Chapter

Development of Nano-Emulsions of Essential Citrus Oil Stabilized with Mesquite Gum

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Abstract

The use of nano-emulsions has great advantages over conventional macro-emulsions since the small droplet size allows to expand the options of applications besides presenting a greater surface area. This chapter focuses on the formulation of nano-emulsions of citrus essential oils in water, stabilized with a natural gum (mesquite gum), using a high pressure microfluidic homogenizer to obtain appropriate physicochemical characteristics and kinetic stability. When establishing the general conditions of the methods for obtaining nano-emulsions by high pressure homogenization, several formulations presented stability and size corresponding to nano-emulsions, and these were monitored during 4 months in order to study their stability as a function of time. Taking into account the results of size and stability, the best nano-emulsion obtained had a composition of Persian lemon oil (9.86%), mesquite gum (4.93%) Tween 80 (4.89%), Span 20 (1.45%), and deionized water (78.86%) with an average droplet size of 40 nm. In addition, the antibacterial activity studies also showed that this formulation had the best performance against common bacteria such as *Staphylococcus aureus* and *Escherichia coli*. The analysis of the minimum inhibitory concentration (MIC) shows that it is possible to prevent the growth of these particular bacteria using 6.25% of the best nano-emulsion formulations.

Keywords: nano-emulsions, essential oil, mesquite gum, high pressure microfluidic homogenizer, antibacterial

1. Introduction

1.1 Nano-emulsions

Nano-emulsions can be defined as ternary systems composed of nano-sized droplets (20–500 nm) dispersed in a continuous phase and stabilized by amphiphilic molecules known as surfactants [1–4]. The nature of the droplets and continuous phase can be aqueous or oily, forming oil-in-water (O/W) or water-in-oil (W/O) nano-emulsions. Regarding surfactants, these molecules must possess a

double affinity, a hydrophilic polar head and a hydrophobic non-polar tail, for example. The surfactant allows decreasing the oil/water interfacial tension (e.g. ~5–10 mNm^{-1}), leading to formation and dispersion of very small droplets. The decrease in interfacial tension can be enhanced by the addition of a co-surfactant, which acts in similar way than surfactants, but is added in a much lower content. The singularity of nano-emulsions is its small droplet size range, kinetic stability, and the lack of surfactant (5–15% is usually needed) compared to other emulsion systems such as microemulsions [5, 6]; but, in order to be formed they require an external stimulus (being kinetically stabilized) [7] as:

a. Mechanical input: applied with a special equipment such as ultrasonic probe (UP) or high pressure homogenizer (HPH). In a typical HPH (**Figure 1**), a pump pushes a pre-formulated macroemulsion through a narrow gap, a microfluidic interaction chamber (in the micrometer range), where the large droplets break into smaller droplets due the extreme elongation and shear stresses through the homogenization process. This step can be repeated multiple times until the droplet size becomes constant. On the other hand, the UP can induce the breaking of pre-formulated macroemulsion droplets by cavitation, which can also be repeated numerous times. The size and polydispersity of these droplets depends of the applied energy through the whole nano-emulsification process, as well to the water/oil/surfactant ratio [1, 5].

b. Physicochemical input: requires a change of temperature (PIT: phase inversion temperature method) or the addition of an extra amount of dispersed phase (PIC: phase inversion composition). In the first case, the increasing or decreasing of temperature can induce a phase transition (e.g. O/W ➔ W/O), due the change of the hydrophilic–lipophilic balance (HLB) of the system. In the second case, the increment of the droplet constituent can lead to the dispersed-phase/continuous phase ➔ continuous-phase/dispersed phase transition [8, 9]. Both approaches are based on phase changes that pass through a zero-curvature system such as a bicontinuous microemulsion or a lamellar phase, in order to facilitate phase inversion [3].

Compared with mechanical methods, physicochemical strategies are considered low-energy methods (~103 Wkg^{-1} vs. ~1010 Wkg^{-1}); however, they need

Figure 1.
Typical high pressure homogenizer equipment.

very detailed phase behavior studies, which are time consuming. For this reason, high-energy methods may be preferred. Regarding applications, nano-emulsions can be employed in a wide range of fields such [10]: pharmaceutical, cosmetic, automotive and food industry, depending on their physicochemical properties. In this study, citric oil droplets were formed and dispersed in an aqueous continuous phase, using polysorbates as surfactants and a natural gum (mesquite), through an assisted HPH method. The chemistry of the citric oil and nano-emulsion stability allows the application of the formulated systems for antibacterial applications against bacteria such as *Escherichia coli* and *lactobacillus delbrueckii* at studied conditions.

1.2 Polysorbate surfactants

Tween 80 and Span 20 (**Figure 2**) belong to the polysorbate family, a non-ionic type of surfactants. Chemically, polysorbates are derived from ethoxylated sorbitan, a derivative of sorbitol, which is esterified with fatty acids. Tween and Span are proprietary names from CRODA™ (manufacturer of specialty chemicals); the numeric values are specific to the fatty acid derivative: oleic acid, for Tween 80, and lauric acid, for Span 20. Both surfactants are frequently used as emulsifiers for the food and cosmetic industry, having a very low toxicity and eco-friendly chemistry [11–13]. However, the affinity for polar (water) and non-polar (oil) groups is different for each non-ionic surfactant; according to the hydrophilic–lipophilic balance scale (HLB) [14], Tween 80 is hydrophilic (HLB: 15), while Span 20 is more lipophilic (HLB: 8.6).

1.3 Mesquite gum as emulsifier

Mesquite gum is a vitreous exude, produced by mesquite tree (*Prosopis laevigata*), a widely distributed plant across arid and semiarid zones. This gum is composed of a highly tailored polysaccharide salt, constituted by residues of L-arabinose, D-galactose, 4-O-Methyl-D-glucuronic acid and L-rhamnose (**Figure 3**) [15, 16]. Mesquite gum chemical composition is similar (different molar ratio) to that one of Arabic gum, which is commonly employed in the food and pharmaceutical industry, due its emulsification capacity [17, 18]. In México, mesquite gum is only consumed as a candy, therefore there is a wield field for exploration of this product as emulsifier.

1.4 Citric oil nano-emulsions

A natural antibacterial extracted from plant or fruit origin is the essential oil, many studies have described this effect [19–21]. Pink Grapefruit

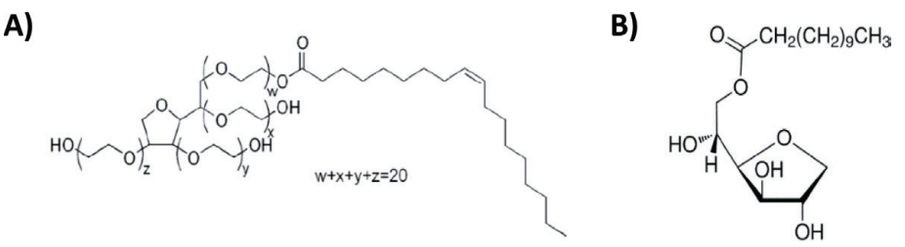

Figure 2.
(A) Tween 80 and (B) Span 20 chemical structures.

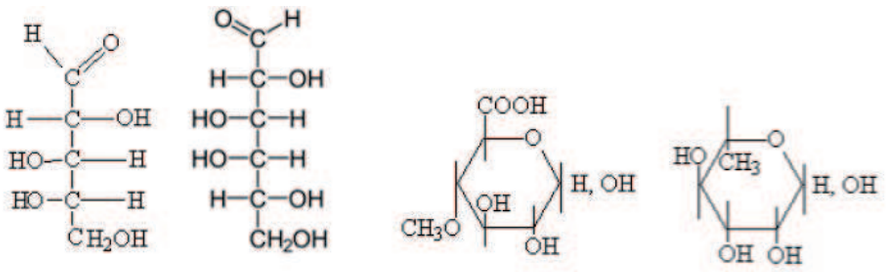

Figure 3.
Main constituents of mesquite gum (from left to right): L-arabinose, D-galactose, 4-O-Methyl-D-glucuronic acid and L-rhamnose.

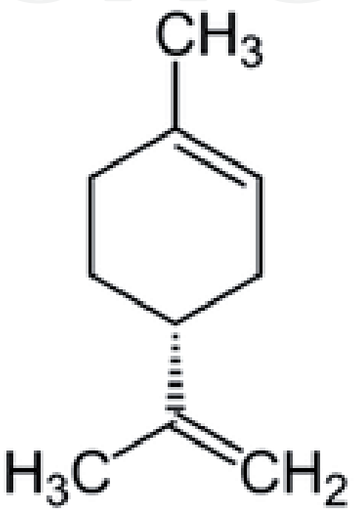

Figure 4.
D-limonene chemical structure.

(*Citrus paradise*) belongs to the Citrus genus, a class of flowering plants in the family Rutaceae [22]. The active constituents exist in this kind of citrus essential oil, such as limonene, a-pinene, b-pinene and a-terpinolene [22, 23]. Its seed and peel extract (essential oil) has shown the antibacterial and antifungal property [24]. Persian lemon (*citrus latifolia tanaka)* is composed of citric, malic and formic acids, as well from pectin, hesperidin, and essential oils as D-limonene (**Figure 4**) and phellandrene, which are the volatile liquid fractions responsible for the characteristic smell of lemon. These compounds can be extracted by distillation, and are commonly employed in the cosmetic, pharmaceutical and food industry [25].

As part of a nano-emulsion, D-limonene has been used as the oil phase of different O/W nano-emulsions. For instance, in the work of Li and Chiang, who have successfully formed D-limonene O/W nano-emulsions phase by an ultrasonic method, using Span 85 and Tween 20 as surfactants [26]. Another example is the work of Donsì et al. [27], who achieved D-limonene nano-emulsions formation through a high pressure homogenizer, using a wide type of emulsifiers. The latter authors have explored the antibacterial properties of these systems by testing against *Escherichia coli* and *lactobacillus delbrueckii*, demonstrating D-limonene nano-emulsion capacity to control and eliminate microbial organisms [27, 28]. As these studies, there are some other investigations about D-limonene nano-emulsions, but none of such studies explores the formulation with a natural emulsifier as mesquite gum.

2. Analysis of the components of a nano-emulsion

The first stage of the research consists of the characterization of the raw materials (gums and citric essential oils) used in the formulation of nano-emulsions.

2.1 Mesquite gum purification

Mesquite gum was extracted from mesquite pearls obtained from a local candy store at Sonora (Sonora, México). The purification process of the mesquite gum begins with the selection of the cleanest pearls by visual inspection as indicated by literature [17]; afterwards the pearls were ground in a ceramic mortar until a powder was obtained. Then a 20% (wt) solution of this powder was prepared, which was filtered (in order to eliminate impurities like dust and pieces of wood), details of the process can be found in literature [12].

Afterwards, several drying processes were experimented, specifically oven and lyophilization. The drying process by oven was discarded since it was very slow, and in addition, the gum obtained was of a brown tone, indicating a possible degradation of the gum or caramelization. Regarding the lyophilization method, several strategies were tested. First, it was tested with a lyophilized sample at 20%, and a gum with a "sponge" texture was obtained, later this "sponge" gum was re-lyophilized in order to obtain a denser version. Therefore, it was decided to test lyophilized 40% mesquite gum solutions in a controlled manner, this last process was selected to be used for the production of gum for the nano-emulsions of this research.

2.2 Infrared spectrum analysis (FT-IR) of the gums

To carry out this analysis, the Thermo Nicolet 6700 FT-IR spectrophotometer with its attenuated total reflectance accessory (Thermo Scientific) was used, using solid samples (powders).

Figure 5 shows the FT-IR spectra of the different gums characterized in this research, which are largely comparable with the FT-IR spectrum of Arabic gum (GA) reported in the literature [17, 18]. Therefore, it follows that the different mesquite gums produced (oven dried procedure, dense lyophilized and controlled Lyophilized) essentially have the same chemical composition in terms of sugars, amino acids and proteins. Absorbance of the —OH and —CH groups are observed at 3375 and 2932 cm^{-1} (similar to that reported in the literature [17]), and a band centered between 1650 and 1600 cm^{-1} that can be assigned to the primary amides. There is a smaller band around 1500 cm^{-1} that is assigned to the secondary or substituted amides. The bands of the primary and secondary amides are characteristic of the presence of peptide bonds and confirm the presence of the protein [17], There is also a band at 1400 cm^{-1} that can be attributed to a carboxylic group. The bands that are around 1000 and 900 cm^{-1} can be attributed to the glycosidic acetal groups of pyranose, according to the literature [17].

2.3 Concentration of aldehydes of citric essential oils

The citrus essential oils used during this research were a generous donation from the FRUTECH company. The determination is made by the hydroxylamine hydrochloride method (ISO 1279: 1973). A Titroline Alpha plus automatic titrator from SI Analytics was used to obtain the aldehyde concentration of the citrus oils of pink grapefruit and Persian lemon. Four grams of oil are weighed in a beaker, 50 mL of hydroxylamine hydrochloride solution are added, stirring for 1 minute at 300 rpm, and then it was allowed to rest for 30 minutes. Subsequently, a titration

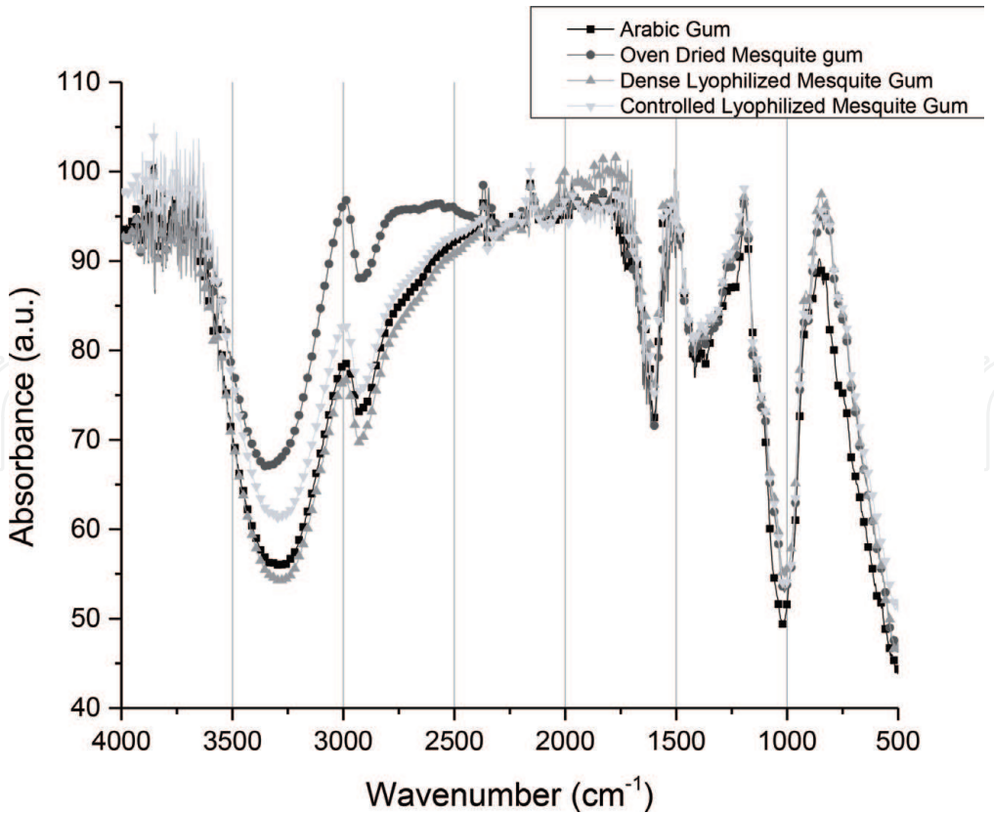

Figure 5.
IR spectra of the different gums.

with methanolic KOH is performed and the amount of mL used is recorded to reach a pH of 3. In order to determine the concentration of aldehydes, the following mathematical formula is used:

$$\%carbonyl\ compounds = (a \times N\,meq \times 100)/P$$

where

a = Volume of the potassium hydroxide solution used in the neutralization of the sample in mL.

N = Normality of the potassium hydroxide solution.

P = Weight of the sample, in grams.

meq = Milliequivalent corresponding to the carbonyl compound in which the result is expressed.

Aldehydes are a family of organic compounds (R—CHO), which are indicative of the quality of essential oils, the higher the concentration of aldehydes, the higher the oil quality [29]. The released HCl is evaluated, which is related to the content of carbonyl groups in the sample and it can be calculated in grams of the aldehyde. The results are shown in **Table 1**, these are within the expected ranges (For Persian lemon is 3.5–7.5 and 0.8 1.5% for pink grapefruit) according to literature [28].

Citric essential oil	%pH	mL	%Aldehydes
Persian lemon	3.89	9.96	3.67
Pink grapefruit	1.07	2.75	1.04

Table 1.
Concentration of Aldehydes.

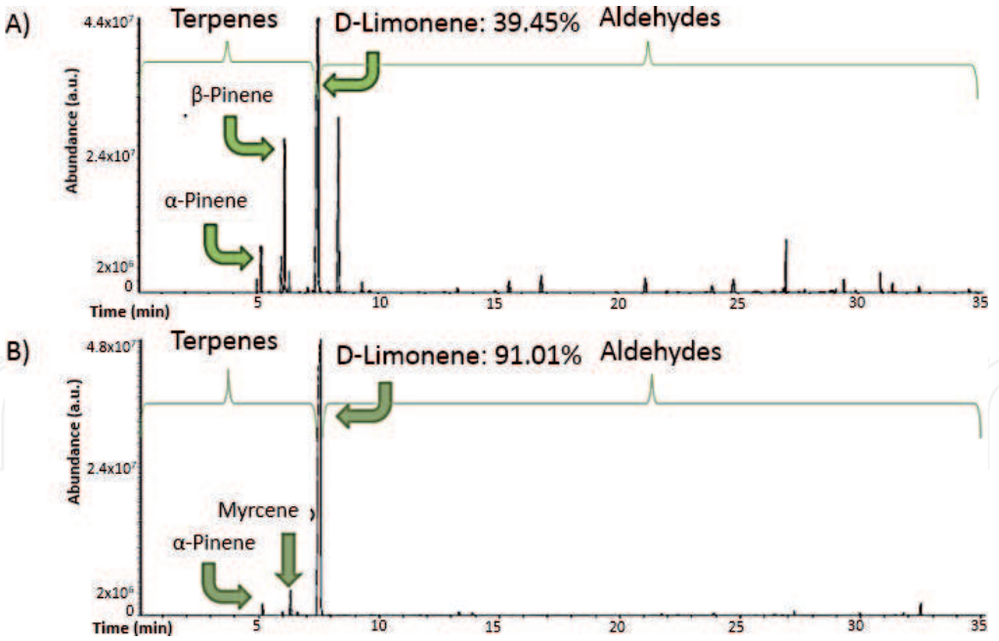

Figure 6.
Chromatographic profile of the sample analyzed: (A) Persian lemon oil and (B) pink grapefruit oil.

It can be seen that Persian Lemon oil has a concentration of aldehydes 3 times greater than pink grapefruit oil, which is why it was a determining factor for the selection of Persian lemon oil as an oil phase. It should be noted that aldehydes are the components with the highest added value since they provide most of the odor, taste and therapeutic qualities, and are therefore the most important components. In addition, some authors attribute the antibacterial activity to aldehydes, although there is controversy in this aspect since other authors give this property to terpenes [30–32].

2.4 Volatile profile by gas chromatography of citric essential oils

The analysis was carried out in a gas chromatograph model 7890ª coupled to a Mass Spectrometer (Agilent Technologies). For the analysis 2 μL of each oil sample is injected, the column HP-5MS (Agilent Technologies) is responsible for passing or retaining each compound of citrus essential oils and uses Helium as a carrier gas at a temperature of 280°C and the Wiley library is used as a database for the identification of each component. This analysis allows the separation and identification of the components of the essential oils. Terpenes have a lower retention time than aldehydes, so this method is used to corroborate the concentration of aldehydes.

As observed in the chromatograms (**Figure 6**) D-limonene is the signal with more abundance in the citrus essential oils used in this research and is a component widely used in microbial inhibition in the food, pharmaceutical and cosmetic industry [33, 34].

The mechanism of the antibacterial function of essential oils is still not detailed, according to the literature [35]. As mentioned earlier there is a controversy regarding antibacterial activity and the relationship with aldehydes and terpenes. As we can see in **Figure 6**, Persian lemon oil has a higher concentration of aldehydes.

3. Methodology for the formulation of nano-emulsions of citrus essential oils

A reproducible process for the formulation of nano-emulsions of essential citrus oils is described. The initial process of preparing the nano-emulsions consists

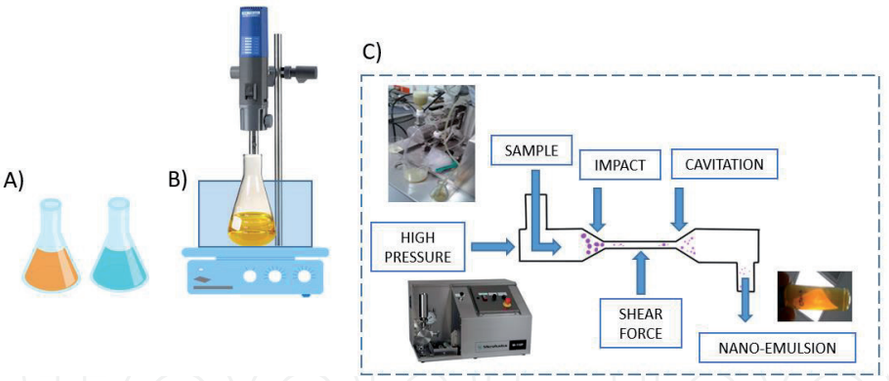

Figure 7.
Process for the formulation of nano-emulsions (A) oil and water phase of nano-emulsions, (B) preparation of pre emulsion by high speed agitation, (C) high pressure process.

of mixing the components in an Erlenmeyer flask using magnetic stirring in a water bath at 40°C for 10 minutes. Followed by high speed mechanical agitation (8000 rpm) for 5 minutes. This first step helps to dissolve the gums and/or surfactants in the aqueous phase as much as possible, and thus it eliminates any possible lumps that could lead to plugging in the microfluidizer interaction chamber. In addition, this step allows to form a pre-emulsion, that is, the oil phase is dispersed as droplets in the aqueous continuous phase; however, at this point, the droplets are micrometric in size and therefore the pre-emulsions have a milky appearance.

This pre-emulsion is then introduced into the high pressure homogenizer (microfluidizer), and subjected to high pressure (the nano-emulsions were subjected to pressures ranging from 10,000 to 30,000 psi) collecting a sample every 1, 3, 5 and 10 laps. In this high pressure process, the droplet size of the nano-emulsion decreases as the number of times the nano-emulsion is introduced to the equipment increases (number of turns or laps or steps), although sometimes the droplet size increases again when the number of laps increases up to a certain value, due to degradation of gums or surfactants. Finally, the nano-emulsion sample is collected and prepared to be sterilized. In **Figure 7**, a summary of the process is presented.

After a series of experiments varying concentrations, applied pressure and number of laps, it was possible to obtain a visually appropriate formulation with relative stability, verified by the characterizations. Therefore, comparison controls are generated, which are described in **Table 2**, replacing mesquite gum with Arabic gum, Tween 80 and Span 20 surfactants, and finally the substitution of mesquite gum for deionized water. These controls were defined in this way to investigate if mesquite gum would have an influence on the characteristics and kinetic stability of the nano-emulsion. Samples of these controls were taken at 1, 3, 5 and 10 steps, **Table 2** shows the samples that resulted in the best size distribution and best visual appearance.

Experiment	Laps	Oil phase	Span 20	Tween 80	Variable	Deionized water	PSI
Delta control	10	9.86%	1.45%	4.89%	Mesquite gum: 4.93%	78.86%	20,000
Control 2	3	9.86%	1.45%	4.89%	Arabic gum: 4.93%	78.86%	20,000
Control 3	10	9.86%	1.45%	4.89%	Span 20: 1.12% + Tween 80: 3.81%	8.86%	20,000
Control 4	5	9.86%	1.45%	4.89%	Deionized water	83.76%	20,000

Table 2.
Details of the formulation of nano-emulsions.

4. Characterization of nano-emulsions

To demonstrate which formulation leads to an optimization of the use of the microfluidizer and natural gums in the formation of these nano-emulsions, it is necessary to evaluate each system previously described in **Table 2**.

4.1 Dynamic light scattering

When comparing these controls against the Delta Control nano-emulsion in **Figure 8** it is observed that Control 3 with 10 turns in the homogenizer presents a smaller size, in this case a single population with a size of 19 nm, but it has a higher polydispersity index (PDI of 0.143). This is attributed to its composition based solely on surfactants Tween 80 and Span 20 with high HLB. On the other hand, Control 4 nano-emulsion, which does not contain gum, and only carries 5% of surfactants Tween 80/Span 20, had a droplet size of 25.3 nm, a lower size than Control Delta but larger than Control 3. With these experiments, it can be implied that the components responsible for the small drop size are the surfactants Tween 80 and Span 20, since greater interfacial activity with the presence of these surfactants was expected. In addition to increasing the concentration of these surfactants (Control 3) produces a greater interfacial area and a smaller drop size. Finally, it is observed that the Control 2 experiment (control using Arabic gum instead of mesquite gum), has a droplet size of 46.8 nm, very similar to Control Delta, which shows that the mesquite gum, in effect, has a very similar performance to that of Arabic gum (in terms of the droplet size). It should be noted that Control Delta nano-emulsion (with mesquite gum) has a narrower size distribution than Control 2 (with Arabic gum).

A small initial droplet size is not a guarantee that the kinetic stability will be better compared to nano-emulsions with a larger droplet size. For this reason, nano-emulsions were monitored in order to see if there was an increase in the droplet size or an increase in the number of populations, which would indicate instability or some other problem such as cremation, sedimentation, flocculation, or some change in coloration or appearance, which would reduce shell life. All the samples were refrigerated at 4°C and were wrapped in aluminum foil in order to prevent the citrus essential oil from oxidizing in the presence of light.

The first monitoring study to be discussed is Control Delta (**Figure 9**), which represents the best formulation that includes mesquite gum. It was observed to be stable for 4 months. The PDI varied between 0.071 and 0.091 and the sizes vary from 41 to 46 nm, therefore it is considered to be a very stable nano-emulsion; besides, its appearance including color did not change.

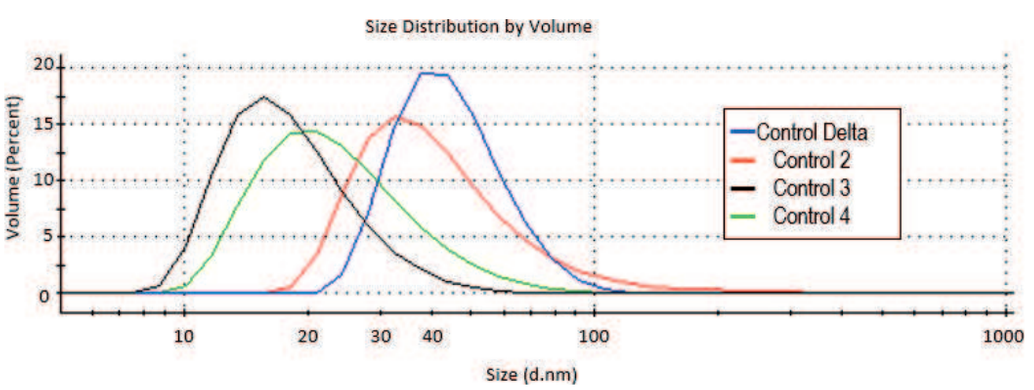

Figure 8.
Graph of volume size distribution in DLS of nano-emulsions.

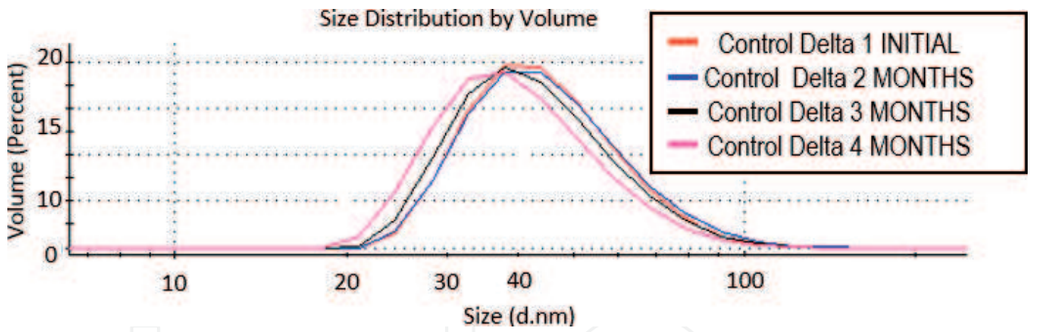

Figure 9.
DLS graph of the 4-month follow-up of Delta Control.

Therefore, a certain percentage of the droplets increased slightly in size, however, there appeared to be no coalescence since the size remained almost constant. On the other hand, the fact that no additional larger populations were produced, unlike the other controls, as shown below, is indicative of the appropriate steric stabilization conferred by mesquite gum.

The next control that was evaluated is the one that incorporates Arabic gum instead of mesquite gum (control 2). From the first series evaluated on this research, we have seen that the nano-emulsions that incorporate Arabic gum developed during this research do not present a very good stability performance, this is confirmed with the results shown in **Figure 10**, where there appears to be an apparent gradual reduction of the droplet size (~25 nm) but with an increase of the PDI from 0.115 to 0.247, to finally increase again at the fourth month (droplet size of ~35 nm). The greatest sign of instability is the presence of other populations with a size around 3 μm that occurs in parallel to the apparent reduction in droplet by the second month, which is attributed to the phenomenon of Ostwald ripening [36], which is one of the main mechanisms of instability in nano-emulsions. This experiment confirms that mesquite gum has advantages over Arabic gum in the formulation of the nano-emulsions of Persian lemon oil. The mesquite gum confers a better steric stabilization as compared to Arabic gum, improving the kinetic stability of the nano-emulsion.

In the follow-up of Control 3 shown in **Figure 11** (nano-emulsion with excess of Tween 80 and Span 20, and without mesquite gum), it seems to be a very stable nano-emulsion, however with each measurement the droplet size, the presence of larger size populations and the PDI increase. For example, from the second month, it evolves from having a single population of 19.0 nm, to having the population of 20.7 nm in coexistence with populations of 467 and 5033 nm, which do not occur in Control Delta. Therefore, it is deduced that the presence of the mesquite gum helps to maintain the stability of the nano-emulsions, providing an additional steric stabilization against coalescence.

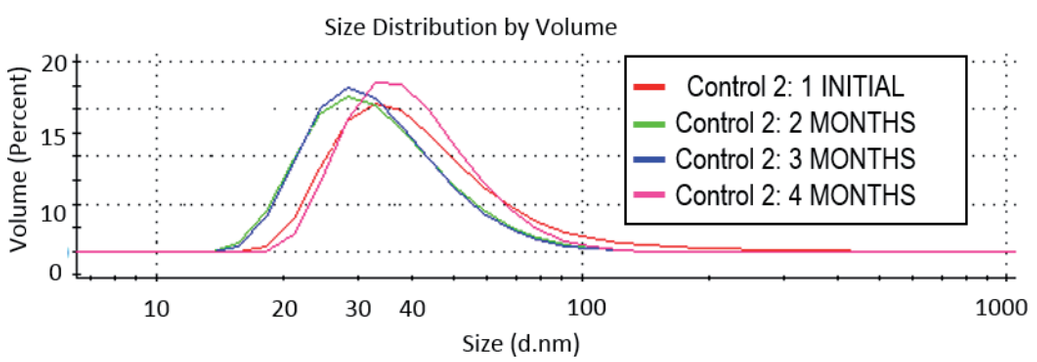

Figure 10.
DLS graph of the 4-month follow-up of Control 2.

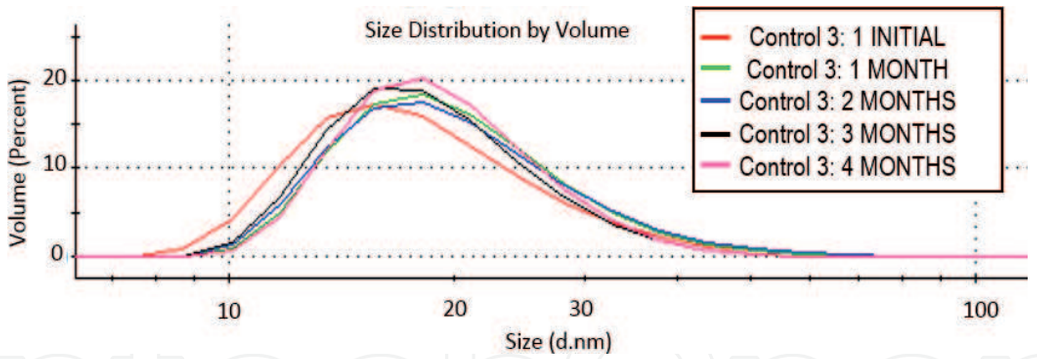

Figure 11.
DLS graph of the 4-month follow-up of Control 3.

Control 4 nano-emulsion incorporates only 5% of surfactant, a mixture of Tween 80/Span 20, and mesquite gum was not included, therefore its water content increases to 85% (as mesquite gum is replaced by water). This control was formulated in order to verify if there is any effect on the size of the nano-emulsion and its stability with the presence of the mesquite gum. This is observed in **Figure 12**, where first, apparent reductions followed by increases in the size of the droplets are seen (droplet size range from ~15 to ~20 nm with PDI range from 0.165 to 0.453), with the presence of large droplet populations, with size around 2000–4000 nm by the fourth month. This may be attributed to a combination of Ostwald ripening and coalescence destabilization phenomena.

In general, the results from the experiments described above showed that nano-emulsions without mesquite gum result in droplet sizes which are smaller than those obtained with samples that include mesquite gum in their formulation, but better kinetic stability and smaller PDI are obtained with the nano-emulsions that contain mesquite gum. This is attributed to the additional steric stability conferred by mesquite gum, which results it a better kinetic stability, since there is virtually no droplet size growth. An arrangement of the different surfactants and stabilizers at the droplet interface is proposed in the scheme of **Figure 13**. It is proposed that the larger hydrodynamic size obtained with mesquite gum is due to its location at the surface of the droplet, where carbohydrate chains of the gum can interact with the sorbitan groups and EO groups of Tween 80.

4.2 Minimum inhibitory concentration

Next, the results of the antibacterial activity tests of Control Delta, Control 2, Control 3 and Control 4 nano-emulsions are shown (in this study, each

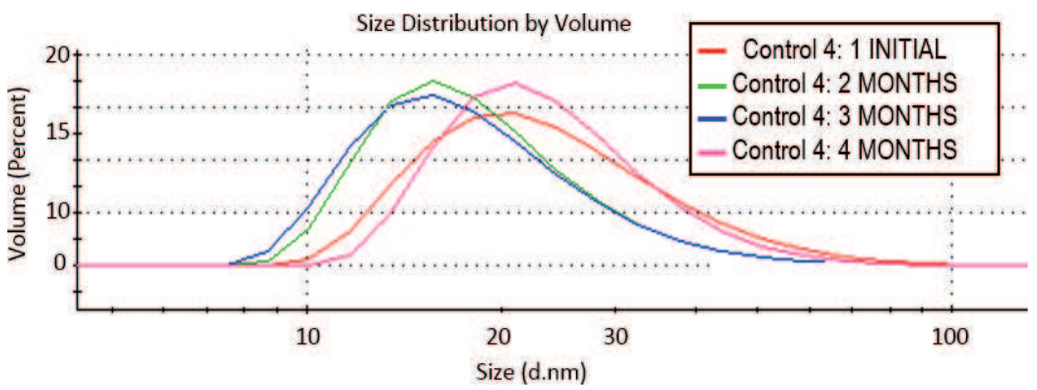

Figure 12.
DLS graph of the 4-month follow-up of Control 4.

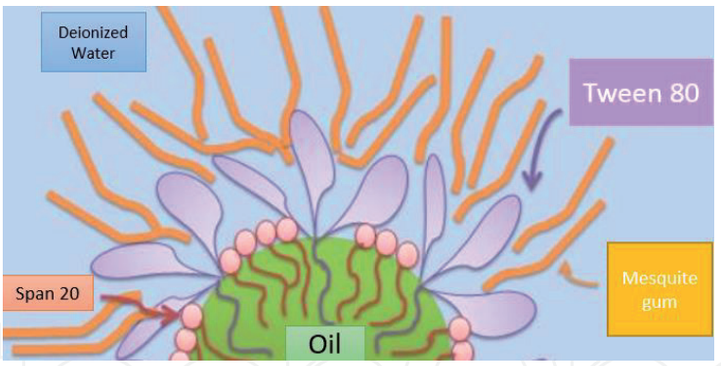

Figure 13.
Schematic of the proposed arrangement of surfactants and stabilizers at the interface of the nano-emulsion droplets.

nano-emulsion was evaluated at 1, 3, 5 and 10 steps into the microfluidizer). To determine the MIC (minimum inhibitory concentration) of the nano-emulsions against the test organisms *Escherichia coli* and *Staphylococcus aureus* the broth micro-dilution method was used as recommended by the National Committee for Clinical Laboratory standards. This test was performed in sterile 96-well microplates. The nano-emulsions were properly prepared and transferred to each microplate into two lines in order to verify reproducibility. The inoculate (10 μL) containing 5x105 CFU (colony-forming unit) of each microorganism was added to each well. A number of wells were reserved in each plate to test for sterility control (no inoculate added), inoculate viability (no nano-emulsion added), and the nano-emulsion inhibitory effect. Plates were aerobically incubated at 35°C. After incubation for 18–24 h, bacterial growth was evaluated by the presence of turbidity and a pellet formed at the bottom of the well. MIC was defined as the lowest concentration of nano-emulsions that had no macroscopically visible growth. A sterilization process was applied to the nano-emulsion samples prior to MIC studies, in order to ensure that no previous contamination was present in the samples.

Table 3 shows the MIC results for nano-emulsions sterilized during 40 minutes under UV light corresponding to *Escherichia coli* and **Table 4** shows the MIC results corresponding to *Staphylococcus aureus*. Additionally, a nano-emulsion with the same composition and processing of Control Delta was prepared, but replacing the Persian lemon essential oil with industrial D-limonene, since this component could be the active bactericidal component.

In general, the best result of MIC was obtained with *Staphylococcus aureus*. The delta control nano-emulsion resulted in a MIC of 6.25% for both bacteria. Therefore, with these results it was confirmed that the nano-emulsions of Persian lemon oil developed under the method described in this research have an antibacterial effect against *Staphylococcus aureus* and *Escherichia coli*.

Nano-emulsion	Steps in Homogenizer	MIC (% of concentration of the nano-emulsion)
Control Limonene	All steps	25
Control Delta	All steps	6.25
Control 2	All steps	25
Control 3	1, 3, 5 10	6.25 12.5
Control 4	All steps	6.25

Table 3.
MIC results for nano-emulsions sterilized during 40 minutes. Bacterium used: ***Escherichia coli.***

Nano-emulsion	Steps in Homogenizer	MIC (% of concentration of the nano-emulsion)
Control Limonene	All steps	25
Control Delta	All steps	6.25
Control 2	All steps	25
Control 3	1	1.56
	3	3.12
	5	6.25
	10	12.5
Control 4	1, 3	3.12
	5, 10	6.25

Table 4.
MIC results for nano-emulsions sterilized during 40 minutes. Bacteria used: ***Staphylococcus aureus****.*

Considering that the nano-emulsions contain 10% Persian lemon oil, the MIC of this essential oil could be considered as 0.625% for both *Staphylococcus aureus* and *Escherichia coli* and (taking into account the composition of Control Delta samples). Considering the best results, we have a MIC of 0.625% for Control Delta, Control 3 and Control 4 for *Escherichia coli* and 0.156% for Control 3 and *Staphylococcus aureus*; it is inferred then, that these nano-emulsions (Control 2, 3 and 4), which present smaller droplet sizes, their antibacterial power can be attributed to a greater interfacial area, since there is a greater contact area between Persian lemon oil and bacteria. However, due to the better kinetic stability of Control Delta nano-emulsion, it is considered as more promising. Some adjustments could be made to improve these results, such as increasing the concentration of Tween 80/Span 20 in Control Delta in order to reduce the size, but maintaining the presence of mesquite gum in order to preserve the steric stability conferred by it.

When carrying out the antibacterial activity tests, it was observed that when the nano-emulsions were subjected to treatment with UV light, they became slightly more transparent, so it was suspected that the UV light radiation can cause a reduction in the droplet size. In **Figures 14** and **15**, we observe the effect of the UV light treatment on the two nano-emulsions with better kinetic stability behavior and droplet size. In the case of Control 3 nano-emulsion at 10 steps (formulation without mesquite gum, but with additional Tween 80 and Span 20), there was no significant reduction in size (from 1 to 0.5 nm reduction), after sterilization treatment at different exposure times. However, for Control Delta nano-emulsion there was a reduction of approximately 10 nm after treatment with UV light, which may indicate that the mesquite gum is being broken into smaller carbohydrate chains, or

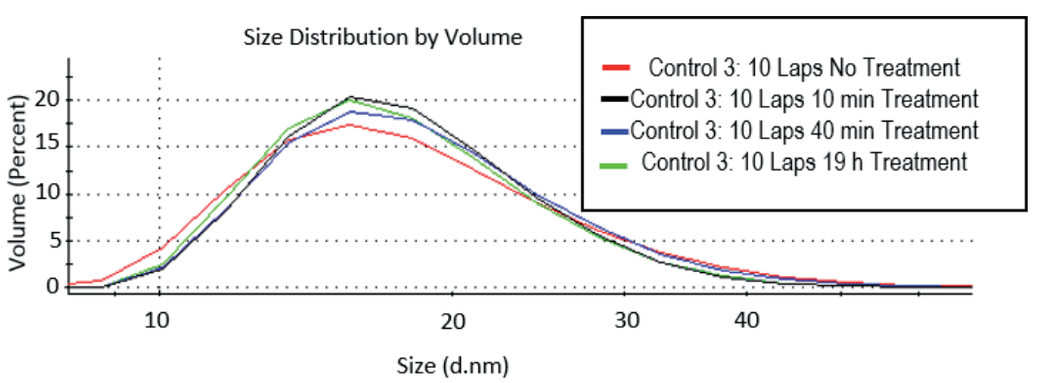

Figure 14.
Size distribution chart by volume of control 3 10 steps with UV treatment.

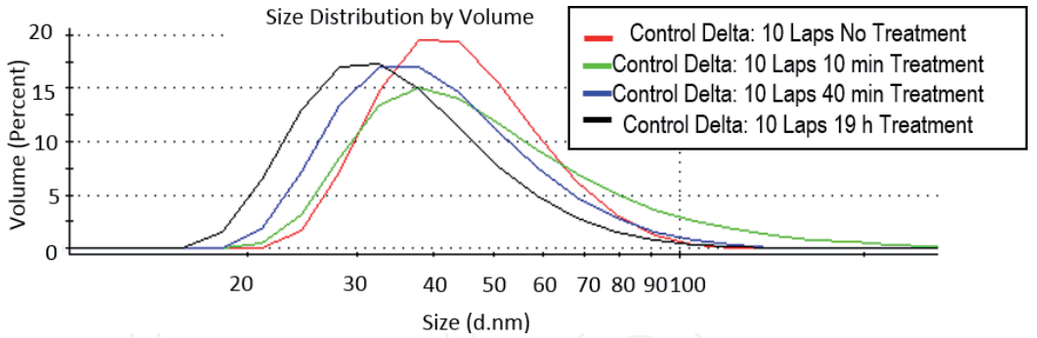

Figure 15.
Size distribution graph by volume of control delta 10 steps with UV treatment.

that rearrangement of the carbohydrate chains is taking place, thereby reducing the hydrodynamic droplet size.

On the other hand, although the literature indicates that it is not that clear which of the components of the citrus essential oils is the cause of the antibacterial effect, the nano-emulsion with industrial D-limonene results in a higher MIC than Control Delta which is prepared with Persian lemon oil; thus, it may be inferred that the aldehydes of the Persian lemon oil could be the components that are mainly responsible for this effect, as compared to the terpene components.

4.3 Volatile profile by gas chromatography of nano-emulsions

After the analysis of the previous results was carried out, it may be inferred that the aldehydes present in the Persian lemon oil may be the components that potentiate the antibacterial power. Nano-emulsions of Control Delta (Combination of Surfactants—Span 20: 1.45%, Tween 80: 4.9%, Mesquite Gum 4.9%), both unsterile and sterilized (with 10 and 40 minutes of UV light treatment) were investigated in order to perform a profile analysis of the volatiles by gas chromatography. As observed in **Figure 16**, when the nano-emulsion Control Delta was exposed to different UV sterilization times, the D-limonene signal becomes smaller in comparison with the nano-emulsion sample that was not exposed to UV, so it can be implied that the D-limonene component does not appear to be the main component that acts as an antibacterial agent in this particular sample.

Once the retention time of D-limonene passes (**Figure 17**), the region of the aldehydes begins at higher retention times, and as observed in the previous analysis

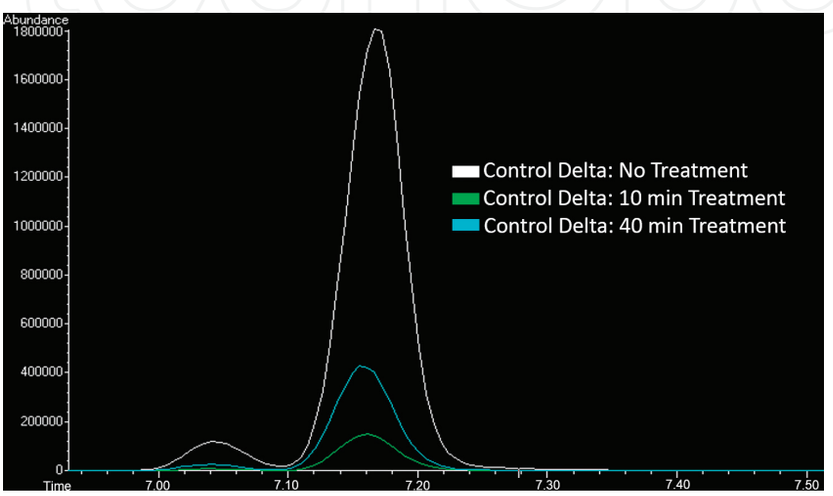

Figure 16.
D-limonene signal in the Control Delta Chromatogram.

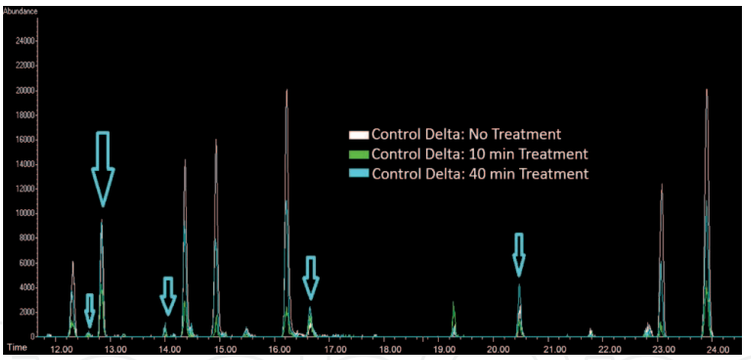

Figure 17.
Central area of the Control Delta Chromatogram.

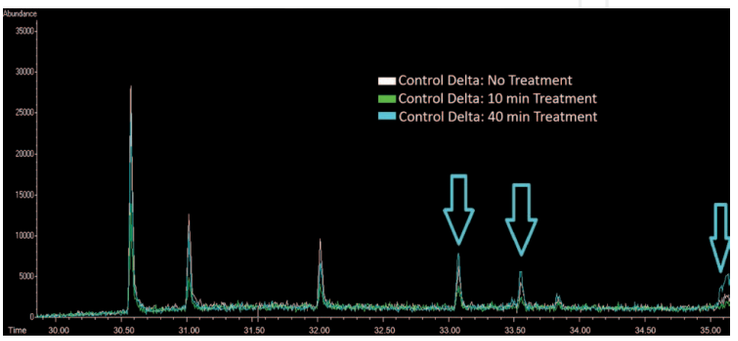

Figure 18.
Oxides area in the Delta Control Chromatogram.

of the Persian lemon essential oil, it contains a higher concentration of aldehydes than other citrus essentials oils. There are several aldehyde signals that increase when the sample is subjected to 40 minutes of UV. It was observed that the nano-emulsions contain a higher percentage of aldehydes and a better antibacterial response after UV treatment.

The oxides zone can be detected after a retention time of 30 minutes (**Figure 18**). In this area, other signals increase when the nano-emulsions are subjected to 40 minutes sterilization process. Thus, it may be inferred that these oxides may also contribute to the antibacterial effect.

5. Conclusions

This chapter examines the influence of nano-emulsion composition and high pressure homogenization conditions on droplet size and stability. Nano-emulsions with a droplet size smaller than 100 nm (diameter) can be produced by precise conditions of pressure, a specific number of steps in the high pressure homogenizer, and the presence of a combination of surfactants and emulsifiers that is capable to perform ideally under those conditions without degrading. Surfactants and natural gums were used to produce nano-emulsions with small droplets of Persian lemon oil, which has antimicrobial properties, with potential applications in the areas of cosmetics, pharmaceuticals and the food industry. In this particular study mesquite gum was used for the first time to maintain the kinetic stability of the Persian lemon oil droplets.

With the results of the MIC characterization it was confirmed that the nano-emulsions of Persian lemon oil developed under the method described in this research have an antibacterial effect against *Staphylococcus aureus* and *Escherichia*

coli. And although there is controversy about which components of the citric essential oils increase the antibacterial activity, our study suggest that aldehydes have an important role for the antibacterial effect of Persian lemon oil nano-emulsions.

Considering the series of experiments described in this research and the study of stability as a function of time it is clear that the Control Delta formulation composed of Span 20 (1.45%), Tween 80 (4.9%) and mesquite gum (4.9%) was the best. It was also shown that mesquite gum is superior to Arabic gum for the kinetic stability as shown by the behavior of the nano-emulsion that replaces mesquite gum with Arabic gum (Control 2). Indeed, when an excess of surfactant (Tween 80 and Span 20) is used, it is possible to obtain a smaller droplet size as observed in other control (Control 3 with excess Tween 80 and Span 20), but mesquite gum provides additional steric stabilization that confers a better stability over time against coalescence. The sample that replaces mesquite gum with water (Control 4) confirms the additional steric stability of mesquite gum.

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Conflict of interest

The authors declare no conflict of interest.

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