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<https://doi.org/10.1016/j.foodhyd.2018.03.001>

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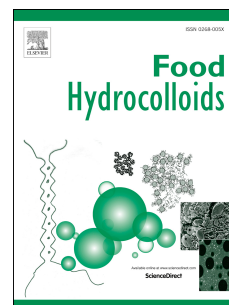


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# Accepted Manuscript

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PII: S0268-005X(17)31959-8

DOI: [10.1016/j.foodhyd.2018.03.001](https://doi.org/10.1016/j.foodhyd.2018.03.001)

Reference: FOOHYD 4312

To appear in: *Food Hydrocolloids*

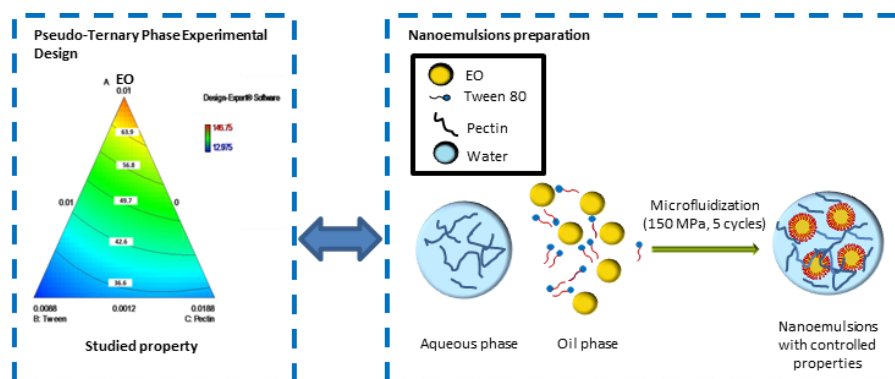
Received Date: 22 November 2017

Revised Date: 31 January 2018

Accepted Date: 1 March 2018

Please cite this article as: Artiga-Artigas, M., Guerra-Rosas, M.I., Morales-Castro, J., Salvia-Trujillo, L., Martín-Belloso, O., Influence of essential oils and pectin on nanoemulsion formulation: A ternary phase experimental approach, *Food Hydrocolloids* (2018), doi: 10.1016/j.foodhyd.2018.03.001.

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# Influence of essential oils and pectin on nanoemulsion formulation: a ternary phase experimental approach.

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**Abstract**

A pseudo-ternary phase experimental approach was used to model the influence of the mixture components concentration on the nanoemulsions properties as ternary systems. For this, several types of essential oils (EO) were used as lipid phase, being oregano (OR-EO), thyme (TH-EO), lemongrass (LG-EO) and mandarin (MN-EO), while pectin and Tween 80 were studied as emulsifiers. All formulations were processed by microfluidization at 150MPa and 5 cycles. Polynomial models were fitted to experimental data and their adjusted  $R^2$  and p-values were obtained. Remarkably, a pectin concentration of 1 % (w/w) allowed the formation of submicron emulsions between 350 and 850 nm in the absence of Tween 80 for all the studied EOs, thus confirming its emulsification capacity. In general, increasing the pectin concentration up to 2 % (w/w) enlarged the particle size of emulsions and their viscosity thus suggesting decreased emulsification efficiency during microfluidization. Nonetheless, nanoemulsions with particle sizes below 500 nm were obtained when a minimum Tween 80 concentration of 1.8% (w/w) was used, regardless the pectin or EO concentrations. The modest decrease in the  $\zeta$ -potential that was observed depending on the type of EO at increasing pectin concentrations indicated that pectin is not or weakly adsorbed at the oil-water interface. All nanoemulsions were transparent at high surfactant and low EO concentrations due to a weak light scattering of the nano-sized oil droplets. Thus, this work contributes in elucidating the role of pectin and small molecule non-ionic surfactants on the formation of submicron emulsions and nanoemulsions containing essential oils.

**Keywords:** Nanoemulsions, essentials oils, droplet size,  $\zeta$ -potential, pseudo-ternary phase diagram

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## 1. Introduction

Essential oils (EOs) are natural compounds that contain a complex mixture of terpenoids, with non-volatile and volatile nature produced by aromatic plants as secondary metabolites (Fisher & Phillips, 2008; Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2014). EOs have been traditionally used as natural flavorings and more recently as natural antimicrobials for food preservation (Guerra-Rosas, Morales-Castro, Ochoa-Martínez, Salvia-Trujillo, & Martín-Belloso, 2016). Due to their lipophilic nature, they are able to interact with biological membranes of microbial cells causing the leakage of cytoplasmatic content and the subsequent cell collapse (Burt, 2004; Guerra-Rosas et al., 2016). Besides the benefits of adding EOs to food matrices, their poor water solubility, their intense aroma or their potential toxicity at high concentrations needs consideration (Svoboda, Brooker, & Zrustova, 2006). Therefore, the design of adequate delivery systems able to encapsulate, protect and release lipophilic bioactive compounds into food matrices more efficiently represents a challenge for the food technology field.

Recently, nanoemulsions have been described as colloidal dispersions of oil droplets with particle size diameters lower than 500 nm, which are suspended within an aqueous phase (Otoni, Avena-Bustillos, Olsen, Bilbao-Sáinz, & McHugh, 2016). Nanoemulsions seem to be a promising tool for incorporating antimicrobial EOs in foods and they have been reported to present several potential advantages in comparison with conventional emulsions. Nanoemulsions present a higher active surface area/volume ratio due to their small droplet size, thus enhancing the transport of active compounds through biological membranes. Therefore, the use of nanoemulsions as carriers of antimicrobial essential oils would allow reducing the concentration to be used in order to achieve equivalent microbial inactivation levels of those of conventional emulsions or bulk oils. This,

would help overcoming the low threshold values of essential oil incorporation for consumer acceptance (McClements, 2012; Salvia-Trujillo et al., 2014a; Solans, Izquierdo, Nolla, Azemar, & Garcia-Celma, 2005).

The fabrication of nanoemulsions requires the use of surfactants in order to stabilize the oil droplets in the aqueous phase. Surfactants are able to adsorb at the oil-water interface thus lowering the interfacial tension of oil in water, which facilitates the emulsification process and prevents different destabilization phenomena such as aggregation, flocculation or coalescence (Iva Kralova & Sjöblom, 2009). In addition, natural biopolymers are gaining importance for their use as emulsifiers and thickening agents. The increase in viscosity of the aqueous phase prevents the destabilization of emulsions and nanoemulsions due to a diminished gravitational movement of oil droplets, which may retard or avoid droplets coalescence (Guerra-Rosas et al., 2016). However, some biopolymers may also present surface active properties thus having emulsifying capacity. For instance, pectin is a natural biopolymer mainly present in fruits and vegetables that has shown certain adsorption capacity at oil-water interfaces and may enhance the stability of emulsions (Alba & Kontogiorgos, 2017; Chan, Choo, Young, & Loh, 2017; Ozturk & McClements, 2016). The lipid fraction also plays an important role in the physicochemical properties, which depend on the characteristics of the different EOs including their chemical composition and the hydrocarbon chain length (Hopkins, Chang, Lam, & Nickerson, 2015). In fact, it is reported that short chain fatty acids, such as EOs, are prone to destabilization phenomena due to Oswald ripening effect since they consist on an aromatic carbon ring, which makes them slightly water-soluble oils (Suriyarak & Weiss, 2014). In this context, differences between nanoemulsion stability incorporating different EOs may be rather affected by their



chemical composition (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2015).

The selection of the appropriate concentration of each individual ingredient in the formulation of nanoemulsions is of crucial importance in order to obtain systems with the desired physicochemical characteristics (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2014b). Building conventional pseudo-ternary phase diagrams has been explored as a strategy to optimize the formulation of multi-component emulsions. However, determining the region where nanoemulsions are formed requires performing a large number of experimental combinations and formulations. Oppositely, the ternary phase experimental design, and specifically D-optimal models, is a methodology which allows to evaluate the effect of multiple factors, alone or in combination, with a minimum number of experiments (Ramsey, 1997; Salvia-Trujillo et al., 2014b). Consequently, the combination of pseudo-ternary phase diagrams and response surface methodologies can improve the understanding of the influence of the emulsion components concentration and their interactions as well as predicting optimized multi-component formulations with controlled physicochemical and functional properties (Ren, Mu, Alchaer, Chtatou, & Müllertz, 2013). To the best of our knowledge, there is a lack of research works using this strategy for the formulation of nanoemulsions containing several emulsifying agents, such as Tweens and pectin. This would allow to fully map the behavior of the different components and their interactions on nanoemulsion characteristics.

Thus, the aim of this work was to study the influence of four different essential oils (EOs) such as oregano (OR-EO), thyme (TH-EO), lemongrass (LG-EO) and mandarin (MN-EO) essential oils on the properties (oil droplet size,  $\zeta$ -potential, viscosity and

whiteness index) of nanoemulsions. Moreover, the use of a small molecule surfactant (Tween 80) and a natural biopolymer (pectin) were studied as emulsifiers.

## 2. Material and Methods

### 2.1. Materials

Oregano (*Origanum compactum*), thyme (*Thymus vulgare*) and lemongrass (*Cymbopogon citratus*) essential oils (EOs) were purchased from Essential'aroms® (Dietetica Intersa, Lleida, Spain) and had a 100% purity. Oregano EO was composed of 26-45% carvacrol, 9-30% thymol, 9-26% p-cymene, 12-20%  $\gamma$ -terpinene and traces of  $\alpha$ -terpinene and  $\beta$ -mircene. Thyme EO mainly contained thymol (30-47%) and p-cymene (15-35%) with traces of carvacrol and linalool. Lemongrass had 39-47.1% of geranial, 28.9-35.7% of neral, 0.5-8.3% of limonene, 0.9-6.9% of geranyl acetate, 0-5.3% of geraniol and traces of citranelol, eugenol and linalool among others. Mandarin EO (*Citrus reticulata*) 100% pure, was kindly donated by Indulleida, S.A. (Lleida, Spain) and contained 74.4% of D-limonene, a 13.6% of oxygenated monoterpenes and traces of cis-oxide limonene, cis-para-mentha-2,8-dien-1-ol, carvone, trans-carveol and z-patchenol (>1%). Food-grade high methoxyl pectin (Unipectine QC100 from citrus source) with a degree of methylesterification (DM) from 69 to 75% and a particle size of the dry powder at least 99% less than 315  $\mu$ m (ASTM Screen N°45) was kindly provided by Cargill Inc. (Reus, Spain). Tween 80 (Polyoxyethylenesorbitan Monoesterate) (Lab Scharlab, Barcelona, Spain) was used as food-grade non-ionic surfactant. Ultrapure water, obtained from Millipore Milli-Q filtration system (0.22 $\mu$ m) was used for the formulation and analysis of nanoemulsions.

## 2.2. Pseudo-Ternary Phase Experimental Design

An experimental mixture design, specifically a D-optimal design, was used to study the influence of the essential oil (EO), Tween 80 and pectin concentrations on the oil droplet diameter (nm),  $\zeta$ -potential (mV), viscosity (mPa·s) and color of emulsions. For the experimental design, the software Design Expert 7.0.0 (Stat Ease Inc., Minneapolis, MN) was used. Posteriorly, pseudo-ternary phase diagrams were built in order to predict and identify the regions where stable nanoemulsions are formed. Firstly, for the experimental design setup, the concentration of each component was set according to the following constraints expressed as weight fraction in the aqueous phase (% w/w):  $0.12 \leq \text{EO} \leq 1$  in case of OR-EO and TH-EO but  $0.1 \leq \text{EO} \leq 2$  for LG-EO and  $0.05 \leq \text{EO} \leq 3$  in case of MN-EO;  $0 \leq \text{Tween 80} \leq 6$ ,  $1 \leq \text{pectin} \leq 2$  and for all the cases:  $\text{EO} + \text{Tween 80} + \text{pectin} + \text{water} = 1$ . The minimum concentration of EO was set above its minimum inhibitory concentration against the most common pathogenic microorganisms that proliferate in foods such as *Escherichia coli*, *Listeria monocytogenes* or *Staphylococcus aureus* (Burt, 2004; Hammer, Carson, & Riley, 1999), whereas the maximum concentration was set regarding the toxicity of EOs at high concentrations (Svoboda et al., 2006). Tween 80 concentration was set to reach a maximum oil:surfactant ratio of 1:3 (expressed in weight), which ensures that enough surfactant molecules are available to be adsorbed at the oil-water interface (Qian & McClements, 2011; Salvia-Trujillo et al., 2014b). Pectin is generally recognized as safe (GRAS) thus its only limitation regarding its addition to nanoemulsions is the increase of viscosity of the mixtures in order to pass through the microfluidizer (Laurent & Boulenguer, 2003).

After running the statistical software, a series of mixture combinations were generated as an output of the D-optimal design. All oil-in-water emulsions were prepared in

random order and analyzed the same day of preparation without further storage. Three replicate analyses of each formulation and physicochemical parameter were performed, and the mean value with its corresponding standard deviation was calculated and used for modeling. The empirical data of the experimental designs related with high methoxyl pectin, Tween 80 and EOs – oregano (OR-EO), thyme (TH-EO), lemongrass (LG-EO) and mandarin (MN-EO)- are shown in the supplementary material (Tables 2, 3, 4 and 5). Afterwards, experimental data were represented graphically with pseudo-ternary phase diagrams and modeled with a Scheffe polynomial equation (Eq. 1) for the four components – EO, Tween 80, Pectin and water- in each formulation. Nevertheless, in some cases data did not follow a normal distribution and a power transformation recommended by de Box-Cox method was needed to stabilize variance and improve the validity of measures of association between variables (Table 1). A power transform is a family of functions that are applied to ensure the usual assumptions for linear model hold (Li, 2005). The statistical significance of the models for each response was evaluated regarding their adjusted  $R^2$  and  $p$ -values that were obtained through the ANOVA analysis.

$$Y = \sum \beta_i A + \sum \sum \beta_{ij} AB + \sum \sum \sum \beta_{ijk} ABC + \sum \sum \sum \sum \beta_{ijkl} ABCD \quad (1)$$

where Y is the response variable, i, j and k are the number of ingredients in the mixture,  $\beta_i$  is the first-order coefficient,  $\beta_{ij}$  is the second-order coefficient,  $\beta_{ijk}$  is the third-order coefficient,  $\beta_{ijkl}$  is the fourth-order coefficient...; A, B, C and D are the corresponding EO, Tween 80, pectin and water concentrations, respectively.

Contour plots obtained from the ternary phase diagrams were generated by setting the concentration of water at 0.98 (Figures 1, 3, 4 and 5). However, for contour plots of particle size, water content was also fixed at 0.94 to highlight the regions where smallest particle sizes values were found (Figure 2).

### 2.3. Nanoemulsions formation

All the formulations used for the D-optimal experimental design were prepared and their physicochemical properties tested. To achieve this, powdered pectin was added slowly to ultrapure water at 70°C until its total dissolution and stored under refrigeration overnight to ensure the complete hydration of the biopolymer. Afterwards, a lipid phase was formed by adding the required Tween 80 amount to the corresponding EO. Then, the lipid phase and the aqueous phase were mixed by using a laboratory mixer (T25 digital Ultra-Turrax, IKA, Staufen, Germany) at 9600 rpm and 2 minutes, which led to the formation of a coarse emulsion. Nanoemulsions were obtained by passing the coarse emulsions through a microfluidizer (M-110P, Microfluidics, USA) at 150 MPa for 5 cycles (Qian & McClements, 2011) and were cooled down at the outlet of the microfluidization unit through an external coil immersed in a water bath with ice, so that temperature was kept at 10 °C.

### 2.4. Nanoemulsions characterization

#### 2.4.1. Particle Size and $\zeta$ -Potential

The emulsion droplet diameter (Z-average in nm) was measured by dynamic light scattering technique (DSL) with a Zetasizer laser diffractometer (NanoZS, Malvern Instruments Ltd., Worcestershire, UK) working at 633 nm at 25 °C and equipped with a back-scatter detector (173°) (Brar & Verma, 2011). Average droplet diameter (nm) and size distribution curves in intensity (%) were used to characterize the nanoemulsions. Particle size was calculated with the Mie Theory, which correlates the Brownian motion of the dispersed particles with their particle size by using the optical properties of the oil and the aqueous dispersant phase. Refractive indexes of OR-EO, TH-EO, LG-EO and MN-EO measured with a refractometer (ABBE-2WAJ, optic ivymen system<sup>®</sup>, Comecta

SA, Barcelona, Spain) were 1.501, 1.497, 1.484 and 1.475, respectively; and their absorbance measured with a spectrophotometer (Jasco V-670, Tokyo, Japan) at 633 nm were 0.002, 0.002, 0.024 and 0.004, respectively.

The electrophoretic mobility of oil droplets, also reported as  $\zeta$ -potential (mV), was measured by phase analysis light scattering (PALS) with a Zetasizer laser diffractometer (NanoZS, Malvern Instruments Ltd., Worcestershire, UK). It determines the surface charge at the interface of the droplets dispersed in the aqueous solution. Samples were diluted prior to particle size and  $\zeta$ -potential measurements in ultrapure water with a dilution factor of 1:9 sample-to-dilutant.

#### 2.4.2. Viscosity

A vibro-viscometer (SV-10, A&D Company, Tokyo, Japan) vibrating at 30 Hz was used to measure the viscosity (mPa·s) of 10 mL aliquots of the emulsions. Moreover, the viscosity of water, which was used as dispersant phase, was 0.89 mPa·s. The viscosity of water was considered as the viscosity of the aqueous phase for the particle size measurements, conducted at  $25 \pm 2$  °C.

#### 2.4.3. Whiteness Index

The color of emulsions and nanoemulsions was measured with a colorimeter (Minolta CR-400, Konica Minolta Sensing, Inc., Osaka, Japan) at room temperature set up for illuminant D65 and 10° observer angle and calibrated with a standard white plate (Y=94.0; x=0.3133; y=0.3194). CIE  $L^*$ ,  $a^*$  and  $b^*$  values were determined, and the whiteness index (WI) was calculated with Eq. 2 (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2013):

$$WI = 100 - ((100 - L)^2 + (a^2 + b^2))^{0.5} \quad (2)$$

### 3. Results and Discussion

#### 3.1. Particle Size

The experimental values for the average oil droplet size of emulsions containing OR-EO, TH-EO, LG-EO and MN-EO are shown in the supplementary material (Tables 2, 3, 4 and 5, respectively); and the coefficients of the polynomial equation regarding particle size are shown in Table 1. On the one hand, a special cubic model fit the droplet size diameter experimental values of OR-EO whereas a linear or a quadratic model without transformations was needed for nanoemulsions containing TH-EO or LG-EO, respectively. On the other hand, for nanoemulsions with MN-EO, a quadratic model with a  $\log_{10}$  power law transformation was required. The polynomial model to predict the particle size for each type of oil was statistically significant ( $p$ -values  $\leq 0.01$ ) and with a good fit as indicated by the coefficients of determination ( $R^2$ ) for OR-EO, LG-EO and MN-EO formulations with values of 0.99, 0.97 and 0.94, respectively; being lower in nanoemulsions containing TH-EO (0.62). The minimum average droplet size observed for OR-EO, TH-EO, LG-EO and MN-EO nanoemulsions was  $120 \pm 9$ ,  $113 \pm 3$ ,  $146 \pm 6$ , and  $29 \pm 1$  nm, respectively. Contour plots obtained from the ternary phase diagrams regarding particle size were represented setting the concentration of water at 0.98 to facilitate the visualization of the data in a two-dimensional plane (Figure 1). However, in order to highlight the regions in which smallest particle size values were found, enlargements of Figure 1 were also represented setting the water concentration to 0.94 (Figure 2). Tween 80 is a low-mass surfactant, which has a molar mass of 1.310 g/mol and rapidly coats the surface of the created oil-water interface during emulsification (Iva Kralova et al., 2011; Salvia-Trujillo et al., 2014b). Therefore, there must be enough amounts of surface-active molecules to cover the surface area of oil droplets generated during the formation of nanoemulsions (Acevedo-Fani, Salvia-

Trujillo, Rojas-Graü, & Martín-Belloso, 2015). In this regard, the higher the concentration of surfactant, the lower the particle size would be, which is in accordance with previous works (Kralova & Sjöblom, 2009). However, a remarkable decrease in nanoemulsion particle size when concentration of Tween 80 increased only was observed in the contour plot of LG-EO. Actually, which it is shown in Figures 1 and 2 is the formation of submicron nanoparticles (350-850 nm) with concentrations of pectin between 1 and 2% w/w even in absence of surfactant. This highlights the capacity of pectin to act as emulsifier. Furthermore, in contrast with LG-EO nanoemulsions, in Figures 1A, 1B and 1D (OR-EO, TH-EO and MN-EO, respectively) the isolines are oriented parallelly to the pectin axis, which means that the particle size was mainly dominated by pectin concentration. This affirmation agrees with positive coefficients in the pectin concentration for the OR-EO, TH-EO and MN-EO nanoemulsions (Table 1). It is considered that the higher the magnitude of the coefficient parameter of the corresponding terms in the fitted equation, the higher the response effect on the measured parameter (Salvia-Trujillo et al., 2014b). In this regard, an increase in pectin concentration in the aqueous phase caused the enlargement of nanoemulsion particle size of OR-EO, TH-EO and MN-EO nanoemulsions, which might be caused by a number of reasons. Firstly, the increase in biopolymer concentration may raise the viscosity of the continuous phase and therefore low the efficiency of the microfluidizer device in reducing the oil droplet size (Schmidt, 2016). Secondly, pectin might present certain surface activity at oil-water interfaces since it contains functional units including acetyl and methyl groups. Some authors have demonstrated a direct relationship between the DM and emulsifying capacity of citrus pectin by increasing the DM from ~70% to ~80% (Schmidt, Schütz, & Schuchmann, 2017). These groups with interfacial activity are able to act as hydrophobic anchors that facilitate adsorption of pectin chains



at the interface resulting in reduction of interfacial tension (Alba & Kontogiorgos, 2017). Hence, pectin chains present in the bulk aqueous phase can compete with Tween 80 molecules, and cause the displacement of these previously-adsorbed surface active species (Jafari, He, & Bhandari, 2007). And finally, pectin molecules have shown to induce depletion flocculation and subsequent coalescence in emulsions, thus causing an increase in nanoemulsions particle size (Julian McClements, 2005; Neumann, Schmitt, & Iamazaki, 2003; Robins, 2000).

In general, the EOs concentration did not dominate the nanoemulsion particle size especially in the case of MN-EO nanoemulsions, where isolines were closely perpendicular to the oil axis. Thus, differences among the droplet size of EOs-loaded nanoemulsions has been attributed to different chemical composition of the volatile compounds forming each oil phase (supplementary material Figure 6) (Guerra-Rosas et al., 2016; Salvia-Trujillo et al., 2015). Principally OR-EO and TH-EO nanoemulsions, whose major components carvacrol and thymol contain a phenolic group, are prone to suffer Ostwald ripening since are highly water soluble. During this process larger droplets grow at the expense of smaller ones leading to an increase of mean droplet diameters of emulsions (Zeeb, Gibis, Fischer, & Weiss, 2012). Nonetheless, LG-EO nanoemulsions have been reported to be stable against Ostwald ripening (Guerra-Rosas et al., 2016) and pectin seems to contribute positively in their emulsification. In fact, positive coefficients of the quadratic terms of LG-EO nanoemulsions indicate a synergistic effect among oil concentration and the other three components or between Tween 80 and pectin (Ramsey, 1997; Salvia-Trujillo et al., 2014; Cornel, 2002). Being the largest coefficient that of the interaction between the essential oil (component A) and pectin (component C) with an AC value of 31677.86 (Table 5).

### 3.2. $\zeta$ -potential

The electrical charge experimental values of OR-EO, TH-EO, LG-EO and MN-EO nanoemulsions are shown in the supplementary material (Tables 2, 3, 4 and 5, respectively). The  $\zeta$ -potential varied significantly depending on the concentration of the different components of the emulsions and the type of oil used. They ranged between values around  $-5 \pm 1$  mV and  $-18 \pm 2$  mV depending on the type and concentration of EO used and the concentration of pectin.

A linear model was fitted to experimental data in all cases except for the emulsions with LG-EO, in which a quadratic model was used. The p-values and a  $R^2$  for the four types of formulations are shown in Table 1. Consistently, the negative coefficients for Tween 80 (-8.05; -14.69; -3.50 and -12.22; in case of using OR-EO, TH-EO, LG-EO and MN-EO, respectively) as well as pectin coefficients of -18.72, 4.19, -460.82 and -12.27 (OR-EO, TH-EO, LG-EO and MN-EO, respectively), indicated their contribution to decrease the  $\zeta$ -potential of the emulsions and nanoemulsions. On the one hand, it is known that, in the absence of any anionic biopolymer, non-ionic surfactants such as Tween 80 may confer negative charge to oil droplets probably due to the orientation towards the oil/water interface of  $\text{OH}^-$  ions from the aqueous phase or  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  ions from the dissolved atmospheric  $\text{CO}_2$  (Marinova et al., 1996). Moreover, since Tween 80 is a small molecule surfactant, it adsorbs at the oil/water interface very quickly stabilizing the newly created oil droplets. On the other hand, pectin is a linear polysaccharide consisting of covalently linked galacturonic acid (GalA) units with methylester groups along the backbone. The ionization of the carboxylic groups of the methylesters contributes to the negative charge of pectin (Lin, Lopez-Sanchez, & Gidley, 2016). It has been recently described that pectin may adsorb at the oil/water interface but the exact mechanism of adsorption still needs to be elucidated (Alba & Kontogiorgos,

2017). In this regard, a different behavior of the emulsion  $\zeta$ -potential was observed after the addition of pectin into the system depending on the type of EO (Figure 3). In the contour plots of TH-EO and MN-EO,  $\zeta$ -potential values remained practically unaltered after increasing the concentration of pectin from 1 to 2% w/w, ranging from -12 to -15 mV (Figure 3B) and from -10 to -11 mV (Figure 3D), respectively. This suggests that, despite the anionic nature of pectin, it may not be adsorbed at the oil/water interface due to a preferential adsorption of Tween 80 at the oil droplet surface. Oppositely, contour plots of OR-EO and LG-EO showed a decrease of  $\zeta$ -potential values becoming more negative with an increase in the concentration of pectin. For instance, the  $\zeta$ -potential of OR-EO or LG-EO nanoemulsions decreased from -10 to -15 mV (Figure 3A) or from -9 to -15 mV (Figure 3C), respectively, with an increase of the pectin concentration from 1% to 2% w/w. This suggests that pectin might have been partially adsorbed at the oil/water interface with its hydrophilic part in the continuous water phase and the hydrophobic part in contact with the oil drops (Nambam & Philip, 2012). This is in accordance with other studies that had described a competitive adsorption between negative biopolymers and low mass surfactants on the oil/water interface. If the concentration of surfactant molecules is not enough to cover the oil water interface, it might lead the adsorption of polymer molecules conferring a more negative  $\zeta$ -potential (Gasa-Falcon, Odriozola-Serrano, Oms-Oliu, & Martín-Belloso, 2017; Goddard, 2002; Salvia-Trujillo et al., 2014b).

### 3.3. Viscosity

The viscosity values are presented in the supplementary material (Tables 2, 3, 4 and 5 for OR-EO, TH-EO, LG-EO and MN-EO, respectively). A linear model with a  $\log_{10}$  transformation power law, recommended by the Box-Cox method was successfully fitted experimental data of OR-EO nanoemulsions (Table 1). Those formulations

showed p-values lower than 0.0001 and a  $R^2$  value higher or equal than 0.98. However, TH-EO, LG-EO and MN-EO fitted to a quadratic model that was based on  $R^2$  values of 0.99 and p-values statistically significant ( $\leq 0.01$ ).

The apparent viscosity values ranged from  $19.9 \pm 0.4$  to  $129.3 \pm 0.5$  mPa·s, from  $24.9 \pm 0.5$  to  $197 \pm 3$  mPa·s; from  $15.15 \pm 0.4$  to  $137 \pm 7$  mPa·s and from  $12.98 \pm 0.05$  to  $147 \pm 1$  in case of using OR-EO, TH-EO, LG-EO and MN-EO, respectively. In fact, the parallel trend of OR-EO, TH-EO, LG-EO and MN-EO contour lines regarding the segment between EO and Tween 80 vertexes indicates a poor influence of these compounds on the nanoemulsion viscosity. Nonetheless, specifically in the case of LG-EO nanoemulsion a slight decrease of viscosity was observed when the concentration of surfactant increased (Figure 4C), which was linked with a smaller particle size in those regions (Figure 1C). It is well known that an increased concentration of surfactant may lead to a decrease in the particle size (Tadros, Izquierdo, Esquena, & Solans, 2004) and this might have an impact on emulsion viscosity (McClements & Rao, 2011). In addition to this, MN-EO nanoemulsions, present lower overall viscosity values (Figure 4D) in comparison with the rest of the EOs, being also the ones having the smallest droplet sizes (Figure 1D). Thus, the lower viscosity values due to smaller droplets might be attributed to the higher flow properties when a stress is applied. In fact, droplet size has been reported to impact the rheology of emulsions and depends on the properties of the dispersed phase (Pal, 2000). Despite this, our results suggest that nanoemulsion viscosity was mainly determined by pectin concentration as the contour plot isolines are almost parallel to the pectin concentration axis. In this regard, at increasing the weight fraction of pectin in the aqueous phase from 1 % (w/w) to 2 % (w/w) for a given concentration of Tween 80 (6 % w/w) and any concentration of EO, the apparent viscosity remarkably increased regardless the type of EO (supplementary material,

Tables 2, 3, 4 and 5). Guerra-Rosas et al. (2016) also observed that the higher the concentration of pectin, the higher its viscosity due to the thickening properties of pectin, which are related with its ability to holding high quantities of water. In this regard, free pectin molecules in the aqueous phase are able to form gel-like structures, thus increasing the viscosity of the aqueous media (Hansen, Arnebrant, & Bergström, 2001).

### 3.4. Whiteness Index

The whiteness index of the blends understood as an indicator of their milky appearance ranged between  $25.9 \pm 0.1$  and  $79.69 \pm 0.02$  (supplementary material, Tables 2, 3, 4 and 5 for OR-EO, TH-EO, LG-EO and MN-EO, respectively) depending on the formulation. That is, the higher the WI, the whiter, and the lower the WI, the more transparent (Salvia-Trujillo et al., 2013). A quadratic model was fitted to experimental results of all the nanoemulsions regardless the EO used, which was statistically significant with a  $R^2$  values above 0.93 and p values of 0.0009, 0.0026, 0.0015 and 0.0138 for OR-EO, TH-EO, LG-EO and MN-EO respectively (Table 1).

The contour plots of the whiteness index were characterized by their curved isolines, which indicate its dependency to multiple variables (Figure 5A, 5B, 5C and 5D). In this regard, the color of these blends was significantly affected by Tween 80 and EOs concentration. Our results evidenced that the transparency of the EOs nanoemulsions diminished when increasing the oil amount or decreasing the Tween 80 concentration (supplementary material, Tables 2,3,4 and 5), which is in agreement with a previously reported work by Rao & McClements (2011). In fact, it was reflected by the negative coefficients of the quadratic terms AB and BD in the polynomial equation (Table 1).

It is known that emulsion whitish appearance is due to the light scattering of the oil droplets. In turn, light scattering of oil droplets depends fundamentally on the refractive

index of continuous (water) and dispersed phase (oil), oil concentration and droplet size (McClements, 2002a, 2002b). Therefore, OR-EO nanoemulsions, with the highest refractive index, were those less transparent. In MN-EO contour plot parallel isolines to Tween 80 axis indicate the strong influence of surfactant concentration on the whiteness index of nanoemulsions. Thus, WI of MN-EO nanoemulsions decreased while increasing concentrations of Tween 80 regardless EO or pectin concentration. This can be related to the small particle sizes (29-260 nm) of MN-EO nanoemulsions at high concentrations of Tween 80. The whiteness index is closely correlated with the particle size of nanoemulsions since larger particles scatter the light more intensively causing an increase in the lightness, opacity and whiteness index of emulsions (Rao & McClements, 2011).

#### 4. Conclusions

The present work reveals that the pseudo-ternary phase experimental design based on a response surface methodology is useful to obtain significant information about the behavior of the individual components on the formation of nanoemulsions containing lipophilic functional compounds, such as EOs. In all cases, linear, quadratic or special cubic models successfully fit experimental data to describe the behavior of the mixtures. In general, the EOs concentration did not dominate the nanoemulsion particle size, being rather the Tween 80 and pectin concentrations the determining factors. A minimum concentration of Tween 80 of 1.8 % (w/w) was needed to obtain particle sizes in the nano-range ( $d < 500$  nm). This work also evidenced the role of pectin as emulsifier in the absence of small molecule surfactants, since submicron emulsions ( $d \approx 350 - 850$  nm) were obtained at a pectin concentration above 1 % (w/w) regardless the type of EOs. Nonetheless, in general, increasing the pectin concentration enlarged the

particle size and viscosity of emulsions and nanoemulsions. This suggests that high pectin concentrations might reduce the microfluidization efficiency in decreasing the oil droplet size. Moreover, the  $\zeta$ -potential values indicate that pectin is not or weakly adsorbed at the oil-water interface as a modest or negligible decrease in the  $\zeta$ -potential was observed depending on the type of EOs at increasing pectin concentrations. Nevertheless, the transparency of nanoemulsions was achieved mainly when using low EO concentrations, regardless the type of EOs. Therefore, pseudo-ternary diagrams have been used to observe the behavior of nanoemulsions components on their properties. This study has greatly contributed to describe the role of pectin and its interaction with small molecule surfactants in complex systems for the formation of nanoemulsions.

## Acknowledgments

This study was supported by the Ministry of Science and Innovation (Spain) throughout the project ‘Improving quality and functionality of food products by incorporating lipid nanoparticles into edible coatings’ (AGL2012-35635). María Artiga-Artigas thanks the University of Lleida for the pre-doctoral grant.

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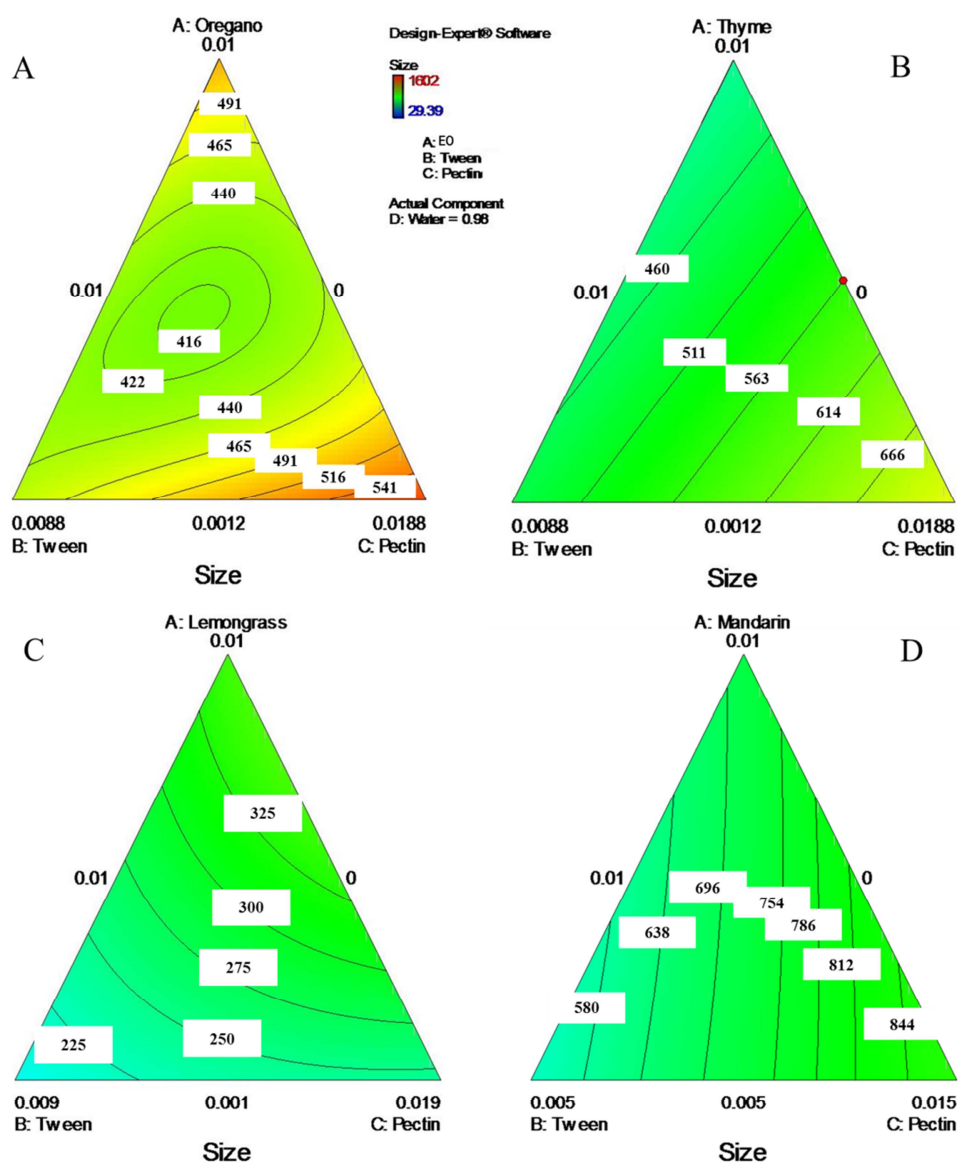
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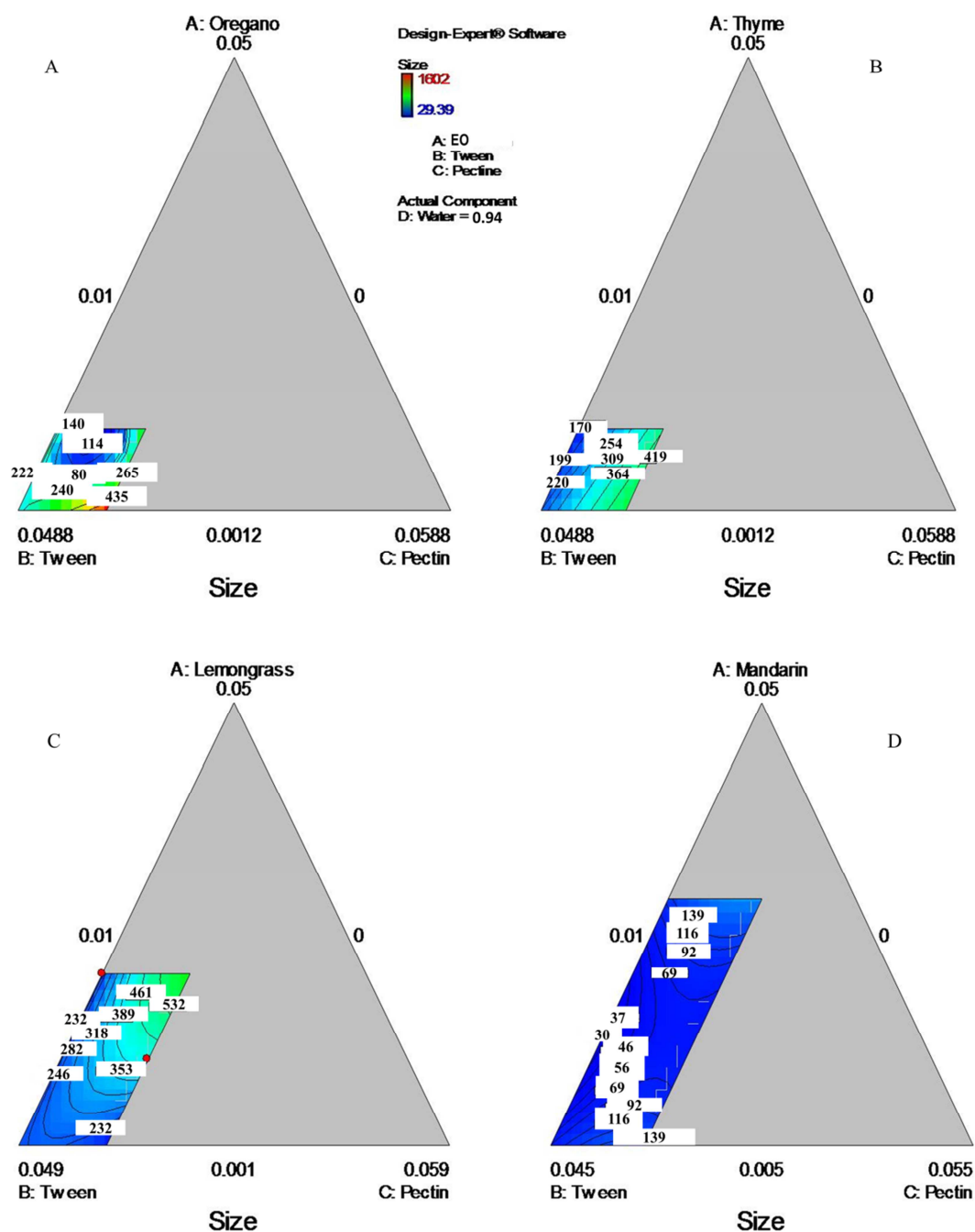
**Table 1.** Coded polynomial equation coefficients: A essential oil, B Tween 80, C pectin and D water; of the Scheffe model for L-pseudo-components and statistical significance of droplet diameter (nm),  $\zeta$ -potential (mV), viscosity (mPa·s) and whiteness index.

Mean Droplet diameter	A	B	C	D	AB	AC	AD	BC	BD	CD	ABC	ABD	ACD	BCD	R <sup>2</sup>	p-value
Oregano	1317.37	317.93	11054.58	537.70	9784.96	2.97·10 <sup>5</sup>	-1040.29	-13580.21	-646.58	-11532.48	-4.66·10 <sup>5</sup>	-29870.35	-3.72·10 <sup>5</sup>	15963.93	0.98895	0.0123
Thyme	-598.90	-6.95	2215.25	532.81	-	-	-	-	-	-	-	-	-	-	0.6184	0.0053
Lemongrass	-2548.84	548.13	-11501.86	510.02	371.28	31677.86	5365.65	11692.84	-1239.98	13109.77	-	-	-	-	0.9714	0.0057
Mandarin (log <sub>10</sub> ) <sup>*</sup>	18.91	3.37	-42.66	2.75	-30.12	22.50	-20.44	51.55	-4.80	52.00	-	-	-	-	0.9435	0.0089
$\zeta$ -potential	A	B	C	D	AB	AC	AD	BC	BD	CD	ABC	ABD	ACD	BCD	R <sup>2</sup>	p-value
Oregano	43.71	-8.05	-18.72	-15.38	-	-	-	-	-	-	-	-	-	-	0.7645	0.0002
Thyme	27.34	-14.69	4.19	-16.36	-	-	-	-	-	-	-	-	-	-	0.4985	0.0257
Lemongrass	-72.70	-3.50	-460.82	-13.62	39.27	783.87	121.35	469.41	-22.43	470.55	-	-	-	-	0.8952	0.0459
Mandarin	5.57	-12.22	-12.27	-11.18	-	-	-	-	-	-	-	-	-	-	0.4689	0.0364
Viscosity	A	B	C	D	AB	AC	AD	BC	BD	CD	ABC	ABD	ACD	BCD	R <sup>2</sup>	p-value
Oregano (log <sub>10</sub> ) <sup>*</sup>	1.59	1.37	8.46	1.12	-	-	-	-	-	-	-	-	-	-	0.9805	< 0.0001
Thyme	333.65	28.52	8813.87	24.33	-229.23	-9788.36	-467.55	-8601.44	-41.96	-8873.03	-	-	-	-	0.9926	< 0.0001
Lemongrass	-329.92	8.82	7751.40	13.24	423.89	-6681.54	440.31	-7423.38	28.59	-7813.82	-	-	-	-	0.9973	0.0003
Mandarin	326.02	-3.25	8906.58	13.66	-369.29	-9011.88	-360.27	-8509.85	71.63	-9025.94	-	-	-	-	0.9939	0.0031
Whiteness index	A	B	C	D	AB	AC	AD	BC	BD	CD	ABC	ABD	ACD	BCD	R <sup>2</sup>	p-value
Oregano	1153.49	36.82	-937.01	33.111	-1440.65	560.80	-868.00	1065.83	-50.96	1092.75	-	-	-	-	0.9777	0.0009
Thyme	484.29	60.26	-1128.68	37.005	-819.41	2001.38	-246.52	1144.33	-102.39	1326.46	-	-	-	-	0.9315	0.0026
Lemongrass	-258.32	67.42	-2007.32	37.067	122.63	3504.76	582.78	2092.66	-131.97	2211.07	-	-	-	-	0.9531	0.0015
Mandarin	334.22	69.14	144.34	53.88	-537.67	-247.98	-269.28	-297.99	-149.81	-56.90	-	-	-	-	0.9580	0.0138

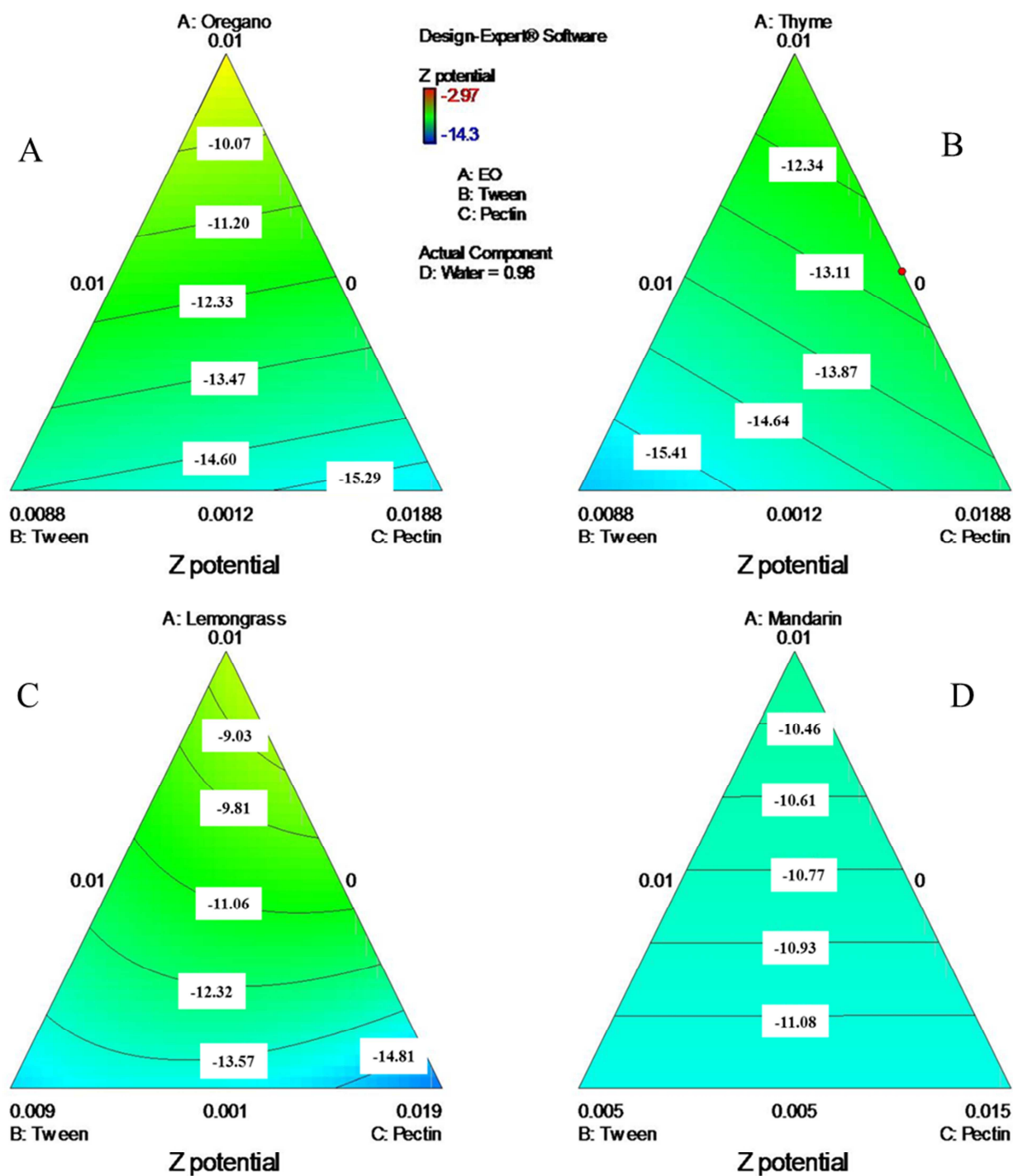
\*Required log<sub>10</sub> transformation recommended by the Box-Cox method.



**Figure 1** Contour plots of average droplet diameter (nm) of mixtures containing four different EOs: oregano oil (A), thyme oil (B), lemongrass (C) and mandarin oil (D), Tween 80 as surfactant and pectin. In the contour plots the water content was set at 0.98.

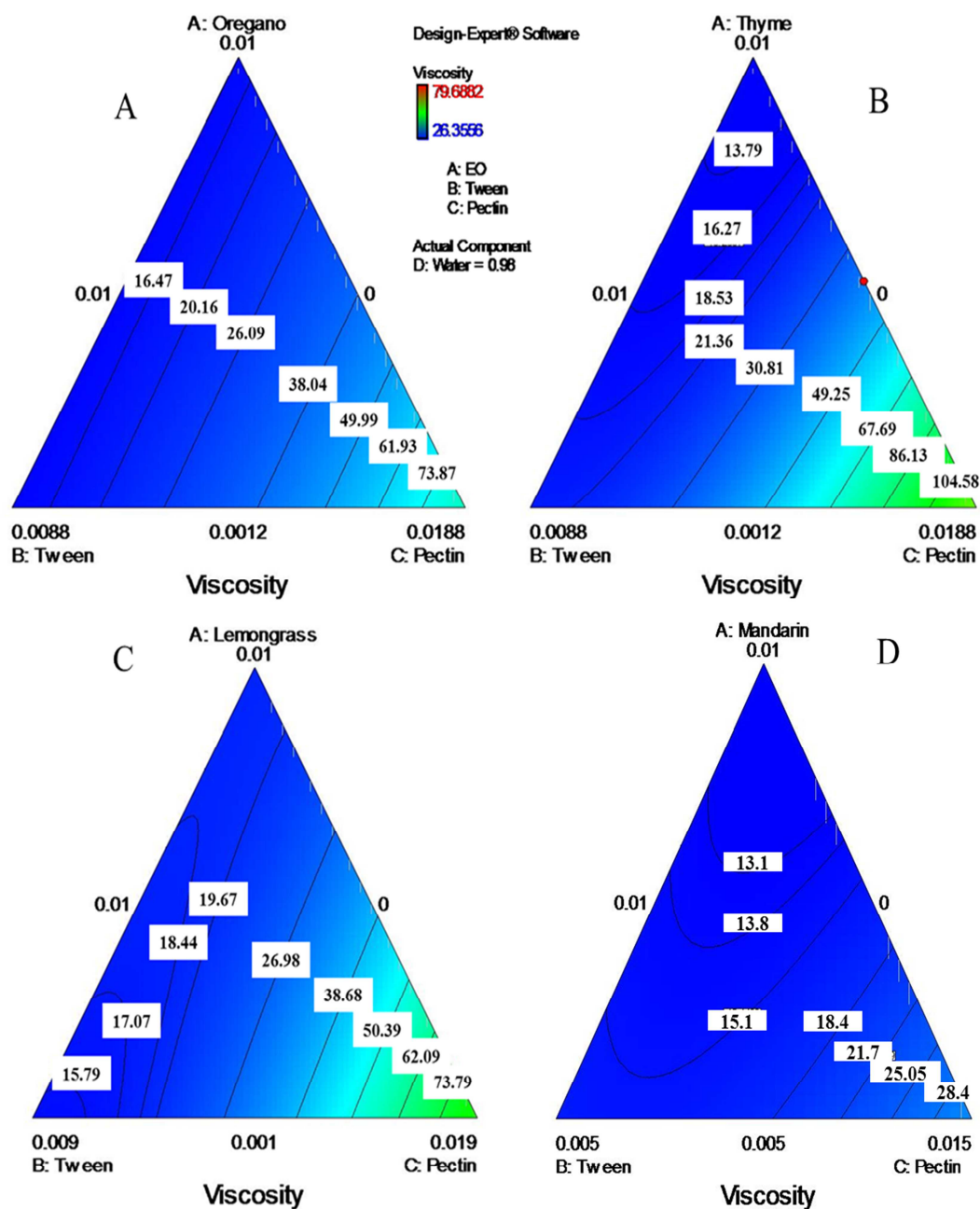


**Figure 2** Enlargements of the contour plots of average droplet diameter (nm) of mixtures containing four different EOs: oregano oil (A), thyme oil (B), lemongrass (C) and mandarin oil (D), Tween 80 as surfactant and pectin. In the contour plots the water content was set at 0.94.

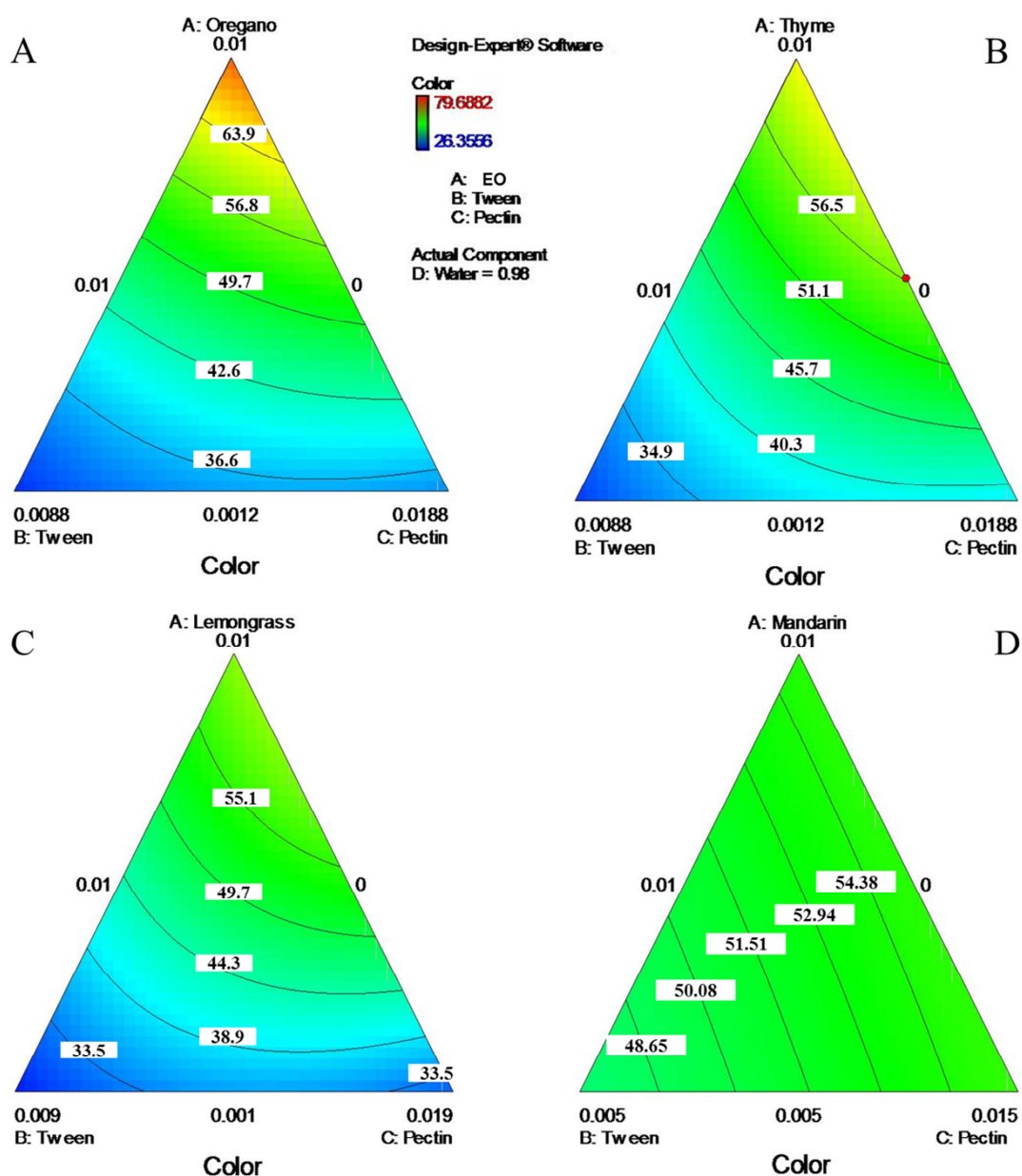


**Figure 3** Contour plots of  $\zeta$ -potential (mV) of mixtures containing four different EOs: oregano oil (A), thyme oil (B), lemongrass (C) and mandarin oil (D), tween 80 as a surfactant and pectin. The water content was set at 0.98.





**Figure 4** Contour plots of viscosity (mPa·s) of mixtures containing four different EOs: oregano oil (A), thyme oil (B), lemongrass (C) and mandarin oil (D), tween 80 as surfactant and pectin. The water content was set at 0.98.



**Figure 5** Contour plots of color (WI) of mixtures containing four different EOs: oregano oil (A), thyme oil (B), lemongrass (C) and mandarin oil (D), tween 80 as surfactant, and pectin. The water content was set at 0.98.

**Highlights**

- Ternary phase experimental design was used to formulate nanoemulsions.
- Nanoemulsion formulation depended on the essential oil, surfactant and pectin.
- The minimum concentration of Tween 80 needed to obtain nanoparticles was 0.015 w/w.
- Pectin effectively contributed in the emulsification of lemongrass nanoemulsions.
- Pectin increased the particle size of thyme, oregano and mandarin nanoemulsions.