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Antioxidant effect of polyphenols extracted from pomegranate peel on vegetable oil/ethylcellulose-based oleogels

Efecto antioxidante de polifenoles extraídos de cáscara de granada sobre oleogeles a base de aceite vegetal y etilcelulosa

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Resumen

Los objetivos del presente trabajo fueron evaluar el uso de polifenoles extraídos de la cáscara de granada como agente antioxidante para el desarrollo de oleogeles a base de etilcelulosa en aceite vegetal y establecer la cantidad óptima de polifenoles necesaria para preservar la estabilidad oxidativa del oleogel y sus propiedades mecánicas. Los polifenoles de cáscara de granada (pp-polifenoles) se extrajeron y purificaron (usando HPLC-flash), se deshidrataron y se pulverizaron; sus componentes se caracterizaron por HPLC-MS/MS. Utilizando la metodología de Taguchi (arreglo ortogonal L9 3^3), se evaluaron los factores etilcelulosa 45 cP (3, 6 y 12 %), pp-polifenoles (0.05, 0.1 y 0.2 %) y temperatura (140, 150 y 160 °C) sobre el oxidación de lípidos producidos

durante el proceso de elaboración del oleogel mediante métodos de peróxidos y dienos conjugados y complementada con FTIR. También se determinaron las propiedades mecánicas de los oleogeles desarrollados. Los resultados mostraron que el principal polifenol identificado en la cáscara de granada fue la punicalagina (anómeros α y β). Con base en el modelo estadístico de Taguchi, se seleccionó la formulación con 3% de EC y 0.1% de pp-polifenoles y el procedimiento térmico a 160 °C durante 2 horas, ya que bajo estas condiciones se obtuvo la mayor inhibición de oxidación (hasta 90%). El índice de peróxidos determinado en esta muestra (5,62 ± 0,4 mEq/kg) estuvo dentro del límite permitido para aceites vegetales comestibles (máximo 10 mEq/kg). Se determinó que ni la adición de polifenoles ni el proceso térmico establecido alteraron las propiedades mecánicas de los oleogeles, independientemente de la adición de pp-polifenoles. Se sugirió complementar este trabajo con la investigación del sinergismo entre los pp-polifenoles y los componentes del oleogel antes de sugerir una funcionalidad o posible aplicación.

Palabras clave: oleogel; etilcelulosa; punicalagina; polifenoles.

Abstract

The objectives of the present work were to evaluate the use of polyphenols extracted from pomegranate peel as an antioxidant agent for the development of ethylcellulose based oleogels on vegetable oil and stablish the optimal amount of polyphenols necessary to preserve the oleogel oxidative stability and its mechanical properties. Pomegranate peel polyphenols (pp-polyphenols) were extracted and purificated, dehydrated and pulverized; their components were characterized by HPLC-MS/MS and the polyphenols was purified by HPLC-flash. Using Taguchi's methodology (orthogonal array L9 3^3), the factors ethylcellulose 45 cP (3, 6 and 12%), pp-polyphenols (0.05, 0.1 and 0.2%) and temperature (140, 150 and 160 °C) were evaluated on the oxidation of lipids produced during the oleogel elaboration process through peroxide and conjugated dienes methods and complemented with FTIR. Mechanical properties of the oleogels developed also were determined. Based on Taguchi's statistical model, the formulation with 3% EC and 0.1% pp-polyphenols and the thermal procedure at 160 °C for 2 hours was selected, under these conditions the greatest oxidation inhibition was obtained (up to 90%). The peroxides value determined in this sample (5.62 ± 0.4 mEq/kg) was within the limit allowed for edible vegetable oils (maximum 10 mEq/kg). It was determined that neither the addition of polyphenols nor the established thermal process altered the mechanical properties of the oleogels, regardless of the addition of pp-polyphenols. It was suggested to complement this work with the investigation of the synergism between pp-polyphenols and the components of the oleogel before suggesting a functionality or possible application.

Keywords: oleogel; ethylcellulose; punicalagin; polyphenols.

INTRODUCTION

The pomegranate (*Punica granatum*) is a fruit rich in minerals (i.e., potassium, phosphorus, manganese, calcium, iron and magnesium), it contains some vitamins such as C, B_1 and B_2 and high amounts of antioxidants of which

70% is concentrated in the skin and membranes of the fruit. Due to these nutraceutical properties, the pomegranate has managed to take an important place in the industrial production of arils and juices. However, the processing of pomegranate juice generates waste and 78% of it corresponds to the peel, which contains polyphenolic compounds such as ellagitannins, proanthocyanins, as well as alkaloid compounds and glycosides. (Buenrostro et al., 2017; Coronado-Reyes et al., 2020). Among ellagitannins, punicalagin is the main compound found in pomegranate peel (Çam & Hişil, 2010). This polyphenol has important biological capacities such as anti-inflammatory, hepatoprotective, antigenotoxic and also as an antioxidant or free radical scavenger, the latter standing out since punicalagin is responsible for approximately 50% of this activity (Giamogante et al., 2018; Nuncio-Jáuregui et al., 2015; Young et al., 2017). In addition, this compound has shown excellent bioavailability, stability and safety in humans, when administered within food matrices or independently, making it a great alternative for application in oleogels to help prevent or reverse the oxidation process. (Quirós Fernández & López Plaza, 2017). An oleogel is a material made by dissolving gelator molecules (i.e., low molecular weight molecules, lower than 3000 Daltons) in organic solvents such as vegetable oils or mineral oil (López-Martínez et al., 2014). Edible oleogels are structured with vegetable oils, predominantly rich in "healthier" polyunsaturated fats, which provide viscoelastic mechanical properties like those of a solid fat (i.e., lard, butter, vegetable shortening, etc.) (M. Aguilar-Zárate et al., 2019; Mattice & Marangoni, 2018). Because of this, oleogels have been proposed as alternatives to substitute saturated and *trans* fats that are associated with harmful health risks and a negative perception by the consumer. To prepare oleogels high temperatures are required to dissolve gelator molecules and sometimes this process can affect the quality of the systems due to the sensitivity to heat associated with their components (i.e., gelators and polyunsaturated oils). Because of this, the use of an antioxidant agent is important for the development of some oleogels (Singh et al., 2017). This is the case of ethylcellulose (EC) oleogels, which are formed by the interaction of EC polymeric strands through hydrogen bonds (Laredo et al., 2011) after their solubilization in vegetable oils at ~120-140 °C. The temperature to which these solutions are heated causes decomposition of the gelator, surfactants used in the gel and also could cause the oxidation of the oil. The oxidation mechanism that could occur in this type of oleogelled systems is the autooxidation or radical mechanism. This pathway requires an initial activation energy for the removal of a hydrogen atom, thus it is enhanced by high temperatures and the presence and position of double bonds (Barriuso et al., 2013; Taghvaei & Jafari, 2015; Villeneuve et al., 2021). As consequence, the degradation of the molecules of the system (i. e., unsaturated and polyunsaturated fatty acids) can lead to a deterioration in gel quality (texture properties) and safety for use (Gravelle et al., 2012). Due to this, one of the most used ways to control and reduce autooxidation has been the incorporation of synthetic antioxidants as butylated hydroxytoluene (BHT), that has been applied to try to prevent the effects of oxidation caused by temperature on its mechanical properties (Davidovich-Pinhas et al., 2015). In the present work, the possibility to use a natural antioxidant to prevent the oxidation has been studied, in EC oleogels, presenting as alternative the polyphenols extracted from pomegranate peel. It has been reported that punicalagin is the main pomegranate polyphenol (P. Aguilar-Zárate et al., 2017), is not toxic (Kulkarni et al., 2007) and it is resistant to temperatures of up

to 121 °C under vacuum (Qu et al., 2014). In a recent study, in which was compared punicalagin antioxidant effect with the BTH in canola oil, presented similar reduction effect of primary oxidation products than BHT, so the authors proposed punicalagin as suitable replacements of synthetic antioxidants for canola oil (Alsufiani et al., 2020). Because the above, in the present work it was hypothesized that pomegranate polyphenols can act as an antioxidant in EC oleogels in canola oil, since it is necessary to subject the solutions to high temperatures to complete dissolution of the gelator. The study of natural antioxidants using in edible oleogelled systems is limited due to some of them are unrecognized as safe or assesses long term stability because in the most studies they have been examined in model systems. Only few natural antioxidants have demonstrated being effective in oleogels. For example, it has been reported that the addition of curcumin to beeswax oleogels increased their oxidative stability but only during refrigerated storage. (Ramírez-Carrasco et al., 2020). On the other hand, it was obtained that in β-sitosterol and lecithin oleogels also added with curcumin, the crystalline network of molecules served to inhibit the chain of oxidation reactions and, in turn, protected the antioxidant activity of curcumin, it was deduced because diminished the formation of hydroperoxides (Li et al., 2019). But the preparation temperature of these oleogels in the last two cases was 90 °C and their studies were carried out at 60 °C, so there was no evidence that curcumin acted effectively at high temperatures, but rather it turned out that the oleogel served as vehicle for curcumin. In this context, the objectives of the present work were to evaluate the use of polyphenols extracted from pomegranate peel as an antioxidant agent for the development of ethylcellulose based oleogels on vegetable oil and stablish the optimal amount of polyphenols necessary to preserve the oleogel oxidative stability and its mechanical properties.

MATERIALS AND METHODS

Materials

The pomegranate peel was extracted from ripe fruits obtained from a local farm market in Ciudad Valles, San Luis Potosi, Mexico. For the development of the oleogels, ethylcellulose (EC) with a viscosity of 45 cP was used (Ethocel Standard 45 Premium, DUPONT, Suiza) and canola oil (high in polyunsaturated fatty acids) Canoil brand that was purchased from a local supermarket. To filter, Whatman No. 41 brand filters were used; Amberlite XAD – 16 was used in the separation columns. All reagents used for primary and secondary oxidation analyzes (acetic acid, isooctane, potassium iodide, sodium thiosulfate, sodium laureate, starch indicator, tween 20, ethanol, and sodium hydroxide) were purchased from various commercial sources.

Experimental methods

Extraction and purification of polyphenols from pomegranate peel

The extraction and purification was carried out according to the methodology reported by Ascacio-Valdés et al., (2013) with some modifications, from dehydrated and pulverized residues of pomegranate peel 40 grams of powdered sample was weighed into a container and 400 mL of water was added. For extraction, the mixture was heated at 60 °C for 30 min, and then filtered. After this, the obtained extract was taken to centrifugation for 2 min at 2000 rpm and vacuum filtered using filter paper (Whatman No. 41) to remove larger debris particles. The extraction process was applied 3 times to the same plant material. Afterward, purification was performed using a column packed with Amberlite XAD-16, previously washed with ethanol and then with distilled water. Two hundred mL of extract were emptied into the

column and a first wash with water was performed to discard undesirable compounds, then a wash with ethanol was performed to obtain a fraction rich in ellagitannins, and finally the recovery of the sample was carried out. The process was repeated until the desired volume was achieved. The purified sample was taken to a rotary evaporator to remove as much ethanol as possible, then placed in Petri dishes and dried in an oven at 50 °C for 48 h. Finally, the dried powder was recovered and stored in an aluminum-lined glass jar in a cool, dry place until use.

Characterization of polyphenols by HPLC

The analyzes of the fractions (200 ppm) were conducted according to the methodology of Aguilar-Zárate et al. (2017) by reverse-phase high-performance liquid chromatography (RP-HPLC), including an autosampler (Varian ProStar 410, Palo Alto, CA, USA), a ternary pump (Varian ProStar 230I) and a photodiode array detector (PDA) (Varian ProStar 330). Ten µL of sample was injected onto a Denali C18 column (150 mm × 4,6 mm, 3,1 µm, Grace, Columbia, MD, EE.UU.). The oven temperature was 30 °C. The eluent was a gradient of aqueous acetic acid (3%, v/v; solvent A) and acetonitrile (solvent B). Solvents were filtered through 0.45 µm nylon membranes. The flow rate was kept at 0.2 mL/min, and the elution of the phenolic compounds was monitored at 280 nm. Data processing was performed with the Workstation Multi instrument (V. 6.2). The following gradient was applied: initial, 3% B; 5-15 min, 16% linear B; 15-45 min, 50% linear B. The column was then washed and reconditioned.

Determination of ESI-MS parameters

A liquid chromatography ion trap mass spectrometer (Varian 500-MS IT Mass Spectrometer) equipped with an electrospray ion source was used. Samples were submitted in tandem from HPLC to ESI-MS. All MS experiments were performed in the negative mode $[M-H]^{-1}$. Nitrogen was used as the nebulization gas and helium as the buffer gas. The ion source parameters were nebulization voltage 5.0 kV and capillary voltage, and temperature were 90.0 V and 350 °C, respectively. Data was collected and processed with MS Workstation software (V 6.9). Full scan spectra were acquired in the range m/z 50-2000. Samples were first analyzed in full scan mode. MS/MS analyzes were performed on a series of selected precursor ions.

Oleogels development

The oleogels were prepared following the experimental design as in Table 1 by dispersing the percentage (w/w) of EC (3%, 6% or 12%) in vegetable oil and adding the correspondent pomegranate polyphenol amount (0.05%, 0.1% or 2%), to finally heat the solution at the respective temperature (140 °C, 150 °C or 160 °C). The mixture was heated for approximately two hours, in an oil bath inside an oven (Memmert UN30, Germany). Every 30 minutes the temperature of the oil bath was monitored and agitations of approximately 1 minute per glass were performed. When the time was up, they were placed in another incubator at 50 °C for cooling for 40 minutes, then tempered at room temperature (~25 °C) and finally stored in refrigeration (~4 °C) for 24 h.

Optimization of the development of the ethylcellulose oleogel with polyphenols

Table 1 shows the factors and levels that were used in the development of the oleogels. A Taguchi L9 array was used to investigate the effect of these factors in 9 runs, in order to find the combination of elements that gave us the most stable and reliable performance. The L9 orthogonal array was obtained using Statistica 7 software (Statsoft, OK, USA).

Table 1. Factors and their respective levels used to oleogel preparation.

Factor	Level 1	Level 2	Level 3
Ethylcellulose %	3	6	12
pp-polyphenols %	0.05	0.1	0.2
Temperature °C	140	150	160

The experiments were performed combining the factors and levels according to the information presented in Table 2. As a response variable, the percentage of inhibition of lipid oxidation was evaluated. For the statistical analysis of the results, the Statistica 7 software (Statsoft, OK, USA) was used, using the bigger model is better.

Table 2. Experimental matrix L9 of the Taguchi design.

No.	Ethyl cellulose	pp-polyphenols	Temperature
1	1	1	1
2	1	2	2
3	1	3	3
4	2	1	2
5	2	2	3
6	2	3	1
7	3	1	3
8	3	2	1
9	3	3	2

The experimental data were previously transformed using the signal/noise ratio (S/N) using equation 1. In the Taguchi design, the S/N ratio is the ratio of the mean (signal) to the standard deviation (noise), the result is a measure of performance that allows for choosing better levels of control (Minitab LLC, 2022).

$$S/N = -10\log((\sum 1/y^2)/n) \quad \textbf{(Eq. 1)}$$

Where γ represents the experimental value of oxidation inhibition and *n* the number of treatments analyzed. The contribution of each factor was estimated from the analysis of variance (ANOVA) using equation 2.

$$P = \frac{SSi}{SST} * 100 \quad \textbf{(Eq. 2)}$$

Where *SSi* represents the sum of the squares of factor i and *SST* the sum of the total squares.

Determination of lipid oxidation

It has been reported that the amount of unsaturated fatty acids and their degree of unsaturation are the most important factors affecting oxidation and other aspects, such as the position of unsaturated fatty acids in the triacylglycerols and the presence of anti- and pro-oxidants controlling oxidation. As peroxides value and conjugated dienes are the indicators of primary oxidative reactions and are rapid and widespread techniques, they were performed in the present work.

Peroxidation

The presence of peroxides in the oleogels was determined using the AOCS acetic acid-isooctane method for quantification of peroxide values (PV) (AOCS official method Cd 8b-90), expressed in milliequivalents of active oxygen per kg of fat in the sample, that cause the oxidation of potassium iodide under the described working conditions. In a 250 mL flask, 50 mL of acetic acid-isooctane solution were added. Separately, 1g of oleogel was weighed and mixed in the previous solution, stirring constantly for one

minute, using a magnetic stir plate (Corning brand), until the sample was completely dissolved. Then, 0.5 mL of saturated potassium iodide solution was added to the same solution and stirring was continued. After 1 minute, 30 mL of distilled water were added, and the titration of this solution was carried out. A 25 mL burette (Pyrex brand) was used, which was calibrated with sodium thiosulfate and little by little aliquots were added to the flask until the yellow color of the iodide faded. At this point, 0.5 mL of sodium laureate and two drops of starch indicator were added. Titration was continued until complete discoloration of the solution. The results were calculated with equation 3 and reported in milliequivalents of peroxides present per kilogram of oleogel. All treatments were performed in triplicate.

$$IP = \frac{V*N*1000}{P} = mEq/Kg^{-1} \textbf{ (Eq. 3)}$$

Where, *V* is the volume of the spent thiosulfate solution, *N* is the normality of the thiosulfate solution, and *P* is the weight of the sample.

Conjugated dienes

Conjugated dienes determination in the oleogels were made using the methodology of Castro-López et al. (2019) with modifications. This test determines the ability and speed of the formation of substances to inhibit the generation of hydroxylated peroxides in the early stages of linoleic acid oxidation, accompanied by stabilization of the radical state via double-bond rearrangement (electron delocalization), which results in conjugated dienes. These relatively stable compounds absorb in the UV at 235 nm, and their absorption can be measured by spectrophotometric techniques to assess oxidation level (Javidipour et al., 2017). The sample was prepared by mixing 0.6 g of oleogel with 1.5 g of Tween 20 in 8 mL of ethanol and 1.5 mL of water. The mixture was homogenized in a vortex and an aliquot of 250 µL was extracted. Each aliquot was processed fresh as follows: a NaOH solution (1 mL, 0.1 M, in 10% ethanol, v/v) was added to stop the oxidation process; then ethanol (2.5 mL, 10%, v/v) was added to dilute the sample. Then, the absorbance of the samples at 235 nm was measured. Water was used as a blank and was processed in the same way as the aliquots in substitution of the oleogel sample. The percentage of inhibition of lipid oxidation was calculated with equation 4.

$$\text{Inhibition of lipid oxidation (\%)} = [(A - B_{24}) - (A - B_0)/A] \times 100 \textbf{ (Eq. 4)}$$

Where *A* is the absorbance of distilled water (as a control), B_{24} is the absorbance of the oleogel with the antioxidants and B_0 the gel control.

Infrared spectroscopy

In order to acquire information on the effect of pomegranate polyphenol extract at the molecular level in inhibiting the oxidation of oleogels, the infrared spectra of the samples previously selected in the Taguchi model were obtained. The Bruker Vertex 70 model infrared spectroscopy equipment (Bruker Optics, Billerica, MA, USA) was used. Spectras were detected in ATR-transmission mode, using the Vertex accessory, through 32 scans in the λ interval from 400 cm^{-1} to 4000 cm^{-1} with a resolution of 4 cm^{-1}. Spectras were obtained using Opus 7.2 software (Bruker Optics, Billerica, MA, USA). The principle of infrared (IR) spectroscopy is to detect the absorption of light by a compound in the IR region of the electromagnetic spectrum. In order to absorb light, a molecule must have a bond within its structure that can exhibit what is known as a "dipole moment," meaning that the electrons within a bond are not shared equally. When energy is applied in the form of infrared radiation, it causes vibration between the atoms of the molecules,

and when a certain infrared frequency is applied, a natural frequency of vibration of the bonds that make up the molecule is expressed.

Determination of mechanical properties of the oleogels

Mechanical properties of the oleogels were evaluated as a first and fast indicator of texture quality of the materials developed, after the different processes to which were subjected. Determination was made using the compression technique in a TA.HD.plus texture analyzer (Stable Micro Systems, Texture Technologies Corp., Scarsdale, NY). For this analysis, 20 mL of sample was prepared and poured into 40 mL Pyrex beakers. After 24 h of storage at 20 °C, texture analysis was performed using the cylindrical geometry of stainless steel with a diameter of 20 mm and a height of 80 mm (P/20, Stable Micro Systems) to penetrate 30 mm into each tube with sample at a speed of 1 mm/s. The resulting force response was measured using Exponent software (Stable Micro Systems Ltd., 2000). 5 independent measurements were made at room temperature (~20 °C) and the results were statistically analyzed.

Statistical analysis

The results of all the measurements of each analysis were statistically analyzed by means of an ANOVA and the comparison between the respective means, using the STATISTICA version 7 software (Stat Soft, Tulsa, Oklahoma, USA).

RESULTS AND DISCUSSION

Characterization of polyphenols from pomegranate peel

The yield obtained from the extraction of polyphenols from pomegranate peel (pp-polyphenols) was 12%-14% for every 40 g of sample. Its composition was characterized by HPLC (Fig. 1) and the identified peaks were assigned to the corresponding compounds based on comparisons of data previously reported in the literature (P. Aguilar-Zárate et al., 2017; Fernandes et al., 2017; Hernández-Corroto et al., 2019). In Figure 1a 11 peaks can be seen, of which the signals of interest are: the first peak, at 781 m/z that corresponded to punicalin, peaks 2 and 3 at 1083 m/z that were identified as punicalagin α and β respectively and peak 4 at 799 m/z was ellagic acid derivatives. It is important to note that punicalagin was the main compound identified and in the highest proportion in pomegranate peel followed by its derivatives (i. e., punicalin, gallic acid, ellagic acid, and ellagic acid glycosides). These findings were similar to the P. Aguilar-Zárate et al., (2017) and Çam & Hişil (2010). In Fig.1 peaks 5, 6, 7, and 8 were the ones obtained in the least quantity, these correspond to pedunculagin II (ion 785 m/z), galloyl-HHDP-hexoside (ion 633 m/z), acid ellagic-hexoside (ion 463 m/z) and the last peak was not identified. Finally, in peaks 9, 10 and 11 grenatine B (ion 951 m/z), ellagic acid-deoxyhexoside (ion 447 m/z) and ellagic acid (ion 301 m/z) were detected, respectively, in agreement with the studies made by Fernandes et al. (2017), P. Aguilar-Zárate et al. (2017), Fischer et al. (2011) y Kharchoufi et al. (2018). However, due to the low yields obtained with the technique used for the punicalagin separation, it was decided to use the total polyphenols, assuming that punicalagin was the compound with the highest concentration, as shown in Fig. 1.

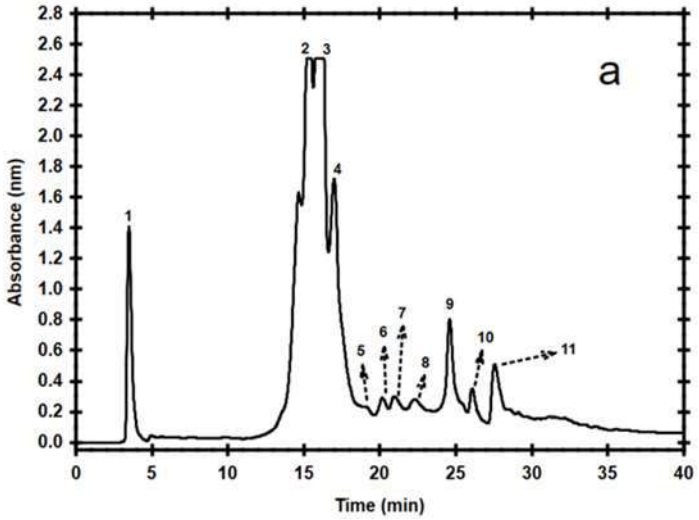

Figure 1. Polyphenols profile identified from pomegranate peel, obtained using HPLC.

Conditions selected to prepare EC oleogels added with pomegranate peel polyphenols

The selection of the adequate conditions to formulate an ethylcellulose (EC) oleogels, added with pomegranate peel polyphenols (pp-polyphenols) as antioxidant agent, was made after analyzing the 9 treatments established in the Taguchi design (Table 2). The results are shown in Table 3, where is observed for each treatment, the percentage of oxidation inhibition, and their respective signal/noise ratio (S/N) resulting from the combination of factors (EC, pp-polyphenols, and temperature). The determination of oxidation in this stage was performed using conjugated dienes technique, because it has been suggested as a better marker of oxidative deterioration than hydroperoxides at higher levels of heat exposure of vegetable oils (Dostálová et al., 2005; Javidipour et al., 2017).

Table 3. Results of the experimental matrix L9 of the Taguchi design on the inhibition of lipid oxidation.

Treatment	Inhibition of lipid oxidation (%)	Signal/noise ratio (S/N)
1	4.00 ± 0.71	11.97
2	30.95 ± 3.37	29.78
3	83.33 ± 6.43	38.40
4	24.62 ± 8.70	27.54
5	29.22 ± 6.46	29.20
6	23.13 ± 1.06	27.28
7	50.81 ± 3.42	34.10
8	79.66 ± 0.00	38.02
9	0.00 ± 0.00	-80.00

The results showed that treatments 3 (3% EC, 0.2% pp-polyphenols, 160 °C) and 8 (12% EC, 0.1% pp-polyphenols, 140 °C) had the best effects with 83% and 79% inhibition of oxidation, respectively. With these treatments, a high value in the signal/noise ratio (S/N) was obtained (Table 3), which implied that the signal is much greater than the random effects of the noise factors (i.e., lipid oxidation). It was also observed that

under the conditions of treatment 3 it was possible to get a better dissolution of the system and consequently a gel with a visually homogeneous consistency was obtained, better than the produced with treatment 8, which had a slightly gritty feel to the touch. It is worth mentioning that for this last treatment a higher concentration of EC was being used and possibly the applied temperature did not allow the gelator completely dissolve during the treatment time, which is why cooling the solution resulted in a system with the consistency that was described above. This gave rise to the oleogel developed under treatment 3 being selected as the best. On the other hand, treatment 9 was the one that had 0% inhibition, this could be attributed to the high concentration of ethylcellulose used to make the gel (i. e., 12%) and to the fact that it was not possible to achieve complete dissolution of this gelator agent (Marangoni, 2013).

Additionally, the optimal conditions were calculated to propose the most favorable results at which an effective inhibition of lipid oxidation was obtained. This was identified using the signal/noise ratio (i. e., the relation between means and standard deviations of the oxidation values). The results shown in Table 4 indicate that the best effect should be obtained using ethylcellulose and pp-polyphenols at level 2 (i.e., 3% and 0.1%, respectively) and at the selected temperature at level 3 (160 °C). These data coincide with the results obtained from the Taguchi L9 experimental matrix, except for the concentration of pp-polyphenols, which in the model turned out to be 0.05%.

Table 4. Effect of the factors in the calculated optimal conditions and in the established conditions.

	a) Optimal conditions			***b) Stablished conditions***		
Factor	**Level**	**Effect**	**Standard error**	**Level**	**Effect**	**Standard error**
Ethylcellulose	2	10.64	15.90	1	9.35	15.90
pp-polyphenols	2	14.97	15.90	2	14.97	15.90
Temperature	3	16.54	15.90	3	16.54	15.90
Expected S/N		59.52			58.23	

Finally, it was decided to establish a level 1 for the EC factor, which corresponded to 3% (Table 4b), because an approximate signal/noise value was obtained with both levels analyzed (Table 4 a and b) and also considering that when using a lower concentration of gelator agent would reduce the cost of the product and avoid possible consequences that could arise from excessive intake of ethylcellulose (Chaves et al., 2018; Co & Marangoni, 2012).

The results of the experimental validation of the lipid inhibition, analyzed by the conjugated dienes technique, indicated that under the established conditions (Table 4 b), 90.4% (± 4.37%) of the oxidation was inhibited. In addition, a signal-to-noise ratio of 48.67 was obtained, which was much lower than statistically predicted (Table 4 a). Additionally, the presence of peroxides, products of the primary oxidation of lipids, in the samples that resulted from the determination of the optimal conditions for the development of oleogels was evaluated experimentally. In these determinations it was found that the peroxide index of an oleogel without and with pp-polyphenols (i. e., 5.41 ± 0.7 mEq/Kg y 5.62 ± 0.4 mEq/Kg, respectively) was statistically equal ($p > 0.05$). It is worth mentioning that the peroxide values that were identified in the developed oleogels are within the limits established in the standards (AOCS Cd-8b-90, 1996) and in

the acceptable quality range for vegetable oils (A. J. Gravelle et al., 2012, 2016). However, Javidipour et al., (2017) have observed that peroxide value is a reliable parameter to evaluate oxidative stability in vegetable oils kept at room temperature but not when they were heated at high temperatures due to the rapid transformation of the peroxides on secondary products. So, this technique could be questionable to be applied in this type of study and it would be necessary to reevaluate and analyze again in future investigations of EC oleogelled systems. Although, the results of the present work it could be said that under the established experimental conditions (Table 4 b) it was possible to obtain oleogels with lower oxidation values, regardless of whether or not pp-polyphenols were added. Other authors have reported favorable results when using phytochemicals as oxidation inhibitors during the development of oleogels. Shi et al. (2014) developed a bigel (mixture of a hydrogel with an oleogel), composed of tea-water/stearic acid-peanut oil polyphenols, in which the formation of peroxides was reduced by 60% by adding only 5% polyphenols to the system, compared to the control made up of peanut oil with stearic acid, where only the formation of peroxides was reduced by approximately 14% over 84 h. It was also shown that the concentration of tea polyphenols in bigel inhibited oxidation 2.5 times more compared to other chemically synthesized antioxidants (i. e., BHT and PG). Besides, Luo et al. (2019) developed an oleogel based on camellia oil structured with particles of tea polyphenol palmitate and citrus pectin and showed that the oleogelation of camellia oil with particles of Tp-palmitate (tea polyphenol-palmitate) and citrus pectin caused an inhibition in oxidation of liquid oil. These authors measured the oxidative effect at high temperature, heating camellia oil for 3 h obtaining its complete oxidation and deterioration, while the oleogel remained acceptable even after heating for 5 h. In this paper, unlike those mentioned above, the studies were carried out after having stored the samples for 24 h at 4 °C and much lower antioxidant concentrations were used (i.e., 0.05% - 0.2%), which are within the range established by the regulations for the use of synthetic antioxidants (i.e., less than 200 ppm) in foods containing fats (U.S. Food and Drug Administration, 2020). It is worth mentioning that Kulkarni et al. (2007) had already reported punicalagin was effective in inhibiting lipid peroxidation of cholesterol, even was twice as effective as the synthetic antioxidant BHT (36.8 ± 0.6 μg/mL and 86.32 ± 1.5 μg/mL of peroxide content, respectively), using concentrations of 10-100 μM/mL. Also, in the study made by Alsufiani et al., (2020) was reported that punicalagin (600 ppm) had similar antioxidant effect than BHT (600 ppm) in canola oil stored during 60 days at room temperature. However, recently the application of some individual natural antioxidants has not been relevant in the edible oil industry, since the separation and purification of an antioxidant compound from a natural extract is not economical, however, these studies provide to the researchers with a better understanding of how phytochemicals act as antioxidants of lipid systems. On the other hand, although they are antioxidants of natural origin, which could be exempt from health risks, it has also been necessary to study whether the addition of polyphenols to edible oils causes alterations in the physicochemical properties of the product that contains them (Davidovich-Pinhas et al., 2014; Gravelle et al., 2012; Gravelle et al., 2014; Zetzl et al., 2014), as a result of its interaction with the molecules that make up the oil. An alternative method to study lipid degradation is FTIR and the investigation of the treated edible vegetable oils revealed that the heating oils caused significant changes in the intensities of their absorption bands and produced no shifts in the position of the bands. These changes have been attributed to the reduction in linoleic (18:2) and linolenic

(18:3) fatty acids content due to the oxidation. These differences are usually observed at 3600-3400 cm^{-1} (stretching mode of –OO-H, due to peroxides formation); 3006 y 1650 cm^{-1} (stretching of -CH in regions *cis* HC=CH y *cis* C=C); between 1800-1700 cm-1(strong vibration of carboxyl groups C = O, due to overlapping of the ester group band of triacylglycerols.) (Barriuso et al., 2013). To verify this, in the present work the infrared spectra of oleogels with and without pp-polyphenols were obtained (Figure 2) and it was observed that there was no difference in the intensity or in the length of the vibrations, that is, in both samples the spectras overlapped in the entire range of wavelengths detected, so it was not possible to conclude any difference in the vibration bands of the functional groups that could reflect the presence of oxidation (Ismail et al., 1993; Ma et al., 2000; van de Voort et al., 1994). This could be attributed to the fact that the samples were analyzed a few hours after preparation (i. e., after 24 hours) and some authors have reported that the presence of oxidation in oils was more easily identified by IR in aged samples (Christy et al., 2003; Rusak et al., 2003). So, there will be needed more detailed FTIR studies to assess the long-term oxidation stability of EC oleogels added with pp-polyphenols and to identify possible synergistic effects between such polyphenols and the components of the oleogel (EC and canola oil constituents fatty acids).

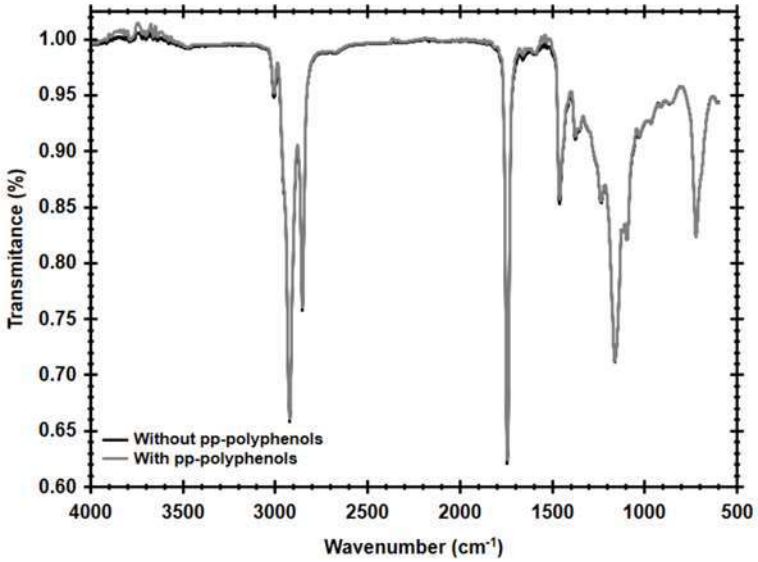

Figure 2. Infrared spectra identified in the oleogel samples with and without polyphenols, obtained at 20 °C by ATR.

Mechanical properties of the oleogels developed

Mechanical properties were identified determining texture parameters (i. e., firmness, consistency and cohesivity) of EC oleogels with and without pp-polyphenols, that were formulated as the selected formulation as the better. The results indicated that both samples analyzed had statistically the same firmness, consistency and cohesivity ($p > 0.05$), independently if was added with pp- polyphenols. This mean that the formulations and processing conditions selected (3% EC, 0.1% pp-polyphenols, 160 °C), and even the degree of oxidation caused during oleogel preparation, did not change the texture quality of the material developed. The importance of determining this properties in structured fats such as oleogels lies in knowing and comparing their mechanical behavior with

that of saturated, hydrogenated or partially hydrogenated fats that are on the market and are used for the industrial or artisanal production of various food products, such as chocolates, bakery products, in the formulation of dairy products, in the preparation of sausages, among others (Davidovich-Pinhas et al., 2016; Rogers et al., 2014; Stortz & Marangoni, 2014; Zetzl et al., 2012).

Table 5. Texture parameters determined in oleogels without and with pp-polyphenols.

Sample	Firmness (g)	Consistency (g.s)	Cohesivity (g)
Without pp-polyphenols	390.28 ± 144.32[a]	4614.07 ± 1619.39[a]	-59.98 ± 7.55[a]
With pp-polyphenols	456.24 ± 46.94[a]	5012.48 ± 338.33[a]	-63.13 ± 1.35[a]

Superscript letters in each column indicate that the samples are statistically equal ($p > 0.05$).

CONCLUSIONS

In this study, under the selected temperature treatment (160 °C) and the addition of pomegranate peel polyphenols (0.05-0.1 %) as antioxidant to prepare oleogels with 3% of ethylcellulose, the primary lipid oxidation was maintained between the limits permitted by the standards and in the acceptable mechanical properties. It is likely that in order to alter the quality of these oleogels through lipid oxidation, it is necessary to storage the systems for long term periods of time, greater than the 24 hours that were applied in this study. As future research, it would be convenient to complement this work with the study of the effect of pp-polyphenols in ethylcellulose oleogels on lipid oxidation during the storage time. In addition, other detailed studies to explain the possible synergism between pp-polyphenols and the oleogel components are required to complement and understand the origin of the behaviors since molecular level to the macrostructure of the system, and finally this could permit to suggest possible applications of the EC oleogels developed and the use of the pp-polyphenols as natural antioxidants for this type of structured oily systems.

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