapsulated antioxidant compounds in different types of food matrices improves the stability of the active ingredients and provides functional properties to foods, thus opening up possibilities of application in other types of products in the food industry.

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Conclusions

The microencapsulation carried out with double emulsion was able to provide protection to the bioactive compounds of the xoconostle pulp extract, as they resisted the heat treatment during the jam making process. In this way, according to the analyses carried out, it can be guaranteed that this product has properties that allow it to be a functional food for daily consumption.

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