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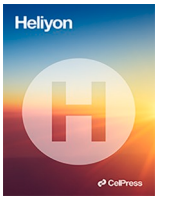

Study of nanoemulsions using carvacrol/MCT-(Oleic acid-potassium oleate)/ Tween 80 ®- water system by low energy method

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A B S T R A C T

Carvacrol is studied in different fields due to its microbial and antioxidant properties. Its use is limited because of the water insolubility and its strong taste. To overcome these problems, carvacrol has been successfully loaded into nanoemulsions. The low-energy emulsification method Phase Inversion Composition (PIC) is used to prepare oil-in-water nanoemulsions in the carvacrol/medium chain triglycerides (MCT)-(oleic acid-potassium oleate/Tween 80 ®)-water system. Oleic acid acts as a co-surfactant when it is neutralized with KOH along the emulsification path changing the spontaneous curvature of the interface when increasing the HLB number from 1 for the oleic acid to 20 for the potassium oleate and, therefore, changing the HLB number of the surfactant mixture. The phases diagrams are studied in order to understand the behaviour of the system and to establish the composition range where nanoemulsions can be obtained. Nanoemulsions are formed when the emulsification path crosses a region of direct or planar structure without excess of oil. Experimental design is performed in order to study the influence of composition variables as carvacrol/MCT ratio and (oleic-oleate)/Tween 80 ® ratio (OL-OT/T80 ratio) on the diameter of the nanoemulsions and their stability. It has been observed the importance of the HLB number of the surfactants mixture in order to obtain small-sized stable nanoemulsions. Surface response graphic shows that (OL-OT)/T80 ratio is a significant parameter in the mean diameter of the nanoemulsions. A minimum diameter is obtained for a (OL-OT)/T80 ratio 45/55 due to the fact that ratio is near the preferred HLB of the oil mixture and the emulsification path contains a wide liquid crystal monophasic region with all the oil incorporated in the structure.

Diameters of 19 nm for carvacrol/MCT ratio of 30/70 or diameters of 30 nm for ratios of 45/55 with high stability values presented a good potential to be incorporated into edible films in the future.

Regarding nanoemulsions stability an optimum value is also observed for a carvacrol/MCT ratio.

The addition of another carrier oil as olive oil instead of MCT showed an improvement of the nanoemulsions stability against Ostwald ripening, probably due to the smaller solubility of olive oil. The use of olive oil does not significantly change the diameter of the nanoemulsion.

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1. Introduction

During the last decade the need to preserve food against bacteria and different food diseases has been increased. The use of edible coatings also could prevent food against oxidation and moisture loss. Several authors have studied the possibility to enhance the shelf life of food products using edible coatings [1–5]. Chaudhary et al. [6] reviewed that a plasticiser, cross-linking agents and additives form edible coatings. The latest ones usually are essential oils (EO) extracted from plants that are known for their properties against a broad range of foodborne pathogens. EO's as canola oil, carvacrol, D-limonene, eucalyptus oil, thyme oil and cinnamon oil are excellent substances to prevent food diseases. They present a main disadvantage in their direct application due to their organoleptic properties, so their use is limited by the consumer taste [7]. Moreover EO, as many hydrophobic compounds cannot be solubilized in water. The use of EO in edible films requires their incorporation using colloidal systems as emulsions, microemulsions, nanoemulsions, solid particles and liposomes [8]. Several authors reported the use of nanoemulsions to incorporate different kind of active compounds for different purposes [9,10]. Nanoemulsions seem good candidates in order to incorporate EO in the edible films ensuring their antimicrobial activity but without noticeable taste. In this work carvacrol oil is used, as it is the main compound of oregano EO. Some authors [11–13] reported that carvacrol has been studied and applied in food as antibiotic alternative due its excellent antimicrobial activity and outstanding antioxidant properties. Carvacrol molecule contains a phenolic ring with methyl and isopropyl substitutions. Its major contributing factor to its excellent antimicrobial capacity is the free hydroxyl functional group. Moreover, carvacrol has been categorized as a generally recognized safe (GRAS) food additive according to Annex II of Council Regulation 2377/90 by the European Commission (2000). The Joint FAO/WHOnExpert Committee on Food Additives by World Health Organization (WHO) (2001), 21 CFR 172.515 by FDA (2010) [14] presented a positive evaluation of carvacrol, which is fundamental for its wide applications as an alternative to antibiotics in food and feeding industries.

Nanoemulsions are a type of emulsions with droplets of small diameters, typically in the range of 20–200 nm [15]. Due to their small diameter their appearance are transparent or bluish translucent, so they are perfect to coat fruits and vegetables maintaining their natural colour and texture.

As non-equilibrium systems, one dispersed phase non-miscible in another phase, to form nanoemulsions an energy input is required. Formation mechanisms can be divided as high-energy methods and low-energy methods. As listed Chaudary et al. [6] in their review a lot of researchers obtained food grade nanoemulsions using high energy methods as sonication, microfluidization [16–19], high pressure homogenization and high shear stirring [20–22]. The authors obtaining nanoemulsions by low energy methods are less although the low energy methods are cost-effective and fast methods for the production of nanoemulsions. Low energy methods include processes like spontaneous emulsification (SE), phase inversion temperature (PIT) and phase inversion composition (PIC). In the first method, SE forms fine oil droplets of small size adding an organic phase that contains a hydrophilic surfactant to the aqueous phase. Most of the nanoemulsions reported using SE technique used Tween surfactants to achieve them, being Tween 80 ® the most used [23–29].

In the second method, the phase inversion temperature (PIT) nanoemulsification method, introduced by Shinoda [30] a macroemulsion is prepared at a temperature higher than the phase inversion temperature of a non-ionic surfactant based in ethylene oxide chains. When the macroemulsion reaches down to normal room temperature, an inversion from water-in-oil emulsions to oil-in-water emulsions occurs due to the hydration of the ethylene oxide chains and the consequent change of the interface spontaneous curvature. The cooling process must be very fast from the HLB temperature. At HLB temperature and surroundings the interfacial tension is very low and the emulsions have a poor stability due to the high coalescence tendency. It is very important that all the oil mixture should be incorporated into the structure.

The third low energy method is the phase inversion composition (PIC). This technique is the less studied to form food grade nanoemulsions [31,32]. In this work, very small diameter nanoemulsions are obtained using the PIC method. It has been chosen as oil phase a mixture of carvacrol (EO) and medium chain triglycerides (MCT) as carrier oil. Several authors [24,25,27] reported the use of MCT for the formation of nanoemulsions with droplet sizes around 100 nm. The surfactant + co-surfactant consisted in a mixture of Tween 80 ® and oleic acid. As described by Solè et al. [33] nanoemulsions can be stabilized by a mixture of non-ionic surfactant (Tween 80 ®) and partially neutralized fatty acid. This technique consists of neutralizing a fatty acid, in this case oleic acid, with an alkaline aqueous solution added progressively along the emulsification path (KOH solution). The resulting fatty carboxylate acts as the ionic surfactant stabilizing the nanoemulsion.

So, the studied system is carvacrol/MCT-(OL-OT)/T80-water. To achieve stable and small droplet-sized nanoemulsions first of all the system phase behaviour is studied, and the phases found along the emulsification path determined.

The influence of composition variables is studied. To ensure the systematic study of these variables an experimental design is carried out. The chosen variables are the carvacrol ratio into the oil mixture, thus the EO is the active component to preserve fruits and vegetables against microbial growth, and the relationship between oleic-oleate/Tween 80 ® (OL-OT/T80).

McClemments et al. [23] proved that EO/MCT ratio has an appreciable impact on particle size and the surfactant required was 20% w/w to obtain a clear and stable nanoemulsion during 30 days. Using the PIC low-energy method, nanoemulsions can be formed with only 10% of surfactant mixture and the final composition and a 5% of oil mixture.

The (OL-OT/T80) ratio is studied in the range 30/60 to 60/40 in order to analyse the influence of the (OL-OT) in the droplet size and stability of the formed nanoemulsions.

2. Experimental

2.1. Materials

Oleic acid pharma grade (142659.1611) with purity 88% is purchased from Panreac. Synthetic non-ionic surfactant Tween 80 ® (P1754) and potassium hydroxide pellets 85% A.C.S Reagent is purchased from Sigma Aldrich. Carvacrol (Merck W224502) with purity >98% food grade is used. Medium chain triglycerides (MCT) oil (Miglyol 812) is purchased from Acofarma. The manufacturer reported a composition of 58.1% of caprylic acid (C8:0) and 41.9% of capric acid (C10:0). Water is deionized and further purified by mili-Q filtration.

2.2. Determination of equilibrium phases

All components are weighed in different tubs, which are sealed, homogenized with a vibromixer, and kept in a thermostatic bath at 25 °C to reach the equilibrium. Liquid crystalline phases are identified using crossed polarizers and small-angle X-ray scattering (SAXS).

2.3. Small-angle X-ray scattering

Small-angle X-ray diffraction scattering (SAXS) measurements are used to determine the structure of the liquid crystals obtained during the phase diagram determination. Measurements are performed in a Hecus X-ray Systems GMBH Graz, equipped with a Siemens Kristalloflex 760 (K-760) generator. The temperature of the samples is fixed using a Peltier Anton Paar (25–300 °C) controller. The radiation wavelength is 1.54 nm.

2.4. Nanoemulsion formation

First, the mixture of surfactants (Tween 80 ® and oleic acid) at desired composition is weighed. The oil mixture, composed by different carvacrol/MCT ratios, is added. According to the phase diagram, for each composition different amounts of water with their corresponding potassium hydroxide content are added drop by drop with continuous agitation with a vibromixer in order to reach a liquid crystal region or microemulsion phase. Several authors [33,34,35] reported the importance of reaching a single planar structure along the emulsification path for obtaining stable, small diameter O/W nanoemulsions. The mixture is hot into a test tube heater at 70 °C for the second formation step. Different SAXS analyses (data not shown) are performed in order to ensure the presence of liquid crystal structure at these temperatures. The raise of the temperature is done to provide lower viscosities to the crystalline phase so the incorporation of the components and agitation is easier. The second formation step is the addition of the remaining water at 25 °C drop by drop to the hot mixture while the tube is stirred with a vibromixer. The addition rate is one of the relevant parameters in nano-emulsion formation [36]. Adding the remaining water drop by drop in 2 min forms the nanoemulsion. After the nanoemulsion is formed the mixture is stirred for 1 more minute. The final water concentration of the emulsions is of 85% w/w, the surfactant mixture (oleic-oleate)/Tween 80 ® of 10% w/w and the oil mixture, carvacrol/MCT, the 5% w/w.

2.5. Droplet size

Dynamic light scattering measurements are performed by the Nanostructured Liquid Characterization Unit. 3D Dynamic light scattering (3DDLS) spectrometer (LS Instruments, Switzerland) equipped with a He–Ne laser ($\lambda = 632.8$ nm) is used, allowing the temperature control (25 °C) at a scattering angle of 90°. The light intensity correlation function is analyzed based on the multimodal method, whereas the z-average diameter is obtained by cumulant analysis. The reported droplet size is the mean of at least three independent measurements.

2.6. Transmittance measurements

The stability of the emulsions was determined by studying the variation of the transmittance (T) light (173°) using a Turbiscan Classic MA 2000. To normalize the T values and compare them independently from their initial values, the relative T after time is calculated as: $\Delta T_{rel} = [(T_t - T_{t0})/T_{t0}] \cdot 100$, where T_{t0} is the transmittance at time = 0 and t_t is the transmittance at a time = t.

2.7. Zeta potential measurements

Zeta potential of the nanoemulsions was measured by the Zetasizer Nano-Z (Malvern Instruments Ltd, Malvern, United Kingdom) equipped with a He-NE laser operated at $\lambda = 633$ nm. Three replicates of each sample were tested. Measurements were done at time 0 days and after 7 days in order to study the stability of the nanoemulsions.

3. Results and discussion

In order to study the formation of carvacrol-loaded nanoemulsions using PIC method the influence of the different phases found

along the emulsification path is studied through the pseudo-ternary phase diagrams. As many composition variables can affect the formation of nanoemulsion, such as final nanoemulsion composition (oil mixture final content, carvacrol/MCT ratio, final surfactant mixture content, (OL-OT)/T80 ratio and final water content) some of them were fixed and some were evaluated. For all formulations the final composition was set 85% w/w of water, 10% w/w of surfactant mixture and 5% w/w of oil mixture. The final oil %, 5%, containing the carvacrol EO, was fixed as high as possible ensuring the formation of stable, small sized-nanoemulsions. The influence of the carvacrol/MCT ratio and (OL-OT)/T80 ratio in the nanoemulsions properties were studied using an experimental design. The different emulsification paths where small-sized nanoemulsions can be obtained have been investigated through the study of the phase behaviour of the system.

3.1. Equilibrium phases

To study the nanoemulsion formation in the ionic system water/(oleic-oleate)-Tween 80 ®/carvacrol-MCT through the PIC method, the equilibrium phases found along of the different emulsification paths used on the experiments are identified. It is noticeable that the presence of different phases along the emulsification path is relevant for the obtaining of stable small diameter nanoemulsions. Morales et al. [35], using PIT (phase inversion temperature method) reported that the main requirement for the formation of O/W

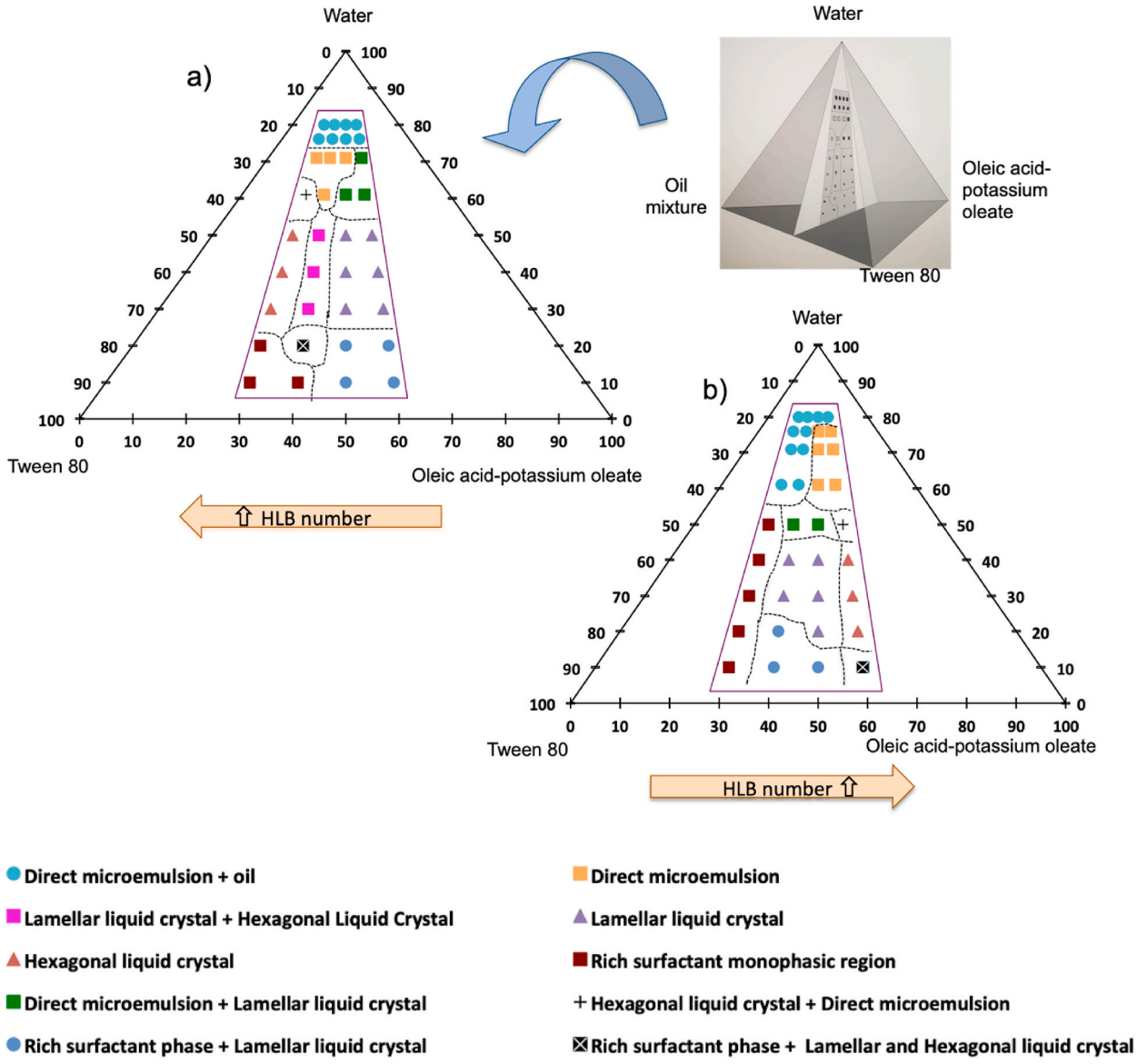

Fig. 1. a) Phase diagram for 30% of carvacrol in the oil mixture and for a 100% neutralization degree. b) Phase diagram for 30% of carvacrol in the oil mixture and for a 150% neutralization degree. For both diagrams oil mixture/total surfactant mass ratio is 0.5/1.

nanoemulsions is to achieve a complete solubilisation of the oil at the HLB temperature, in their case a bicontinuous microemulsion. When all the oil is incorporated before the cooling step high energy is not required for a fine dispersion. Solè et al. [33] and Pey et al. [37] used the phase inversion composition method (PIC), i.e. to prepare a concentrated mixture and progressively added water until the final composition of nanoemulsion. They reported small-sized nanoemulsions when a zone of direct or planar structure was crossed along the dilution process (emulsification path) without excess of oil. Other publications arrived to the same conclusions [38]. Therefore, the study of the phases crossed along the emulsification process (addition of water to the concentrate during the second step, section 2.4) is relevant in order to find suitable formulations and correlate them with their emulsification paths. Thus, partial phase diagrams were studied in order to know the phases crossed when water was added drop-by-drop added to the concentrate. Only for emulsifications paths without oil excess in the crossed structures the nanoemulsions can be obtained. In fact, due to the multiple variables involved in the system only the phase diagrams of the limits of the experimental design, i.e. the carvacrol/MCT ratios 30/70 and 60/40, are analyzed.

For every diagram the relationship carvacrol/MCT is fixed, as well as the oleic acid neutralization degree given by the potassium hydroxide added to the mixture. Previous experiments show that for (OL-OT)/T80 ratio lower than 30/70 of the total surfactant content nanoemulsions could not be formed. The crystal liquid zone for a ratio 20/80 is a narrower region than for higher (OL-OT)/T80 contents (data not shown) and probably the liquid crystal is not completely formed during the emulsification path lasting 2 min. In that short time not all the oil phase is well incorporated to the structure, and as previous authors reported [35,37,38] all the oil should be integrated in the structure and no excess is allowed if small droplet sized nanoemulsions with low polidispersity are required. For (OL-OT)/T80 ratios higher than 60/40 the phases studies shown that a single laminar liquid crystal zone does not appear along the emulsification path and the nanoemulsion formation was, therefore, not possible, according to Morales et al. [35] as not all the oil is incorporated in this phase before emulsification.

Chang et al. [28] reported nanoemulsions using a maximum 40% of carvacrol in the oil mixture, our preliminary experiments could obtain nanoemulsions even using a cavacrol/MCT ratio of 60/40 in the oil mixture although it required higher neutralization degrees. As previously reported [28], carvacrol contents lower than 30% could not form nanoemulsions. In this system, previous experiments showed that nanoemsulsions are not formed when no carvacrol is used.

In Fig. 1 the tetrahedron of the four main pseudo-components is shown. The different vertexes are water content, Tween 80®, oleic-oleate (oleic partly neutralized with KOH) and oil mixture composed by different ratios of carvacrol/MCT. In Fig. 1a and b the oil mixture-total surfactant mass relationship (OL-OT)+T80 is fixed at 0.5/1. In Fig. 1a and b the carvacrol/MCT ratio is fixed to 30/70. The difference between Fig. 1a and b is the neutralization degree used, being a stoichiometric relationship for Fig. 1a and a 50% excess of KOH in Fig. 1b. In both diagrams different (OL-OT)/T80 ratios are studied, such as 30/70, 40/60, 50/50, 60/40. Dotted lines are used to improve the understanding of the figures and the regions limits are no deeply established. The aim of the phase diagram determination is to help to understand the physicochemical changes that lead to the properly nanoemulsion formation.

A rich surfactant monophasic region is present in the further region from the water content and low (OL-OT)/T80 ratio. As increasing the (OL-OT)/T80 ratio, a laminar region appears coexisting with the rich surfactant phase. When the amount of water is increased the presence of different regions of liquid crystals occurs. To understand the behaviour of the system it is important to know the evolution of the HLB (hydrophilic-lipophilic balance) numbers when the (OL-OT)/T80 relationships are changed at the same neutralization degree. Tween 80 ® has an HLB number equal to 15.0 and oleic acid has HLB number of 1.0. When the potassium hydroxide is added to the system the oleic acid is neutralized to potassium oleate. Potassium oleate has a HLB number of 20.0. Due to the fact that oleic acid is a weak acid not all the oleic acid reacts with the potassium hydroxide and becoming potassium oleate. The final pH is measured in order to know the potassium oleate conversion degree and determine the HLB of the mixture. For Fig. 1a the HLB number of (OL-OT) is around 9, so when more Tween 80 ® is used more HLB number the mixture has according to the formula HLB surf mixture $= \sum$xi·HLBi where xi and HLBi are the surfactants mass fraction and pHLB of each surfactant in the surfactant mixture. The HLB number increases from right to the left. So, along the different (OL-OT)/T80 ratios the HLB number changes from 11.4 for a ratio 60/40 to a 13.2 for a ratio 30/70. While in Fig. 1b the HLB number increases form left to right as it will be commented later.

When the HLB number increases the liquid crystal formed passes from laminar, for relationship 50/50 and 60/40, to a hexagonal liquid crystal for a ratio of 30/70. As more HLB number more positive spontaneous curvature have the surfactant structures and the liquid crystal obtained passes from bilayers to cylindrical aggregates ordered in hexagonal structures. When the amount of water is increased the structures become oil-in-water microemulsions to finally lead into two separated phases, region where oil-in-water nanoemulsions could be obtained.

Fig. 1b shows the phase diagram for carvacrol/MCT ratio 30/70 with a neutralization degree of 150% of the stoichiometric needs. For Fig. 1b the HLB number of the OL-OT is 19.9. Nearly all oleic is in oleate form as more potassium hydroxide is used. In that case, unlike in Fig. 1a, when more (OL-OT)/T80 is used the more HLB number the mixture has. So, along the different (OL-OT)/T80 ratios the HLB number changes from 18.0 for a ratio 60/40 to a 16.5 for a ratio 30/70.

In Fig. 1b for 30/70 (OL-OT)/T80 content or lower no liquid crystal region is observed. The behaviour is the same than the reported for Mayer et al. [39] and only rich surfactant region is present. In this region as more water is added the spontaneous curvature of the surfactant structures is gradually increased reaching different kind of microemulsions, from inverse to direct. Finally, when the water content is high, the strong spontaneous curvature and the molecular solubilisation of surfactant in water expel the excess of oil and a two-phases region is found. As more OL-OT is used the HLB number is higher, as mentioned before, and liquid crystal regions appear. First of all, a lamellar + surfactant phase biphasic region appears, followed by a multiphasic region where lamellar and hexagonal liquid crystal are present at low percentage of water. As more water is added multiphasic regions become lamellar crystal liquid and hexagonal liquid crystal, respectively, from left to right. When water is added at high (OL-OT)/T80 ratios a direct microemulsion zone

is achieved. When more water is added, the spare oil is expelled and two separated phases are observed, direct microemulsion and excess of oil.

Fig. 2 shows the same tetrahedron cut but in this case the oil mixture is composed by a carvacrol/MCT ratio of 60/40. The same two different neutralization degrees are used as in Fig. 1. Fig. 2a corresponds to 100% of neutralization degree and Fig. 2b–a neutralization degree of 150%. Rich surfactant regions are composed by different kind of microemulsions, that probably passing from inverse to direct ones.

In Fig. 2, the behaviour of the system regarding the HLB number is the same than explained in Fig. 1. In Fig. 2a it can be observed that the presence of the rich surfactant zone is extended to higher amount of water than in Fig. 1a. At low water content, probably the phase corresponds to an inverse microemulsion but while increasing the water content, around 50%, birefringence at movement is observed, indicating a bicontinuous microemulsion. As more water is added a multiphasic region with lamellar liquid crystal where not all the oil has been incorporated to the structure is observed. In the case of a neutralization degree of 100% at all (OL-OT)/T80 studied ratios no monophasic crystal region appears.

For a 30/70 oleic acid ratio when increasing the water content it can be observed neither a single liquid crystal region nor a direct microemulsion. Therefore, the capacity of the oil phase to be retained in a stable structure is limited. When the amounts of water and oil are similar a bicontinuous phase with birefringence at movement appears. In the region where in Fig. 1a a single laminar liquid

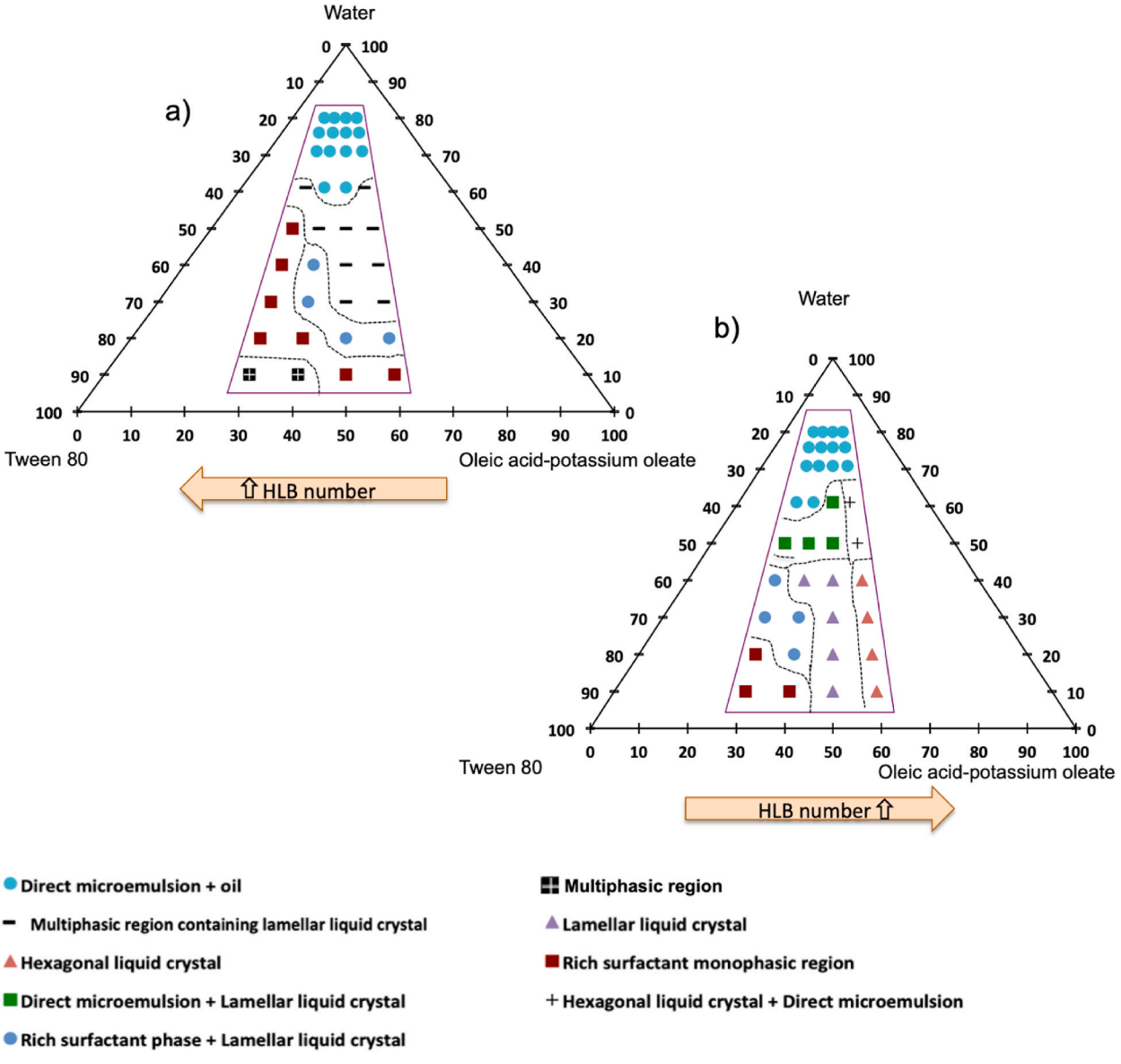

Fig. 2. a) Phase diagram for 60% of carvacrol in the oil mixture and for a 100% neutralization degree. b) Phase diagram for 60% of carvacrol in the oil mixture and for a 150% neutralization degree. For both diagrams oil mixture/total surfactant mass ratio is 0.5/1.

crystal exists, in Fig. 2a a multiphasic region appears with a lamellar crystalline structure. When 70% of water amount or higher is used the two-phase region where nanoemulsions could be obtained appears. However, as the neutralization degree is increased (Fig. 2b) monophasic regions are obtained only for (OL-OT)/T80 ratios 50/50 and 60/40. These regions are identified as lamellar liquid crystal, and as the spontaneous curvature is increased, because the HLB number increases, hexagonal liquid crystal appears.

Fig. 3 shows the SAXS patterns for some points of the different diagrams. In Fig. 3a a hexagonal structure can be observed with four peaks with a relationship between them of 1/0.57/0.7/0.25 indicating a hexagonal structure corresponding to the hexagonal region showed in Fig. 1a. As the (OL-OT)/T80 relationship is increased from 30/70 to 40/60 the peaks are less defined and a second relationship appears indicating the presence of different structures (lamellar and hexagonal) with a mixed relationship of the peaks corresponding both structures (Fig. 3b). Fig. 3c corresponding to a lamellar liquid crystal for the 40% w/w of surfactant mixture ((OL-OT)/80 ratio 50/50), 40% w/w of water and 20% w/w oil mixture with 60% w/w carvacrol and a neutralization degree of 150% presented a Bragg's repetition distance double than for the same composition but with half of carvacrol content (Fig. 3d). This data pointed out the fact that doubling the amount of essential oil the laminar liquid crystal obtained changed significantly resulting in a swollen structure.

3.2. Formation of the nanoemulsions

Nanoemulsions are formed by the PIC low-energy emulsification method described above, in which KOH aqueous solution is added to oil + surfactant mixtures. First, the minimum necessary amount of KOH aqueous solution is added in order to form a monophasic liquid crystal or a surfactant region (microemulsion). After the monophasic region is obtained water is progressively added drop-by-drop to obtain the nanoemulsions. Preliminary studies have shown the relevance to properly reach the liquid crystal zone in order to

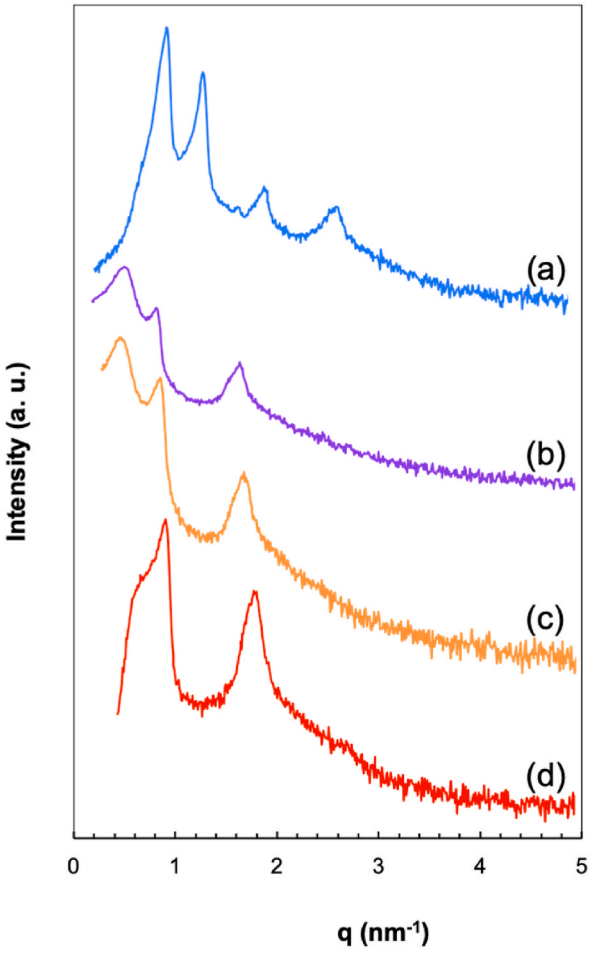

Fig. 3. SAXS pattern for composition (a) 40% w/w of surfactant mixture ((OL-OT)/80 ratio 30/70), 40% w/w of water and 20% w/w oil mixture with 30% w/w carvacrol and a neutralization degree of 100% (b) 40% w/w of surfactant mixture ((OL-OT)/80 ratio 40/60), 40% w/w of water and 20% w/w oil mixture with 30% w/w carvacrol and a neutralization degree of 100% (c) 40% w/w of surfactant mixture ((OL-OT)/80 ratio 50/50), 40% w/w of water and 20% w/w oil mixture with 60% w/w carvacrol and a neutralization degree of 150% and (d) 40% w/w of surfactant mixture ((OL-OT)/80 ratio 50/50), 40% w/w of water and 20% w/w oil mixture with 60% w/w carvacrol and a neutralization degree of 100%.

obtain nanoemulsions. If the emulsification path does not have a monophasic region without oil excess the nanoemulsion cannot be formed.

The importance of the oleic acid neutralization degree appears as a factor to be considered due to the produced changes in HLB number. Phase diagrams have shown that it is relevant the (OL-OT)/T80 ratio and the neutralization degree to assure the appearance of that monophasic region which provides the properly nanoemulsion formation.

For carvacrol/MCT ratio 30/70 and a neutralization degree of 100% nanoemulsions are obtained for a range of (OL-OT)/T80 ratios including 30/70 to 60/40. For (OL-OT)/T80 ratio 30/70 the mean diameter of the obtained nanoemulsions is 19 nm, as increasing the (OL-OT)/T80 ratio to 45/55 the mean diameter increases to 36 nm and for (OL-OT)/T80 ratio 60/40 to 40 nm. The phase diagram shows wide monophasic crystal regions that allow entrapping all the oil in the structure. All the retained oil in the lamellar structure is trapped in the droplets that appear when the liquid crystal bends to form those droplets. For (OL-OT)/T80 ratios away that range (i.e 20/80 and 70/30) macroemulsions are obtained instead of nanoemulsions. It is related to the fact that the liquid crystal monophasic region is too small and during the nanoemulsion formation there is not enough time to reach the equilibrium and properly form the liquid crystal, entrapping all the oil mixture into the structure. While using lower (OL-OT)/T80 ratio there is not enough oleic acid to change the spontaneous curvature of the system when water + KOH is added to obtain the PIC. When more carvacrol/MCT ratio is used, 60/40, macroemulsions are obtained instead of nanoemulsions because no monophasic liquid crystal or rich surfactant region is formed as previously discussed in Fig. 2a. In order to obtain a nanoemulsion using high carvacrol/MCT ratios the neutralization degree is increased to a 150%. For all (OL-OT)/T80 ratios nanoemulsions are formed but they presented very low stability and higher mean diameters 51–177 nm depending on the (OL-OT)/T80 ratio used.

3.3. Experimental design for formulation variables

Experimental design is used in order to obtain information about which composition variable has a significant behaviour for the obtaining of stable, small diameter nanoemulsions. As the phase diagrams showed, multiple variables could affect the resulting nanoemulsion. Therefore, an easier way to find out the relevance of each variable and their possible interactions is the development of an experimental design. A factorial 3^k + central point design is carried out, being k the carvacrol/MCT ratio in the oil mixture and the (OL-OT)/T80 ratio in the surfactant content.

Table 1 shows the performed experiments. The composition variables studied are carvacrol/MCT ratio and (OL-OT)/T80 ratio. Final oil mixture content (5 %w/w), surfactant content (10 %w/w) and water content (85 %w/w) are fixed as the neutralization degree (150%) for all experiments. Response variables are mean diameter of the nanoemulsions and the transmittance variation. Mean droplet diameter, PDI and zeta potential have been measured at initial time and after 7 days. It could be observed when the carvacrol/MCT ratio is 60/40 and more neutralization degree is needed higher PDI values presented the nanoemulsions with higher polidispersity. These formulations correspond to the experiments with more carvacrol, which presented during the emulsification path a lamellar liquid crystal with higher Bragg's distance as discussed in Fig. 3.

Nanoemulsions formed using a carvacrol/MCT ratio 60/40 presented poor stability values in terms of zeta potential, mean droplet diameter and increment of relative transmittance. In fact, one of them, (OL-OT)/T80 = 45/55, is completely destabilized after 7 days. For OL-OT)/T80 ratio 60/40 the emulsion at 7 days presented mean droplet diameter higher than 200 nm and the term nanoemulsion is not suitable anymore. Zeta potential measurement values are around −30 mV due the use of a mixture of non-ionic surfactant (Tween 80 ®) and an anionic surfactant (oleic acid-potassium oleate). The nanoemulsions with lower increase of droplet size also presented the lower variation of zeta potential. On the other hand, the nanoemulsions with higher zeta potential variations correlate with that ones with a higher ΔT_{rel}. Therefore, both measurements seem to give equivalent information to be used in the study of the stability of the nanoemulsions.

Table 1
Experiments carried out according to the experimental design. Composition variables are carvacrol/MCT ratio and (OL-OT)/T80 ratio. Response variables are nanoemulsions diameter (D) and transmittance variation days (ΔT rel), polidispersity index (PDI) and Zeta potential (mV) at initial time and after 7 days. Final oil mixture content (5 %w/w), surfactant content (10 %w/w) and water content (85 %w/w) are fixed, as the neutralization degree (150%), for all experiments.

		Time 0 days			Time 7 days			
Carvacrol/MCT ratio	(OL-OT)/T80 ratio	D (nm)	PDI	Zeta potential (mV)	D (nm)	PDI	Zeta potential (mV)	ΔT rel (%)
30/70	30/70	19	0.19	−32.4	26	0.24	−29.8	13
60/40	60/40	177	0.85	−19.7	>200	1.13	−11.1	45
60/40	45/55	51	0.83	−13.4	*	*	*	100
45/55	30/70	29	0.38	−17.8	82	0.43	−12.6	61
30/70	60/40	40	0.62	−32.9	56	0.65	−30.8	5
45/55	45/55	28	0.24	−32.5	29	0.28	−33.5	4
45/55	60/40	110	0.69	−28.6	118	0.67	−29.0	3
30/70	45/55	36	0.16	−27,7	87	0.20	−21.5	26
45/55	45/55	30	0.23	−34.9	33	0.25	−35.8	4
45/55	45/55	30	0.29	−33.4	36	0.31	−33.1	4
60/40	30/70	79	0.31	−13.6	105	0.75	−20.4	72

*Unavailable data due the nanoemulsion is not stable.

Fig. 4 shows the response surface developed using the mean diameter of the nanoemulsion. The mean diameters obtained using PIC method are in the low range of nanoemulsions and a minimum mean diameter is obtained regarding (OL-OT)/T80 ratio around 45/55 for a carvacrol/MCT ratio 30/70 as shown in Fig. 4a. From the equation of the surface response D = 440.18–6.07*carvacrol/MCT ratio – 16.70*(OL-OT)/T80 ratio + 0.05*(carvacrol/MCT ratio)^2 + 0.09*carvacrol/MCT ratio*(OL-OT)/T80 + 0.17*((OL-OT)/T80)^2 it can be calculated that the minimum is displaced to a 35/65 when the carvacrol/MCT increases to 60/40. As some authors reported [26] regarding the carvacrol ratio in the oil mixture exists a carvacrol/MCT ratio which nanoemulsions obtained present a minimum diameter as can be seen in Fig. 4b that shows the main influences of both composition variables. In fact, without carvacrol nanoemulsions cannot be formed. In this experimental design that minimum is out of the selected range because as high carvacrol amount as possible is desired. It is observed as more carvacrol/MCT ratio is used, the higher diameter nanoemulsions have. However, relatively monodisperse small-sized nanoemulsions are obtained.

The obtained nanoemulsions using ratios of (OL-OT)/T80 from 30/70 to 45/55 show very small mean droplet diameters varying from 19 nm for lower carvacrol content to 79 for higher carvacrol content. As reported by Da Silva et al. [40] only nanoemulsions of essential oils obtained by high-energy methods [41–43] reported smaller diameters than the ones reported in the present work. Most of the authors using high-energy methods reported nanoemulsions with higher diameters [18,21,44,45]. Therefore, it seems relevant to explore the use of low-energy methods to reach smaller diameters considering that low-energy methods have great advantages regarding energy requirements for production. Diameters of 19 nm for carvacrol/MCT ratio of 30/70 or diameters of 30 nm for ratios of 45/55 with low polidispersity and high stability presented a good potential to be incorporated into edible films in the future. Other authors [42,43] reported the formation of nanoemulsions using low-energy methods as PIT with an oil mixture of 1–3% w/w. In the present study the oil content is increased until 5% w/w with very small diameters.

As Chang et al. [28] reported nanoemulsions could not be formed using only carvacrol. Presumably carvacrol molecule, which has a phenol group, is preferentially located in the surface of the oil droplet, with the phenol group oriented to the water, as hypothesized by Gong et al. [48]. Carvacrol would provide some flexibility to the interphase, separating the charged or the highly hydrated headgroups of the surfactants with high HLB (oleate and T80), and allowing a stronger compaction of the surfactant mixture. It is known that different sized molecules of amphiphile compounds could stabilize the interfaces better than similar ones, achieving a better packaging [49]. As phase diagrams showed if more carvacrol is used more neutralization is needed. It could be attributed to the fact that with more neutralization, more repulsion leads the oleate molecules and they are more separated. Therefore, carvacrol has more space to be located in the interface.

On the other hand, carvacrol cannot be nanoemulsified without a carrier. MCT is needed to stabilize the nanoemulsions against Ostwald ripening. As reported by other authors [50–52] MCT is less soluble en water than carvacrol and it can help to prevent Ostwald ripening. Therefore, both carvacrol and MCT are required, and a certain relationship between carvacrol and MCT is needed.

Moreover, as it is shown in Fig. 1a, for the optimal ratio the nanoemulsion formation path has a large liquid crystal monophasic region, with all the oil incorporated. As discussed above, it is a requirement to form small and stable nanoemulsions by low energy methods that involve phase inversion. Fig. 4c shows the pareto chart for the studied variables. It can be seen that carvacrol/MCT content and (OL-OT)/T80 ratio have significant influence over the resulting diameters.

In order to analyse the destabilization mechanism, Fig. 5 shows the transmittance values for the nanoemulsion obtained using (OL-

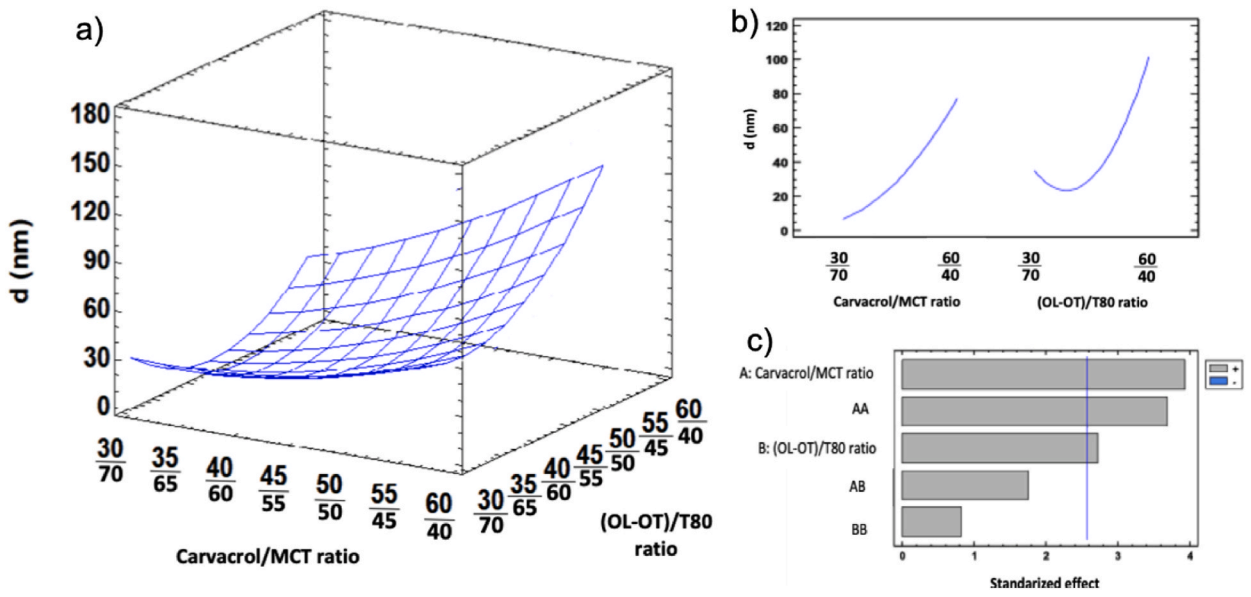

Fig. 4. a) Surface response for nanoemulsions diameters at different carvacrol/MCT ratios and (OL-OT)/T80 ratio b) main effects graphic c) pareto chart for nanoemulsions diameter. Final oil mixture content (5 %w/w), surfactant content (10 %w/w) and water content (85 %w/w) are fixed, as the neutralization degree (150%), for all experiments.

OT)/T80 ratio 30/70 and carvacrol/MCT ratio 30/70. The transmittance values decrease gradually but horizontally, indicating that the main destabilization mechanism is Ostwald ripening. The droplet size increases gradually leading to a lower transmittance value when time advances. As can be seen in Fig. 4, at the longer times, when droplets are big enough, some creaming appears. This result supports the hypothesis that the use of a very low soluble carrier, as the MCT, would stabilize the nanoemulsion against Ostwald ripening.

Once the influence of variables in droplet size is studied, another important response variable, the stability, is analyzed. For this purpose, Fig. 6a shows the surface response for nanoemulsions transmittance variation for all the experiments carried out at seven days after their preparation. At this time the most unstable nanoemulsions are totally destabilized. Lower transmittance variation ΔT_{rel}, indicates higher stability. The transmittance variation decreases until a carvacrol ratio of 45/55 being the nanoemulsions more stable and for higher ratios the transmittance variation rapidly increases.

Nanoemulsions formed using a carvacrol/MCT ratio 60/40 presented poor stability values in terms of zeta potential, mean droplet diameter and increment of relative transmittance. In fact, two of them (OL-OT)/T80 ratios 45/55 and 60/40 are not nanoemulsions after 7 days. For OL-OT)/T80 ratio 45/55 the emulsions is totally separated in two phases and for OL-OT)/T80 ratio 60/40 the emulsion presented mean droplet diameter higher than 200 nm and the term nanoemulsions is not suitable anymore. Zeta potential measurements values are around −30 mV due the use of a mixture of non-ionic surfactant (Tween 80 ®) and an anionic surfactant (oleic acid-potassium oleate). Some nanoemulsions presented quite similar zeta potential values through time indicating a good stability during the first week. The nanoemulsions with zeta potential variations correlate with that ones with a higher ΔT_{rel}. So, both measurements are complementary methods to study the stability of the nanoemulsions.

Mean droplet diameter and PDI have been studied with time as shown in Table 2. Nanoemulsions corresponding to the central point of the experimental design ((OL-OT)/T80 ratio 45/45 and carvacrol/MCT ratio 45/55) are stable during 28 days, increasing their mean droplet size from 30 nm to 44–48 nm approximately. PDI values are in all cases <0.5. Nanoemulsions with (OL-OT)/T80 ratio 30/70 and carvacrol/MCT ratio 30/70 and (OL-OT)/T80 ratio 60/40 and carvacrol/MCT ratio 45/55 are also stable during 28 days. These formulations correspond with the formulations with less ΔT_{rel} and a zeta potential with low variations during 7 days (Table 1).

Therefore, there is an optimum for a carvacrol/MCT ratio 45/55 (Fig. 6b). Probably, according to that discussed above, carvacrol is located in the interface but by itself it cannot form stable nanoemulsions because Ostwald ripening occurs and some amount of a very insoluble carrier oil (MCT) is needed. Fig. 6c shows that (OL-OT)/T80 ratio is not a significant variable but it seems that it could help to stabilize the nanoemulsions. The (OL-OT)/T80 is important as only a range 30/70-60/40 can form nanoemulsions. Out of this range nanoemulsification is not possible. It is well reported by several authors [53–56] that oil-in-water emulsions, O/W, has an optimum stability when the HLB of the surfactant mixture has a value, named preferred HLB, *p*-HLB, that only depends on the composition of the oil mixture. Emulsions prepared with the same surfactants mixture but in ratios giving higher or lower HLB than the preferred one show poorer stability results. This preferred HLB is reported for many oily substances [57]. The *p*-HLB of the oil mixture can be calculated as $p\text{-HLB}_{mixture} = \sum xi \cdot p\text{-HLB}i$ where xi and *p*-HLBi are the mass fraction and preferred HLB of each oil in the oil mixture. Therefore, stable emulsions are best formulated with emulsifiers or combination of emulsifiers having HLB values close to that preferred by the oil mixture. Formulations of the central point of the experimental design presented HLB values of the surfactant

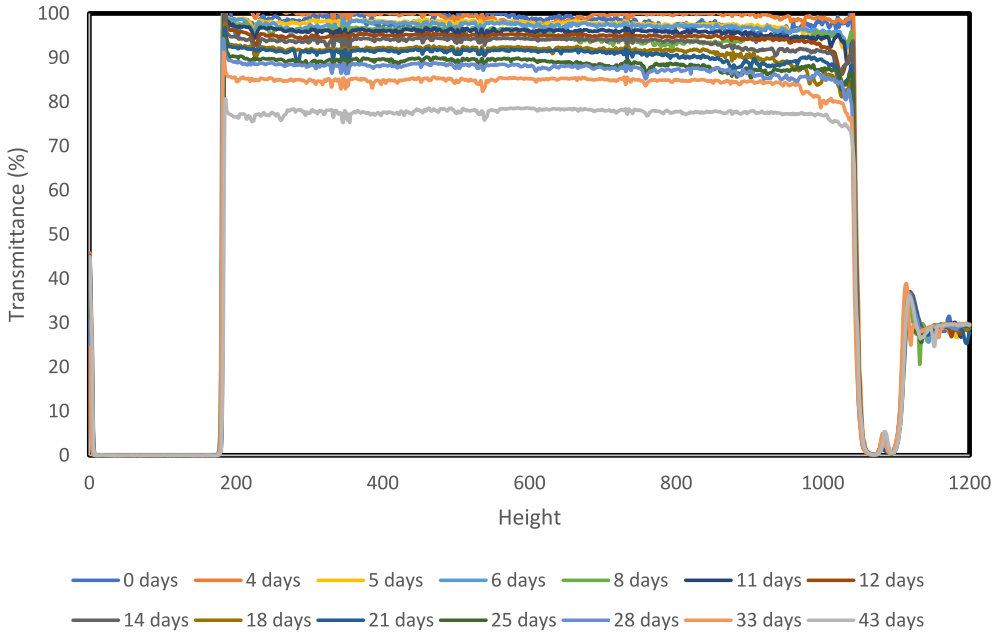

Fig. 5. Transmittance values for the nanoemulsion obtained using (OL-OT)/T80 ratio 30/70 and carvacrol/MCT ratio 30/70. Final oil mixture content (5 %w/w), surfactant content (10 %w/w) and water content (85 %w/w) are fixed, as the neutralization degree (150%), for all experiments.

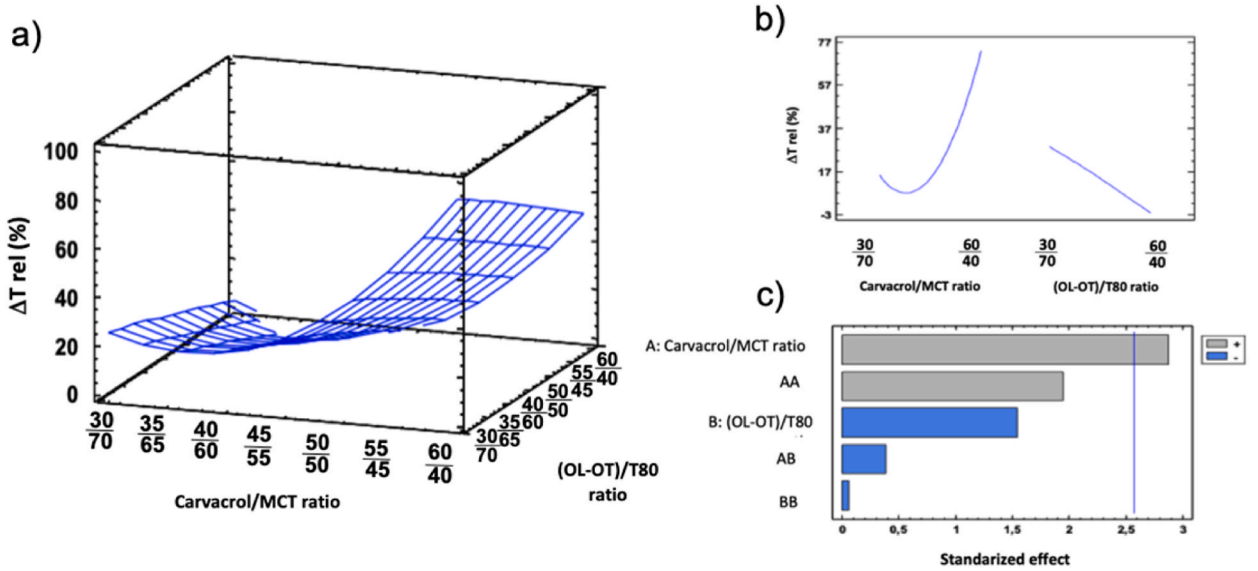

Fig. 6. Surface response for nanoemulsions transmittance variation at different carvacrol/MCT ratios and (OL-OT)/T80 ratio b) main effects graphic c) pareto chart for relative transmittance. Final oil mixture content (5 %w/w), surfactant content (10 %w/w) and water content (85 %w/w) are fixed, as the neutralization degree (150%), for all experiments.

Table 2
Experiments carried out according to the experimental design. Composition variables are carvacrol/MCT ratio and (OL-OT)/T80 ratio. Diameter (D) values and PDI for nanoemulsions though time. Final oil mixture content (5 %w/w), surfactant content (10 %w/w) and water content (85 %w/w) are fixed, as the neutralization degree (150%), for all experiments.

		t = 0 days		t = 7 days		t = 14 days		t = 21 days		t = 28 days	
Carvacrol/MCT ratio	(OL-OT)/T80 ratio	D(nm)	PDI	D(nm)	PDI	D(nm)	PDI	D(nm)	PDI	D(nm)	PDI
30/70	30/70	19	0.19	26	0.24	30	0.25	36	0.32	39	0.34
60/40	60/40	177	0.85	>200	1.13	*	*	*	*	*	*
60/40	45/55	51	0.83	*	*	*	*	*	*	*	*
45/55	30/70	29	0.38	82	0.43	*	*	*	*	*	*
30/70	60/40	40	0.62	56	0.65	95	0.87	*	*	*	*
45/55	45/55	28	0.24	29	0.28	36	0.32	44	0.39	47	0.43
45/55	60/40	110	0.69	118	0.67	133	0.75	135	0.82	141	0.83
30/70	45/55	36	0.16	87	0.20	*	*	*	*	*	*
45/55	45/55	30	0.23	33	0.25	35	0.27	42	0.27	44	0.30
45/55	45/55	30	0.29	36	0.31	37	0.31-	46	0.36	48	0.37
60/40	30/70	79	0.31	105	0.75	*	*	*	*	*	*

*Unavailable data due the nanoemulsions is not stable.

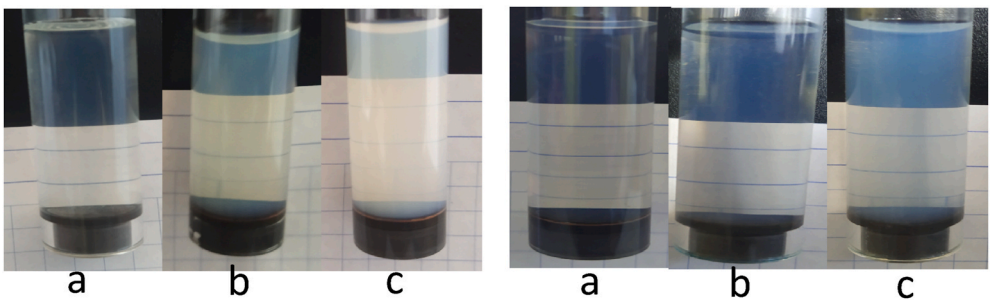

Fig. 7. Nanoemulsions obtained using carvacrol ratio/carrier oil 30/70 and oleic/Tween 80 ® ratio 30/70 for MCT carrier oil (left) and olive oil (right) at different times. a) Initial time b) 7 days and c) 24 days. Final oil mixture content (5 %w/w), surfactant content (10 %w/w) and water content (85 %w/w), (OL-OT)/T80 ratio 30/70 and carvacrol/MCT ratio 30/70 are fixed, as the neutralization degree (150%), for all experiments.

mixture similar to the required *p*-HLB values of the oil mixture providing the nanoemulsions to better stability behavior.

3.4. Comparison between olive oil and MCT as an oil carrier

As listed by Chaudhary et al. [6] several authors have used MCT as carrier oil for the obtaining of suitable food nanoemulsions. Chang et al. [28] reported that any long chain triglyceride oil was able to form spontaneous nanoemulsions using different Tween family surfactants. Olive oil presents less solubility in water than MCT, so if the nanoemulsions could be formed the resistance to Ostwald ripening destabilization would be higher [48–50].

Preliminary experiments showed that only for low oleic contents the nanoemulsion using olive oil could be obtained. Fig. 7 shows the aspect of the nanoemulsions obtained by the two oils at different times. While using olive oil the visual appearance is clearer than when using MCT, especially at long times.

Fig. 8 shows the size distribution of both nanoemulsions with time. It can be observed that when MCT is used as carrier oil the mean diameter of the nanoemulsion increases with time. The mean intensity peak is located at 11 nm at time zero and it magnifies until 32 nm after 28 days. In the case of olive oil that increment is not so noticeable, being the evolution from 7 nm to 16 nm. The shape of the curves also indicates the presence of a relatively wide diameter distribution in the case of MCT, while for olive oil the distribution is narrower. Comparative size distributions show that olive oil is able to slow down Ostwald ripening because the mean diameter does not increases as much that in the case of MCT. Therefore, the size distribution indicates the presence of bigger droplets when MCT is used instead olive oil.

Fig. 9 (left) shows the relative transmittance during 24 days for both samples. The instability is greater when MCT is used, reaching the 33% of variation at day 24. For the sample with olive oil the relative transmittance was changed only 11%. Fig. 9 (right) shows the

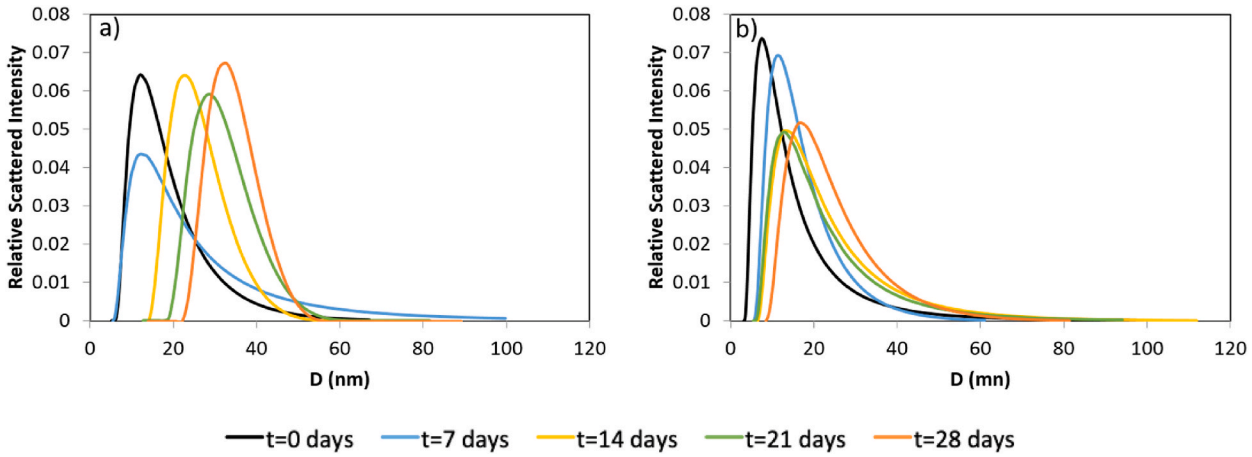

Fig. 8. a) Size distribution of nanoemulsion with time (0, 7, 14, 21 and 28 days) for sample with MCT b) Size distribution of nanoemulsion with time (0, 7, 14, 21 and 28 days) for sample with olive oil. Final oil mixture content (5 %w/w), surfactant content (10 %w/w) and water content (85 % w/w), (OL-OT)/T80 ratio 30/70 and carvacrol/MCT ratio 30/70 are fixed, as the neutralization degree (150%), for all experiments.

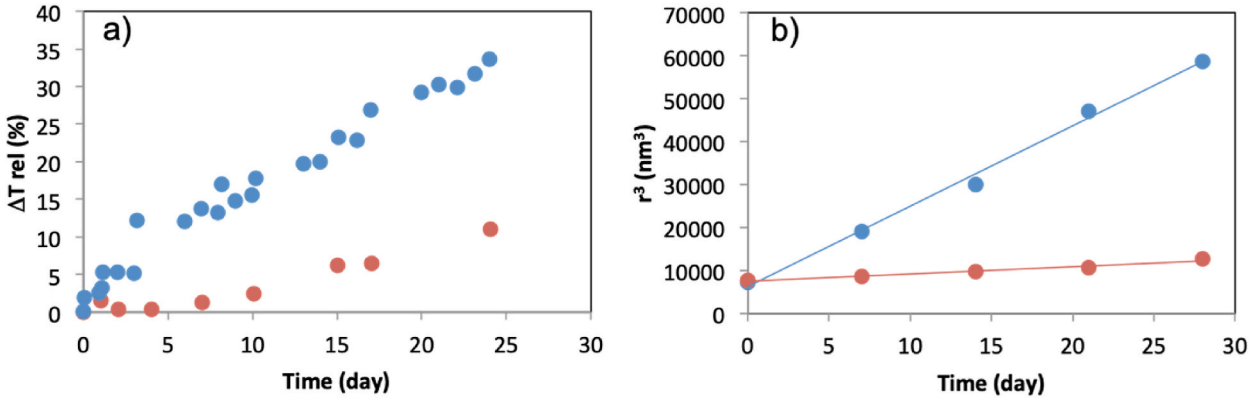

Fig. 9. a) Relative transmittance evolution with time for the sample with MCT (blue circles) and the olive oil (red circles). b) Evolution of the cubic radius of the nanoemulsions with MCT (blue circles) and the olive oil (red circles). Final oil mixture content (5 %w/w), surfactant content (10 %w/w) and water content (85 %w/w), (OL-OT)/T80 ratio 30/70 and carvacrol/MCT ratio 30/70 are fixed, as the neutralization degree (150%), for all experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

evolution of the cubic mean diameter during a month in order to estimate the Ostwald ripening rate. It can be calculated from the slope of the linear fitting, being 1870 nm/day while MCT is used and 168 nm/day when using olive oil. Ostwald ripening is the main destabilization mechanism, the addition of more insoluble oil improves stability, and moreover it is a cheaper and more natural alternative than the MCT.

4. Conclusions

The phase inversion composition low-energy method (PIC) is used for the formation of oil-in-water nanoemulsions in the carvacrol-MCT/Tween 80 ®-oleic acid-potassium oleate/water system. The final oil mixture content is 5 %w/w, which is a percentage higher than most of the nanoemulsions previously reported [19–22,44,45]. Some formulations presented smaller diameters (19–30 nm) than the previously used for edible film formation, including nanoemulsions obtained by PIT method [46,47] and high-energy emulsification methods [44,45].

In order to obtain small-sized, stable nanoemulsions it is necessary that the emulsification path passes through a region where liquid crystal or direct microemulsion appear without excess of oil. When water is added, the induced change in the spontaneous curvature of the surfactant induces the entrapment of all the oil present in the liquid crystal or microemulsion into the droplets, without the requirement of high shear or other high-energy method.

The optimization of the nanoemulsion formulation using experimental design shows an optimal value for a (OL-OT)/T80 ratio regarding the droplet size, that moves to lower ratios when the carvacrol/MCT ratio is increased. Therefore, the influence of one variable depends on the value of the second one. In these cases, the use of surface response obtained by experimental design is especially useful.

Although in the studied range the nanoemulsion mean diameter increases with the carvacrol/MCT ratio, an optimum ratio needs to exist for low values out of the range because nanoemulsions cannot be formed without carvacrol, indicating that carvacrol seems to help the packaging of the surfactant mixture in the droplets interface. MCT strongly slows down the Ostwald ripening destabilization mechanism and without them nanoemulsions cannot be formed either. Experimental design shows an optimum value for the carvacrol/MCT ratio 45/55 in terms of stability measured by relative transmittance changes along time. The results obtained with zeta potential confirm the conclusions reached with the measurement of transmittance.

The addition of another more water-insoluble oil carrier as olive oil, instead of MCT, shows an improvement of the nanoemulsions stability against Ostwald ripening reducing the change of relative transmittance from 33% to 11% in one week. The use of olive oil does not change the diameter of the nanoemulsion.

Author contribution statement

Esther Santamaria: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Alicia Maestro: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper. Susana Vilchez: Analyzed and interpreted the data. Carme González: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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