Self-assembly of Tween 80 micelles as nanocargos for oregano and *trans*-cinnamaldehyde plant-derived compounds

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Abstract

The self-assembly of Tween 80 (T80) micelles loaded with plant-based oregano essential oil (OR) and trans-cinnamaldehyde (TCA) was studied. The effect of different factors, including the surfactant to oil ratio, the presence of sodium chloride, thermal treatment, and dilution on their formation and physicochemical stability was evaluated. The creation of nano-cargos was confirmed by TEM. The self-associated structures had z-average droplet diameters of 92 to 337 nm without any energy input. Whereas addition of 10% (w/v) NaCl prevented the formation of oregano essential oil nano-assemblies of T80, swollen micelles containing TCA were successfully produced. Moreover, the OR or TCA loaded-micelles had only a slight droplet size variation upon thermal treatment. Ultimately, their antibacterial activity analysis against some food pathogens revealed that the encapsulation of OR and TCA within micelles crucially improved their antibacterial activity. These straightforward and cost-effective designed systems can be applicable in different products, including foods and agrochemicals.

Keywords*: Spontaneous emulsification; self-assembly; swollen micelles; Tween 80; oregano essential oil; trans-cinnamaldehyde.*

1. Introduction

Essential oils (EOs) are secondary metabolite compounds of different plants, which can be used as an alternative for synthetic preservatives or flavouring agents in food, cosmetic and pharmaceutical applications (Sedaghat Doost, Van Camp, Dewettinck, & Van der Meeren, 2019). Among these, oregano essential oil (OR) extracted from *Origanum compactum* top flowers and *trans*-cinnamaldehyde (TCA), the major compound present in cinnamon essential oil, have attracted considerable interest due to their strong antimicrobial, antioxidant, and antiradical properties (Sedaghat Doost, Devlieghere, Dirckx, & Van der Meeren, 2018; Sedaghat Doost, Dewettinck, Devlieghere, & Van der Meeren, 2018). Despite their desirable properties, these oils have a very low water solubility and strong scent. Emulsions can be used as a delivery system for hydrophobic bioactive components such as essential oils to increase their solubility, bioaccessibility, and to protect them from environmental stresses (Chang, McLandsborough, & McClements, 2013; Sedaghat Doost, Nikbakht Nasrabadi, Kassozi, Dewettinck, Stevens, & Van der Meeren, 2019). The emulsification methods can be classified into high-energy and low-energy techniques. High-energy methods, which are widely used to prepare conventional emulsions and nanoemulsions, are based on high-power equipment (e.g. high-pressure homogenizer or microfluidizer) used to break the dispersed phase into fine droplets. Low-energy methods, on the other hand, which require no special equipment, are based on controlling the interfacial properties of the oil and aqueous phases. Low-energy methods have recently attracted particular interest due to their low cost, simplicity and suitability for heat-sensitive or volatile compounds. Nevertheless, they are not commonly used in the industry due to the lack of sufficient knowledge about their mechanisms and effective factors. Moreover, these methods are limited to some types of oils and surfactants and high surfactant-to-oil ratios (SORs) (Chang, McLandsborough, & McClements, 2013; Komaiko & McClements, 2015). Spontaneous or self-emulsification is a low-energy method in which the dispersed phase, surfactant, water, and excipients are mixed in different ways (Shahidzadeh, Bonn, Meunier, Nabavi, Airiau, & Morvan, 2000). For instance, a lipid compound and a surfactant are usually mixed together and then are added dropwise to the continuous phase. It is believed that small oil droplets will be spontaneously formed as a result of the interfacial turbulence triggered by the surfactant movement from the oil phase to the aqueous phase due to its affinity. This method has been utilized as a delivery system for essential oils, such as carvacrol (Chang, McLandsborough, & McClements, 2013), cinnamon oil (Yildirim, Oztop, & Soyer, 2017), clove bud oil and thyme oil (L. Zhang, Critzer, Davidson, & Zhong, 2014). However, Yildirim, Oztop, and Soyer (2017) used relatively high SORs (i.e. 1 to 20) and a combination of coconut and cinnamon oil in the lipid phase. In another study by Chuesiang, Siripatrawan, Sanguandeekul, McLandsborough, and Julian McClements (2018) cinnamon oil nanoemulsions containing Tween 80 (T80) were prepared by the phase inversion temperature method at SOR = 1, they had a relatively large z-average droplet diameter larger than 600 nm. A high SOR has an adverse effect not only on the taste; but also from an industrial viewpoint, it is not economic. Furthermore, it is more favoured to formulate a delivery system with a high loading of active compounds because dispersing of an essential oil in another hydrophobic compound may limit its functionality due to the reduction in solubility or hydrophobic interaction which decreases the sensitivity to the bacterial outer layer.

Another way of preparation is mixing the lipid phase with a surfactant dissolved in water (Luo, Zhang, Pan, Critzer, Davidson, & Zhong, 2014; Y. Zhang, Chen, Critzer, Davidson, & Zhong, 2017). In this work, we used this approach by dissolving Tween 80 (polysorbate 80) non-ionic surfactant in the water phase and then adding the lipid phase to the aqueous solution, resulting in the formation of nano-assemblies carrying the lipid phase. Theoretically, an amphiphilic surfactant, T80 in this case, tends to become adsorbed between the oil and water phases due to the affinity for both phases. OR and TCA both have a relatively high water solubility and thereby diffuse through the intervening phase. Thus, oil molecules will be taken up by T80 swollen micelles (SMs) from both the oil layer and the dissolved oil molecules within the aqueous phase.

The novelty of our work lies in the preparation of OR and TCA loaded self-assembly structures without adding a second hydrophobic compound and without mechanical energy input, using a relatively low surfactant concentration. Moreover, to our knowledge, this is the first study to fabricate SMs from oregano EO using spontaneous emulsification, and to investigate their formation and physicochemical stability. Unlike previous studies where at least stirring was applied in the presence of a high emulsifier concentration, an attempt was made to explore a straightforward and economic preparation approach for essential oil colloidal dispersion. The antibacterial activity of the designed nanocarriers against some meat pathogens was investigated to see if these SMs can be potentially used for instance as a washing agent for animal carcasses or as a marinade. These designed systems may also be applicable as an antimicrobial washing agent for fresh vegetables or for hard surfaces.

2. Materials and methods

2.1. Materials

Oregano essential oil (Pranarôm, Belgium) extracted from top flowers of *Origanum compactum* plants was used without further purification. *Trans*-cinnamaldehyde and T80 were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Ultrapure water purified by a Milli-Q filtration system (0.22µm) (Millipore Corp., Bedford, MA, USA) was used for the analyses and preparation of all aqueous solutions.

2.2. Preparation and visual appearance characterization

Either of TCA or OR (0.2 ml) was added to a 3.8 ml aqueous solution containing variable amounts of T80 to produce surfactant to oil ratios (SORs) of 0.001 to 1 (w/w). The SORs were calculated based on the oil's density (0.93 g/ml and 1.05 g/ml for OR and TCA at 25 °C) measured using a density meter (Anton-Paar DM5000).

In order to evaluate the effect of salt on the formation of self-aggregated structures, sodium chloride (10% w/v) was added to the aqueous phase. The samples were stored in glass vials at 25 °C with no stirring or movement prior to analysis.

After recording the visual appearance, the vials were turned over to make a homogenous and representative mixture prior to analyses and were left for at least 10 min to ensure complete separation of left oil molecules.

2.3. Size and surface charge determination

Photon correlation spectrometry (Model 4700, Malvern Instruments, U.K.) was employed to determine the z-average mean droplet diameter of the emulsions at a scattering angle of 150° at 25°C. The z-average diameter was obtained by cumulant analysis of the light intensity correlation function, whereas the intensity-weighed particle size distribution was estimated by multimodal data-analysis. Each individual measurement was an average of 10 runs. The reported z-average particle size is the mean of at least three replicates.

A Zetasizer 2c (Malvern Ltd, UK) was used to determine the zeta potential (ζ) of OR and TCA SMs (at SOR = 1) by converting the electrophoretic mobility using the Helmholtz-Smoluchowski approximation. The samples were diluted in 10 mM KCl solution prior to analysis to prevent multiple scattering.

2.4. Aqueous solubility determination

The aqueous solubility of oregano EO has been evaluated in our previous study (Sedaghat Doost, Stevens, Claeys, & Van der Meeren, 2019), whereas in this work the TCA solubility was determined. The latter was determined using a total organic carbon (TOC) analyser (Shimadzu 5000, Shimadzu Scientific Instruments Inc., Japan) according to the previously reported method by Sedaghat Doost, Stevens, Claeys, and Van der Meeren (2019) with minor modifications. Briefly, mixtures containing 5% (w/v) of TCA in water were prepared in the absence and presence of 10% sodium chloride. They were left to be stirred for five hours at 20 rpm using a rotational stirrer (SB3, Stuart, UK) at 20 °C. In order to separate the oil phase from the aqueous phase, the samples were centrifuged for 20 min at 3076 ×g at 20 °C. The solubility of TCA was calculated from the total organic carbon content (ppm) of the aqueous phase, considering the carbon content of TCA (C_9H_8O), i.e. 81.82%.

2.5. Encapsulation efficiency

The concentration of encapsulated oregano essential oil and TCA within SMs was determined using high performance liquid chromatography (HPLC, Agilent, Belgium) with a C18 kinetex (Phenomenex) column (150 × 4.60 mm, particle size = 2.6 µm, pore size = 100 Å) using a UV-Vis detector (Agilent G1315B Diode Array) set at 280 nm. A small amount of micellar dispersion was collected from the center of the opaque layer using a needle. 10 µL of the sample diluted in acetonitrile was injected. An acetonitrile - water gradient was used based on the method reported by Sedaghat Doost, Stevens, Claeys, and Van der Meeren (2019) with 0.5 ml/min flow rate. The HPLC chromatograms were analyzed using Chemstation (Agilent) software. The calibration curves of pure OR essential oil (0.006 – 0.05 % w/v) and TCA (0.0005 – 0.003 % w/v) dissolved in acetonitrile were obtained in the same manner. The encapsulation efficiency (EE) was determined as the ratio of the amount of oil present within the formed emulsions to the initial added amount (5% v/v). As oregano essential oil contains different components, the carvacrol peak area was considered to calculate its EE.

2.6. Transmission electron microscopy (TEM)

A 2 µL drop of OR essential oil- or TCA-loaded micelles (in water or NaCl) was diluted in Milli-Q water/ NaCl solution and placed on a formvar-coated copper single slot grid (Agar Scientific, Stansted, UK). The copper grid was left to be dried under a fume hood at ambient temperature covered by a lid from a petri dish. TEM photographs were recorded using a JEOL JEM 1010 (Jeol, Ltd, Tokyo, Japan) transmission electron microscope equipped with a Veleta side mounted CCD camera (EMSIS GmbH, Muenster, Germany).

2.7. Impact of thermal treatment and dilution

The stability of the selected formulations at SOR = 1 against heat treatment and dilution was studied. The dispersions were incubated in a water bath at temperatures of 20, 50, and 80 °C for 30 min followed by cooling down to room temperature under running tap water prior to droplet size determination. In addition, the effect of dilution was also studied by diluting into Milli-Q water at different levels (1 – 50 times, v/v). The mean droplet size variation and the optical turbidity at 600 nm were evaluated after 2 h storage at 20 °C.

2.8. Antibacterial activity

The minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) of oregano essential oil and TCA formulation as well as of bulk oregano and TCA oils were evaluated against some food pathogens using the assays adopted from a study by Sedaghat Doost, Dewettinck, Devlieghere, and Van der Meeren (2018) with no modification. *Escherichia coli O157:H7* (Verocytotoxin-wildtype, beef carpaccio, MB 3885), *Listeria monocytogenes* (liver paté, LMG 23192), and *Salmonella* Typhimurium (pork, 2011/01430) were selected. The inhibition zone diameter was also obtained using the previously reported method by Sedaghat Doost, Devlieghere, Dirckx, and Van der Meeren (2018) with minor modifications. Wells with a 10 mm diameter were punched to the Muller-Hinton agar surfaces using a sterile borer and filled up with 70 µl of the concentrated emulsions. To compare the antibacterial activity of bulk oil (OR essential oil or TCA) and the oils encapsulated within SMs, a similar amount of oil present in 70 µl of emulsions dispersed in water was added to the wells. This amount was determined based on the obtained encapsulation efficiency using HPLC analysis for SOR = 1.

2.9. Statistical analysis

The results are expressed as mean ± standard deviation. The statistical analyses were performed using SPSS software (IBM® SPSS® Statistics, Version 26, 2019). ANOVA one-way analyses was done to compare the significant ($p < 0.05$) differences followed by Tukey post hoc test between the means. To compare the significant difference between two means, independent t-test was used.

3. Results and discussion

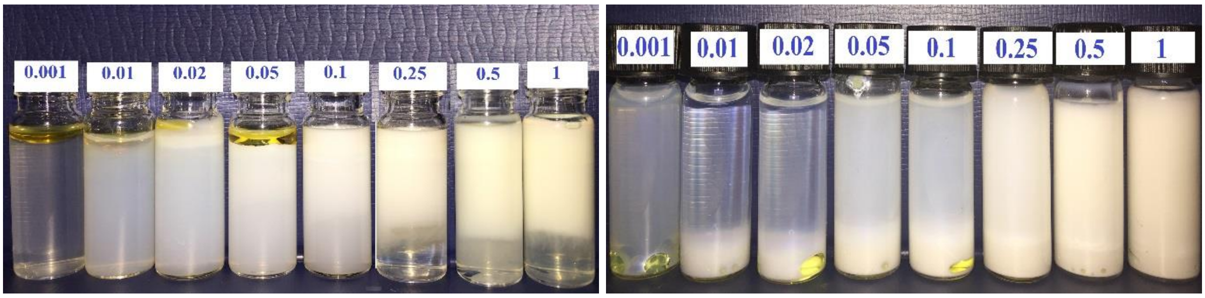
3.1. Influence of SOR, oil type and salt

One of the driving factors for the properties of dispersed swollen micelles is the surfactant to oil ratio. Initially, the effect of SOR (0.001 – 1 w/w) on the visual appearance of OR essential oils and TCA formulations with a constant lipid content (5% v/v) was monitored. It should also be mentioned that stirring has no considerable influence on the spontaneous emulsification process, except from the fact that the rate of the formation increases by providing a larger interfacial area.

It was evident from the visual appearance that the interfacial area became cloudy either upward or downward along the vials during incubation (Figure 1a&b); TCA and OR essential oil have a higher and lower density compared to water, respectively, whereas both have a lower density than 10% NaCl. The rate of cloudy layer growth was faster and more pronounced by increasing the SOR up to 1, meaning that as more micelles were provided to the oil molecules, more and faster solubilisation of the oils within T80 micelles occurred.

a) Oregano EO + T80 in water

b) TCA + T80 in water



c) Oregano EO + T80 in 10% NaCl

d) TCA + T80 in 10% NaCl

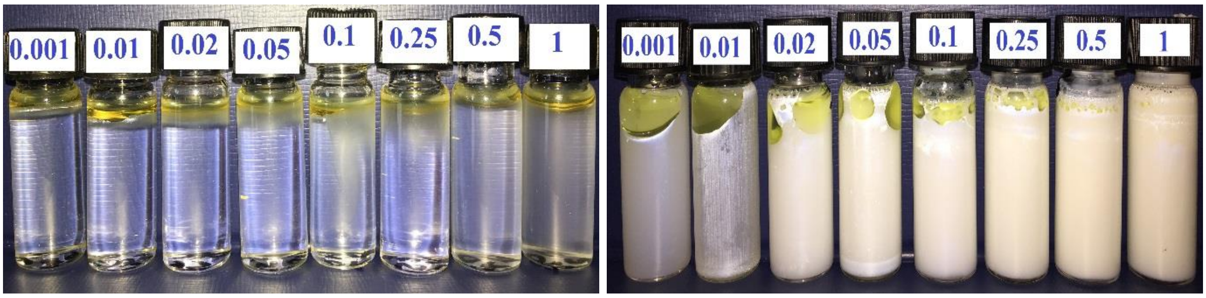


Figure 1. Visual appearance of mixture of either oregano EO (a,c) or TCA (b,d) and an aqueous solution of T80 at different surfactant to oil ratios (0.001 – 1 w/w) in the absence (a,b) and presence of NaCl (10% w/v) (c,d) after 14 d storage at 25 °C.

Although after 14 days of incubation, a yellow layer of oil was still observed, this layer was significantly less pronounced at the highest studied SOR value (i.e. 1). It was also visible that at SOR = 0.001, the relevance of oil-loaded micelles was limited, irrespective of the oil composition because at this SOR the number of micelles present to solubilize the oil phase is limited. Nevertheless, these samples looked hazy showing that also in these samples SMs entrapped a fraction of the oil phase.

Sodium chloride salt is often used to improve some properties of particularly food products. We therefore studied whether it was possible to add NaCl to the aqueous phase during encapsulation of the oils. Surprisingly, addition of sodium chloride prevented the creation of OR essential oil loaded-SMs as there was no similar turbidity gradient that was observed in the mixtures without added salt even at the highest SOR (Figure 1c). In terms of TCA, on the other hand, turbid mixtures were formed which became more opaque and milky by increasing the SOR, particularly starting from SOR > 0.01 (Figure 1d). This indicated that the formation of SMs in the presence of salt depends mainly on the lipid phase composition. These observations were implicitly consistent with our previous studies, revealing that oregano EO nanoemulsions emulsified by T80 and prepared using a high energy method were immediately broken down if sodium chloride was added to the system, whereas TCA nanoemulsions were resistant to the addition of NaCl (Sedaghat Doost, Dewettinck, Devlieghere, & Van der Meeren, 2018; Sedaghat Doost, Sinnaeve, De Neve, & Van der Meeren, 2017).

It is well known that in the mixture of aqueous T80 solution and a lipid phase, T80 surfactant molecules organize themselves into aggregates. Theoretically, three possible mechanisms have been proposed for the self-organization of the oil and surfactant molecules through spontaneous emulsification (Davies & Rideal, 1961; López-Montilla, Herrera-Morales, Pandey, & Shah, 2002; Shahidzadeh, Bonn, Aguerre-Chariol, & Meunier, 1999). First of all, one of the substances may diffuse into the other one. Moreover, interfacial turbulence may occur, leading to a reduction of interfacial tension (IFT) in the interface region.

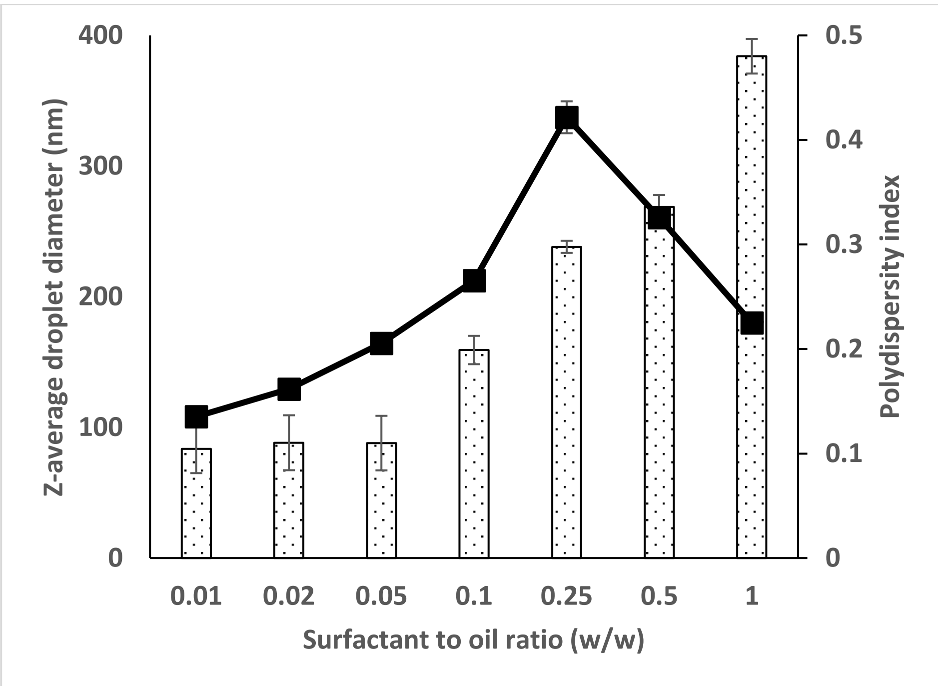
Last but not least, a negative IFT may be obtained when the surface pressure is higher than the IFT between the two pure phases ($\gamma = \gamma_0 - \Pi$). It is usually critical to discriminate which mechanism is responsible for the formation of self-aggregate structures because they may simultaneously occur (Riehm, Rokke, Paul, Lee, Vizanko, & McCormick, 2017). The lack of self-emulsification in oregano essential oil mixtures in the presence of NaCl is surprising for two reasons. Despite the fact that salt reduces the aqueous solubility and diffusivity of oil molecules, it has been shown in our previous study (Sedaghat Doost, Stevens, Claeys, & Van der Meeren, 2019) that oregano EO has still a considerable solubility in water, even in the presence of 10% NaCl. Thus, the second above-mentioned mechanism should still play a role but to a lesser extent. In case of TCA, we determined its aqueous solubility based on the TOC content of water that was equilibrated with TCA. The addition of 10% sodium chloride led to a reduction in the TCA solubility in water from 1.62 to 1.12 g/L. It is obvious that sodium chloride lowered the aqueous solubility of both studied lipid compounds but only OR essential oil was not able to be taken up through the micelles. Secondly, the head groups of T80 molecules turned out to become dehydrated in the presence of salt in previous studies. Hence, the T80 molecules acquired a higher affinity towards hydrophobic surfaces by making the aqueous phase a less appropriate solvent. It means that the surface pressure increases by the accumulation of T80 molecules at the interface and the interfacial tension might become more negative, which is a promoting condition for spontaneous emulsification. However, the addition of salt has been shown to increase the IFT. For instance, the IFT between palm oil and T80 aqueous solution was found to become substantially increased by sodium chloride addition (Ramly, Zakaria, & Naim, 2016). In another study, the presence of salt in the aqueous phase led to an increase in IFT between water and hydrocarbons (Lima, Melo, Baptista, & Paredes, 2013). Moreover, it seems that there are contradictory reports in the literature about the effect of salt on the critical micelle concentration (CMC) of non-ionic surfactants. For instance, we found that NaCl did not change CMC of T80 but an increase in IFT between a triglyceride and T80 aqueous solutions was observed (Sedaghat Doost, Stevens, Claeys, & Van der Meeren, 2019).

Next, the mean particle diameter of the SMs was evaluated and the results are given in Figure 2. The oregano essential oil-loaded SMs had a z-average mean diameter ranging from 108 to 337 nm, whereas smaller micelles (i.e., 92 to 185 nm) were self-organized when the lipid phase was TCA (Figure 2a&b). A sharp decrease in particle size from SOR = 0.25 to 1 occurred when the lipid phase was oregano essential oil, which was opposite to the size distribution of TCA-loaded micelles where an increase was observed. It has been shown in previous studies that smaller particles are formed at a higher SOR (Davidov-Pardo & McClements, 2015; Saberi, Fang, & McClements, 2013), which is opposite to what our results indicated for TCA-loaded SMs and to some extent for oregano essential oil-loaded SMs. Moreover, Davidov-Pardo and McClements (2015) reported that at higher SORs there was an increase in the polydipersity index (PDI). They attributed the particle size decrease to the lower interfacial tension and the PDI increase to the formation of a bimodal size distribution due to the simultaneous formation of nanoemulsions and microemulsions. However, the studied SOR in most of these previous studies was higher than 1, which is different from our designed system, in which excess of oil was present in all samples. In agreement with our results, Zeng, Zhou, Wang, Huang, Zhan, Liu, et al. (2010) also observed a high PDI (0.5) when essential oil loaded O/W microemulsions were formulated by polysorbate 80 micelles. Nevertheless, our experiments did not show an overall logical relationship between the particle size and SOR. This controversy has been previously reported by several studies (Mora-Huertas, Fessi, & Elaissari, 2011; Saberi, Fang, & McClements, 2013).

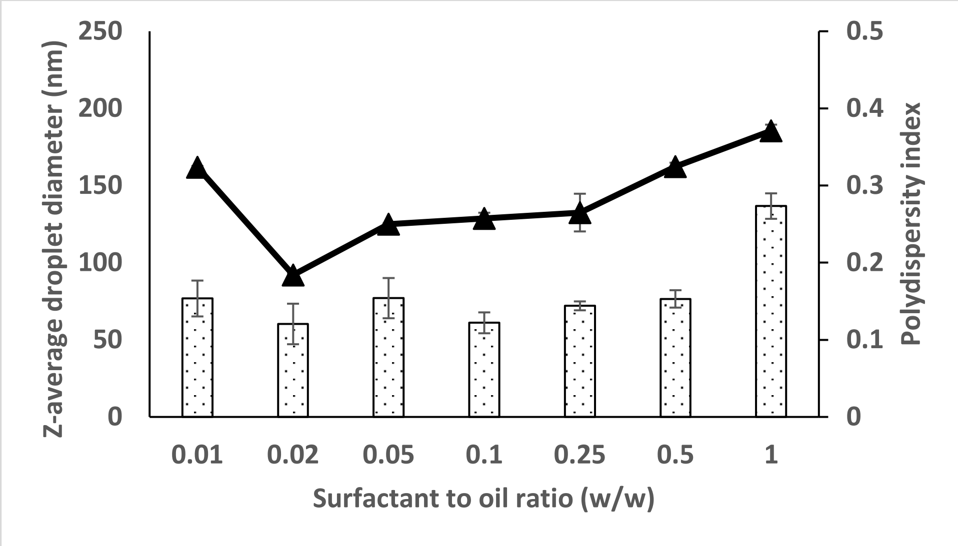
In fact, this is a self-assembly phenomenon driven by different factors, including physicochemical properties, composition, and structural organization that may differ over time and space (Saberi, Fang, & McClements, 2013) and can hardly be controlled. As no mixing was applied in our preparation procedure, it means that the uptake of oil by the micelles was largely a diffusion-driven process, which typically takes a very long time for equilibration, as can be clearly seen from the turbidity gradient in most samples in Figure 1, even after 14 days of incubation. Whereas faster equilibration could have been realised by stirring, the latter was not applied to be sure that the uptake of essential oil (components) was due to spontaneous uptake into surfactant aggregates, rather than by shear-induced emulsification.

Particle size analysis also revealed that there was no clear trend when sodium chloride was added to the aqueous phase as the z-average size varied between 140 and 206 nm (Figure 2c), indicating that smaller aggregates were formed when NaCl was present. An explanation that may be put forward is the lower aqueous solubility of TCA which reduces the micellar solubilisation. This could be visually observed as the oil layer was thicker and more pronounced in comparison to TCA-loaded formulations in the absence of NaCl. Moreover, the addition of NaCl led to higher PDI values.

a)



b)



c)

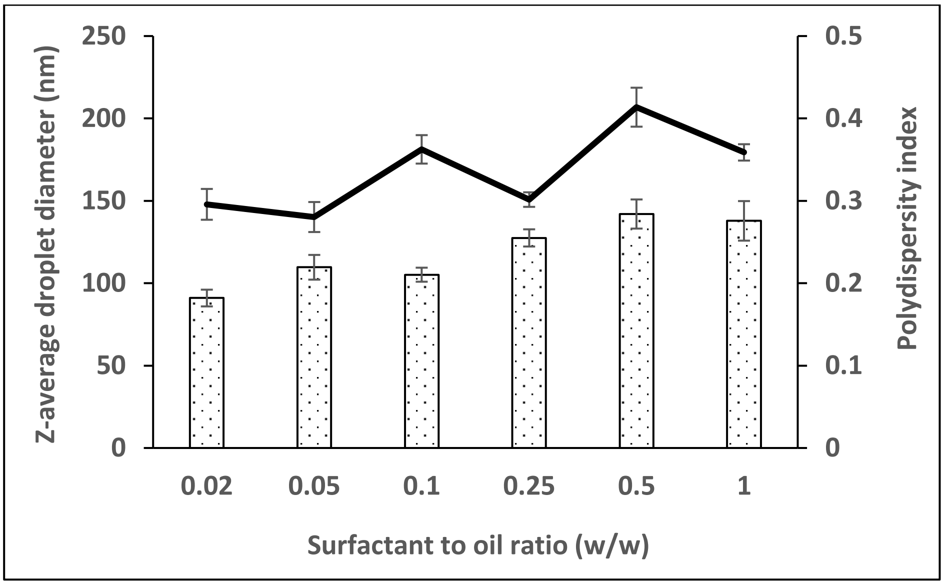


Figure 2. Z-average particle size variation at different Tween 80 (T80) surfactant to oil ratios (0.01 to 1 w/w). Oregano + T80 in aqueous phase (a) and trans-cinnamaldehyde + T80 in aqueous phase with 0 (b) and 10% w/v (c) sodium chloride. The bar charts and scatter line plot represent polydispersity index and z-average mean diameter variation, respectively. Standard deviation error bars are included.

The surface charge properties of the nano-assemblies, which have a great influence on the stability and functionality of the colloidal dispersions, were evaluated by electrophoretic light scattering. The zeta-potential of the swollen micelles loaded with oregano essential oil and TCA was -5.8 ± 0.6 and -5.1 ± 0.2 mV, respectively, indicating that most likely due to the free fatty acids resulting from the production of T80 molecules, the nano-assemblies were slightly negatively charged. These values were in a good agreement with the outcomes reported by Zeng, et al. (2010) and indicated that the surfactant aggregates were stabilised by steric, rather than electrostatic interactions.

The morphology of the formed nano-assemblies was also studied using TEM. It was observed that oregano essential oil- and TCA-loaded self-assembled aggregates were successfully formed (Figure 3a&b). The aggregate structures were almost spherical in shape. It was also evident that SMs were formed in the presence of salt (Figure 3c). TEM image analysis also revealed that the average size of the nano cargos seems smaller than the values measured by DLS. In this respect, it has to be realized that electron microscopy provides number-based information, which typically favors smaller structures, whereas DLS yields intensity-weighed distributions, which favor the larger structures (more strongly light scattering). The later was visible from the particle size distribution of oregano EO-loaded microemulsions at SOR = 1, which was in agreement with the large obtained PDI values (Figure S1 supplementary information). It should be mentioned that the irregular shape of the SMs in Figure 3c might be due to the long exposure of this specimen to the electron beam, inducing the complete evaporation of water. Overall, it can be concluded that relatively small self-associated surfactant structures loaded with plant-based lipophilic compounds could be formed without any mechanical energy input.

a) b) c)

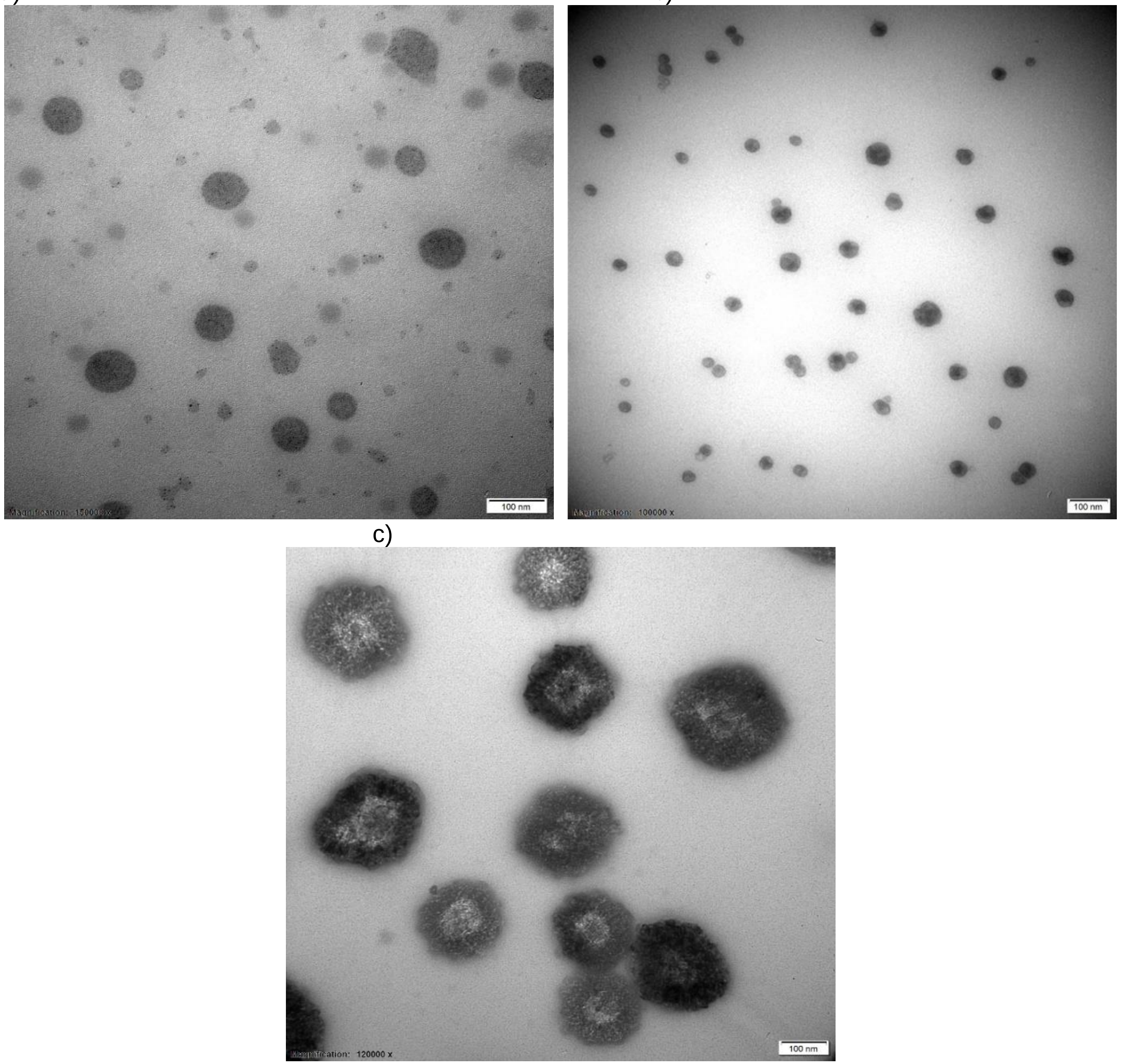


Figure 3.TEM photographs of self-assembled micelles containing oregano essential oil (a) and trans-cinnamaldehyde in the absence (b) and presence (c) of salt at SOR 1. The scale bars are 100 nm.

3.2. Encapsulation efficiency (EE)

From an application and functionality point of view, in addition to a small mean droplet size and a low surfactant concentration, a high content of essential oil is desired to provide effective activity (Sedaghat Doost, Nikbakht Nasrabadi, Kassozi, Nakisozi, & Van der Meeren, 2020). As mentioned earlier, a layer of oil was still seen at the end of the observation period, indicating that this system was unable to encapsulate all the added lipid phase. Therefore, the EE of these formulations was established using HPLC (Figure 4). The highest EE was obtained for the mixtures containing SOR = 1: for OR- and TCA-loaded formulations, the EE was 50 and 79%, respectively, meaning that the final OR and TCA content in the aqueous phase was 2.5 and 4.0%. Considering the limited aqueous solubility of OR and TCA, this amount was most likely solubilized into surfactant aggregates. The EE results also suggested that more cinnamaldehyde than oregano essential oil can be taken up by the micelles, which may be explained from the fact that the former is more water-soluble. Considering the loading capacity, the initial points of Figure 4 follow a linear relationship, which corresponds to about 10 g of hydrophobic compound solubilised per gram of surfactant. This capacity became lower upon further increasing the surfactant concentration and approached a value of 0.5 and 0.8 g/g for OR and TCA at SOR = 1.

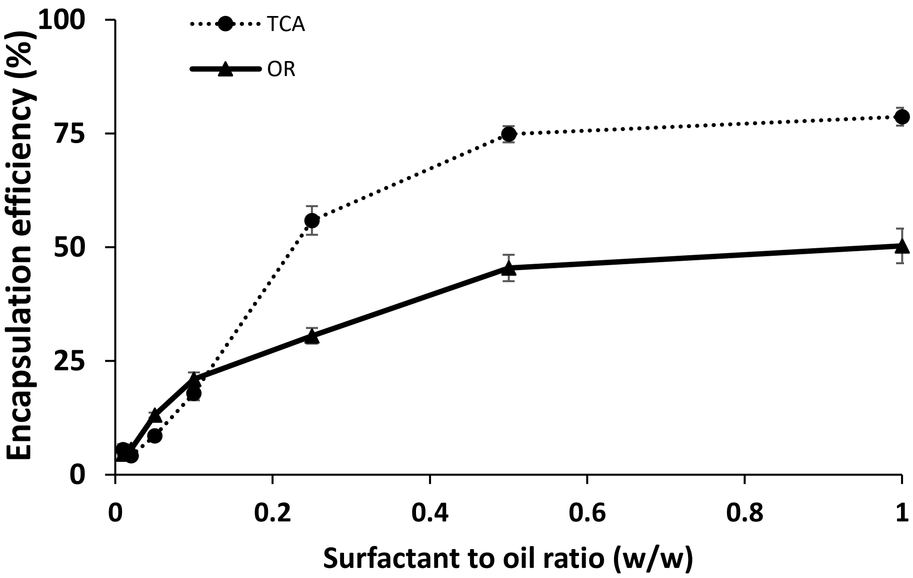


Figure 4. Encapsulation efficiency of Tween 80 self-assemblies in water in the encapsulation of trans-cinnamaldehyde (TCA) and oregano (OR) essential oils at different surfactant to oil ratios after two weeks of incubation at 25 °C.

3.3. Impact of thermal treatment and dilution

There are different stages where a product may be exposed to temperature variation, including processing, packaging, and transport. It is important from a functionality and consumer point of view that the formulated delivery system can retain its stability against these thermal shocks. This was examined by incubating the formulations at 50 and 80 °C for 30 min (Table 1). The particle size of micelles carrying cinnamaldehyde was not significantly ($p < 0.05$) influenced by the incubation temperature. By contrast, there was a significant ($p < 0.05$) size growth by heating swollen micelles loaded with oregano essential oil from 253 nm (at 20 °C) to 316 nm after 30 min at 80 °C. The lower susceptibility of particle size of the cinnamaldehyde-loaded micelles to the elevated temperature conditions is likely due to the fact that the micellar solubilisation of cinnamaldehyde at the preparation temperature of the swollen micelles was already close to the maximum: the encapsulation efficiency results indicated that about half of the added oregano essential oil (EE = 50%) was left in the mixture (and hence could be additionally solubilised upon heating) as compared to only 21% for cinnamaldehyde (EE = 79%). It has indeed been previously shown that the micellar solubilisation of a lipophilic compound increases upon heating (Ganguly, Kunwar, Dutta, Kumar, Barick, Ballal, et al., 2017; Ganguly, Kunwar, Kota, Kumar, & Aswal, 2018). Thus, it was found recently that the T80 micellar solubilisation of curcumin increased about two-fold upon heating to 80 °C, which was similar to the results obtained for lavender essential oil (Ganguly, Kumar, Nath, & Aswal, 2019). In a different study, the higher incorporation of oil molecules into microemulsion delivery systems has been also reported by Warisnoicharoen, Lansley, and Lawrence (2000), who showed that higher levels of different oil types were entrapped in polyoxyethylene-10-dodecyl ether aggregate structures upon increasing the temperature from 298 to 310 K. Thus, the droplet size growth of oregano essential oil enriched self-aggregates could be explained by the increasing micellar solubilisation, considering the fact that a larger excess of oregano essential oil was present to be taken up into the surfactant aggregates upon heating.

Table 1. Effect of incubation temperature during a 30 min thermal treatment on the z-average droplet diameter of self-assembled particles with Tween 80 to oregano essential oil and trans-cinnamaldehyde ratio of 1.

Oil loaded in swollen micelles	Temperature (°C)		
	20	50	80
Oregano essential oil	253 ± 2[a]	245 ± 10[a]	316 ± 24[b]
cinnamaldehyde	186 ± 10[a]	193 ± 3[a]	193 ± 14[a]

* Different symbols represent significant ($p < 0.05$) differences between different temperatures.

As one of the proposed applications for such systems is their use as washing agent for food, such as meat carcases or fresh vegetables, it might be necessary to dilute them for different reasons and thus they should remain in their original state to a desired extent. SMs containing OR were found to have an increase in their size distribution after dilution, irrespective of the dilution ratio. For instance, the initial z-average particle size increased from 253 to 355 nm after one-fold dilution which was larger than the z-average particle size after 50-times dilution (320 nm). A similar trend was also observed for TCA encapsulating micelles, with a z-average particle size growth from 185 to 212 nm. Nevertheless, the optical absorbance of 50-fold diluted TCA SMs became very small (0.037 at 600 nm), showing that a big fraction of the micelles was broken down by dissolution in the aqueous phase. Although the dispersion of these micelles was transparent, the intensity-based size distribution implicitly showed that there were still some micelles loaded with TCA, considering the fact that the size of native T80 micelles is only a few nanometres. On the other hand, the decrease in optical turbidity for SMs containing OR was limited: they still had an opaque appearance after 50 times dilution. Hence, dissolution of the formed OR-loaded SMs might occur, but to a lesser extent, as the absorbance decreased only from about 3 for the original sample to 2.08 after 50-times dilution. These results suggested that the formed microemulsions retained their structure to a certain extent of dilution. It is indeed well-known that by dilution of a microemulsion system, the surfactant concentration decreases which may result in a thermodynamically unstable colloidal dispersion due to a lack of surfactant to induce the very low interfacial tension required ($\gamma < 10^{-2}$ N/m) for thermodynamic stability (I. Solè, Solans, Maestro, González, & Gutiérrez, 2012). Although the increase in the interfacial tension is typically from less than 10^{-4} up to around 10^{-2} N/m, the microemulsion droplets tend to grow to decrease their large interfacial free energy (Taylor & Ottewill, 1994). Moreover, the interfacial tension shift caused by dilution leads to the formation of nanoemulsions from microemulsions, which has been previously reported in different studies (Pons, Carrera, Caelles, Rouch, & Panizza, 2003; Isabel Solè, Maestro, González, Solans, & Gutiérrez, 2006; I. Solè, Solans, Maestro, González, & Gutiérrez, 2012). However, the nanoemulsions created by the dilution of microemulsions (swollen micelles) are suffering from a low stability mainly due to Ostwald ripening (Taylor, 1998). The latter is especially pronounced when the lipid phase has a relatively high water solubility which is the case for oregano essential oil and cinnamaldehyde.

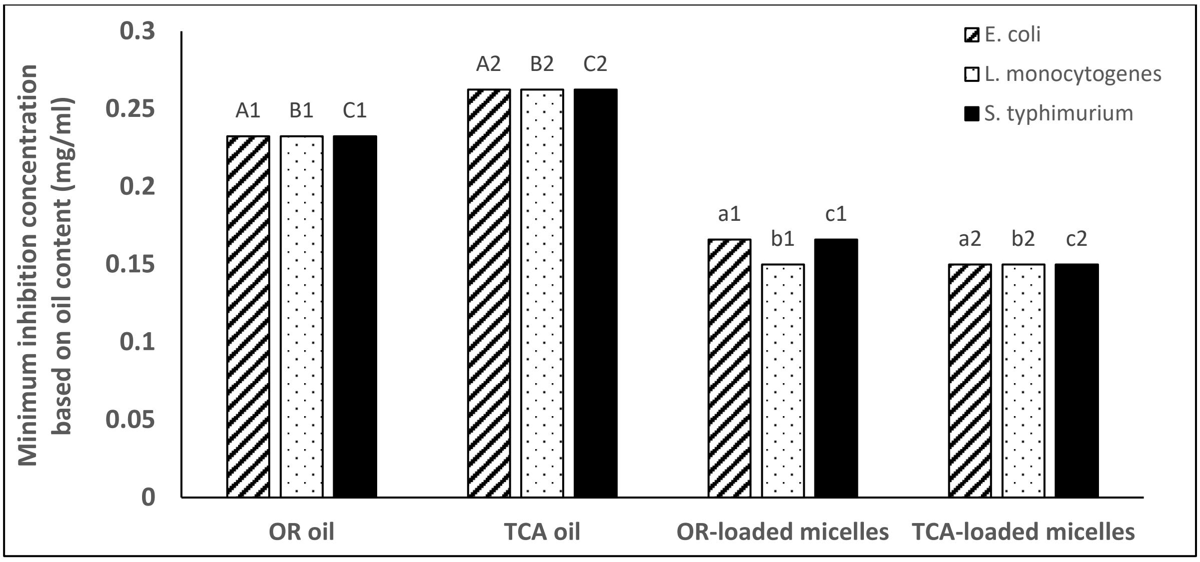
3.4. Antibacterial efficacy

In the last section, the antibacterial activity of the designed colloidal dispersion carrying OR or TCA was determined against three food pathogens, including *E. coli*, *L. monocytogenes*, and *S.* Typhimurium using two assessment approaches with different principles. The antibacterial activity measured by the MIC increased as OR and TCA were encapsulated within T80 micelles, meaning that a lower amount of OR and TCA was required to fully inhibit the growth of bacteria (Figure 5a). For example, OR bulk oil at 0.23 mg/ml concentration could inhibit the growth of the bacteria while this value was significantly ($p < 0.05$) lower, i.e., 0.16 mg/ml, when OR was present into self-assembled surfactant structures. TCA showed also a similar trend as 0.15 mg/ml (Figure S2, supplementary material) TCA loaded within the micelles was appreciably lower ($p < 0.05$) in comparison to TCA bulk oil (0.26 mg/ml) in order to inhibit bacterial growth. These values were comparable with our previous studies and other previously reported values (García-Salinas, Elizondo-Castillo, Arruebo, Mendoza, & Irusta, 2018; Sedaghat Doost, Devlieghere, Dirckx, & Van der Meeren, 2018; Sedaghat Doost, Dewettinck, Devlieghere, & Van der Meeren, 2018). This clearly indicates that the encapsulation of OR and TCA through self-aggregated micelles substantially ($p < 0.05$) enhanced their antibacterial activity, which has been previously shown (Anwer, Jamil, Ibnouf, & Shakeel, 2014). In agreement with our results, the reduction of the droplet size of EO emulsions (lemongrass and clove) has been found to cause a faster and enhanced microbial inactivation (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2015). This effect could be attributed to the fact that the smaller size of the carriers promotes their aqueous solubility, and enhance their penetration through the bacterial cell wall. The antibacterial improvement of the essential oils through emulsification was also found in a study by Y. Zhang, Chen, Critzer, Davidson, and Zhong (2017). They prepared cinnamon essential oil emulsions stabilized by a combination of gum Arabic, whey protein and lecithin as alternative washing solutions for carrots. Their MIC outcomes also suggested a lower oil concentration for the emulsions in comparison to free cinnamon oil.

The MIC determination assay revealed that a higher TCA oil content is required for an efficient bacterial activity in comparison with OR bulk essential oil, which could be attributed to the different oil composition. It has been previously shown by GC-MS analysis that oregano essential oil consists of different components with carvacrol and thymol as its major constituents (Sedaghat Doost, Stevens, Claeys, & Van der Meeren, 2019). Therefore, one would expect a broader bacterial inactivation spectrum from OR than TCA, which contains only one single compound. However, TCA-loaded SMs performed better in the inhibition of *E. coli* and *L. monocytogenes* than SMs containing oregano essential oil. The results also suggested that SMs carrying OR essential oil were slightly more efficient against *L. monocytogenes* in comparison to *E. coli* and *S.* Typhimurium, which is consistent with the outcomes obtained by Siroli, Patrignani, Montanari, Tabanelli, Bargossi, Gardini, et al. (2014), who also reported a better antibacterial activity of oregano EO for *L. monocytogenes* than *E. coli*. In fact, *L. monocytogenes* is a gram positive food pathogen while *E. coli* and *S.* Typhimurium are gram negative bacteria. It is believed that the lipopolysaccharide outer layer of gram negative bacteria protects them more from hydrophobic phenols than gram positive bacteria whose outer membrane consists of peptidoglycan (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013). Therefore, the transfer of hydrophobic compounds, in this case essential oils, through the cell walls of gram positive bacteria is easier which can explain the obtained lower MIC value of swollen micelles loaded with oregano essential oil. It should be noted that the MBC values were equivalent to the MIC values. It has been corroborated by previous studies that the determined minimum amount of oil as MIC is sufficient to kill 99.9 % of the bacteria (Benthotage, Hossain, Wimalasena, Senarath Pathirana, Sepala Dahanayake, & Heo, 2018; Sedaghat Doost, Devlieghere, Dirckx, & Van der Meeren, 2018).

A similar trend was also observed in the antibacterial properties of oregano essential oil and cinnamaldehyde and their SMs established by measuring the diameter of the inhibited bacterial growth zone. In this assay, a certain amount of the sample was placed in a well punched into an inoculated agar medium and thus the capability of the active compound through diffusion is evaluated. Our results indicated that the IZD was considerably ($p < 0.05$) lower for a comparable amount of OR essential oil and TCA oil in comparison to the oils encapsulated within self-assembled structures (Figure 5b). Moreover, the results presented that no significant difference ($p > 0.05$) in effect between swollen micelles loaded with oregano essential oil and cinnamaldehyde on *L. monocytogenes* growth was found (Figure S3, supplementary material). On the other hand, the IZD outcomes revealed a higher ($p < 0.05$) antibacterial activity of cinnamaldehyde-loaded than OR-loaded SMs against *E. coli* and *S.* Typhimurium.

a)



b)

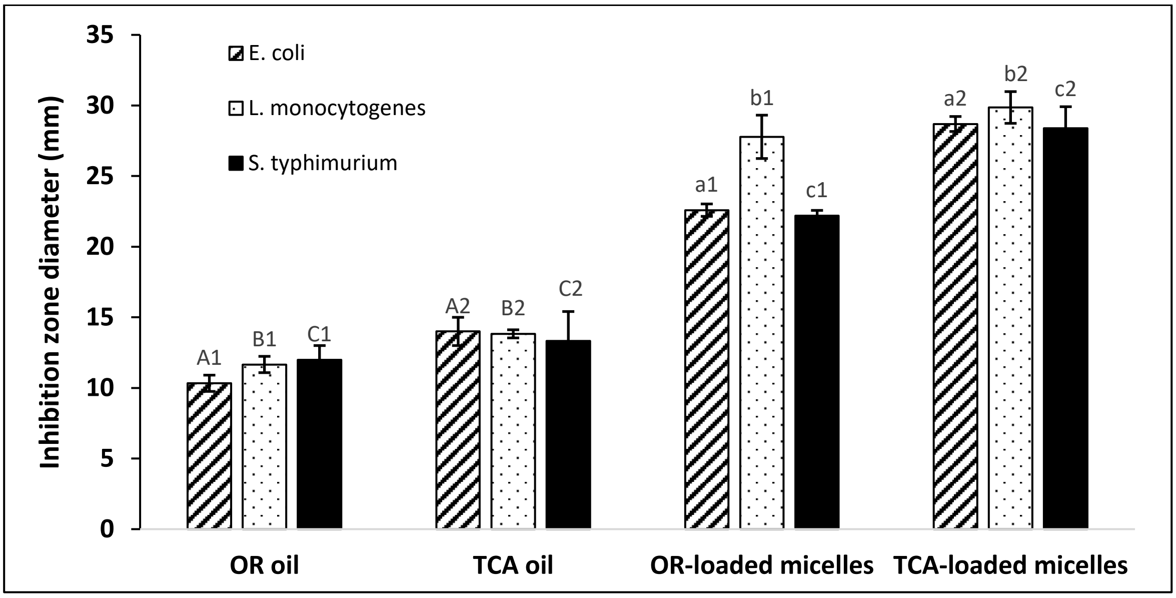


Figure 5. Evaluation of antibacterial activity of bulk oregano (OR) and trans-cinnamaldehyde (TCA) oil and OR- and TCA-loaded swollen micelles at a surfactant to oil ratio of 1 (w/w). Different symbols are significantly ($p < 0.05$) different (symbols with similar subscripts and bacterium type should be compared).

The antibacterial properties evaluation of the formulations showed an improved efficiency of both OR and TCA in the presence of T80 in the growth inhibition of three food pathogens. Overall, it can be concluded that in addition to the fact that these essential oil-based compounds need to be encapsulated in a delivery system to control their scent and taste, emulsification improves their desired antibacterial activity.

4. Conclusions

In this study, we investigated the usefulness of T80 self-assemblies loaded with oregano EO or *trans*-cinnamaldehyde through the straightforward and cost-effective self-emulsification method. The formed micelles were shown to have a z-average diameter ranging from 92 to 337 nm, depending on the studied SOR (0.01 – 1 w/w) and lipid composition (OR or TCA). Whereas previous studies indicated that smaller droplets were formed by increasing SOR, larger TCA-containing structures were created at the highest SOR considered in our study (i.e. 1). TEM analysis revealed spherical aggregates of T80 and TCA even in the presence of 10% sodium chloride. Furthermore, the outcomes obtained from encapsulation efficiency experiments presented that the self-emulsified oil content of OR and TCA was 2.5 and 4.0%, respectively, at SOR = 1. Ultimately, the antibacterial properties evaluation of the formulations showed a significantly ($p < 0.05$) improved efficiency of both OR and TCA in the presence of T80 determined by well diffusion and minimum inhibitory concentration tests against three food pathogens. The practical information obtained in this study is applicable in the design of nano-carriers for plant-based oils which can be used in different products, including foods and agrochemicals. However, the optimization of the processing and formulation parameters needs further investigation.

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