

Effect of Nanoemulsion Formulation on Permeation of Essential Oils through Biological Membranes

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The encapsulation of essential oils in nanoemulsions represents an effective approach not only to disperse them in the aqueous systems where microorganisms proliferate but also to enhance their permeability through biological membranes.

In this work, different food-grade emulsifiers, such as whey proteins, lecithin, Tween 80, alginate or zein, were tested in the preparation of carvacrol nanoemulsions in water. In order to prevent the occurrence of instability phenomena associated with Ostwald ripening, carvacrol was mixed at different ratios with peanut oil or medium chain triglycerides. The high-pressure homogenization method (200 MPa, three passes, using an orifice valve) was used to reduce the droplet size in the nanometric range.

The nanoemulsions were tested for their stability over time at varying the main formulation parameters, and then carvacrol permeability across dialysis membranes was investigated, using a Franz cell assembly.

The results showed that, in order to prevent droplet coalescence due to Ostwald ripening, the minimum ripening inhibitor oil-carvacrol ratio in the dispersed oil phase should be 3:1.

In addition, despite comparable droplet sizes (< 150 nm), the nanoemulsions stabilized by lecithin or whey proteins enabled a higher carvacrol diffusion through the cellulose membranes, which was one order of magnitude higher than Tween 80-based systems.

1. Introduction

Essential oils are secondary plant metabolites, characterized by a strong antimicrobial activity (Burt, 2004). They are made of volatile hydrophobic molecules, which are able to strongly interact with the lipids of the cell membrane, increasing membrane permeability, disturbing the original cell structures, breaking homeostasis, and causing the leakage of ions and cytoplasmic content (Seow et al., 2014). Therefore, their antimicrobial activity is non-specific and broad-spectrum, preventing the development of bacterial resistance, differently from many commercial antibiotics (Solórzano-Santos and Miranda-Novales, 2012). Because of their mechanism of action, the antimicrobial activity of essential oils requires that the active molecules get in contact with the microbial cell membrane, and therefore are efficiently delivered in the aqueous-based environment of microorganisms proliferation (Donsi and Ferrari, 2016). In particular, nanoemulsions are especially suitable for food applications, because they can be fabricated with food-compatible ingredients and by scalable approaches, such as high-pressure homogenization (Donsi et al., 2011). In the last decade, the exploitation of food-grade nanoemulsions for the delivery of essential oils has become a consolidated research field, with both fundamental and applicative studies (Donsi and Ferrari, 2016). For example, the application of essential oil nanoemulsions, within edible coatings (Torrieri et al., 2015), enabled to extend the shelf-life of rucola leaves, through the microbial stabilization of their surface (Sessa et al., 2015). However, microorganisms might represent also a direct threat to living plants, when they penetrate in the plant tissue.

For example, the *Plasmopara viticola* oomycete is responsible for the vine disease commonly called "Downy mildew". It is able to penetrate the plant tissues through the stomatal openings and, if not promptly eradicated, it causes the necrosis of the leaves (Jackson, 2008). To date, the only effective remedy to prevent this disease consists of sprinkling the plants with broad-spectrum protectant fungicides, such as chlorothalonil, mancozeb, and fixed copper. However, these products, over long periods of use, accumulate in the soil,

Paper Received: 21 March 2018; Revised: 15 June 2018; Accepted: 17 October 2018

Please cite this article as: Donsi F., Ferrari G., 2019, Effect of Nanoemulsion Formulation on Permeation of Essential Oils Through Biological Membranes, Chemical Engineering Transactions, 75, 247-252 DOI:10.3303/CET1975042

causing severe environmental issues. Essential oils, as natural, broad-spectrum antimicrobial and antifungal agents (Ribes et al., 2017), represent the primary choice for a more sustainable approach to the control of *Plasmopara viticola*, provided that they are delivered through suitable carriers.

In this work, the fundamental aspects of encapsulation and delivery of essential oils in nanoemulsions are elucidated selecting carvacrol as model essential oil component, and investigating the effect of different nanoemulsion formulations on the diffusion through cellulose membranes, with the final goal of developing a sufficient body of knowledge to support the application in real systems (i.e. grape leaves).

2. Materials and Methods

2.1 Materials

Carvacrol was used as model essential oil component, medium chain triglycerides (MCT, from Sigma- Aldrich, Germany) or peanut oil (PO, from Olio Sagra, Italy) were used as ripening inhibitors, Tween 80 (T80, from Sigma- Aldrich, Germany), soy lecithin Solec IP (LEC, a kind gift from Solae Italia s.r.l., Milan, Italy) or whey protein isolates Volactive UltraWhey 90 (WP, a kind gift of Volac International Limited, UK) were used as emulsifying agents, sodium alginate (A, from Sigma- Aldrich, Germany) or zein (Z, from Sigma- Aldrich, Germany) were used as stabilizing agents. All the chemicals were obtained from Sigma- Aldrich (Germany).

2.2 Methods

Nanoemulsions were produced with different approaches, depending on formulations. In the case of T80-based emulsions, the method of spontaneous emulsification was applied (Chang and McClements, 2014). Briefly, T80 was mixed with the oil phase (carvacrol and ripening inhibitor, MCT or PO) by vortexing for 1 min. Subsequently, the oil phase was added, dropwise, to the aqueous phase (pure water or a stabilizer aqueous solution) under magnetic agitation to produce a primary emulsion. In the case of LEC or WP, the primary emulsions were produced by adding dropwise the oil phase in the aqueous phase containing the emulsifier, under agitation with an Ultra Turrax T25 blender (IKA Labortechnik, Germany) at 24,000 rpm for 5 min, in an ice bath, to prevent carvacrol degradation. The nanoemulsions were produced by high-pressure homogenization (HPH) of the primary emulsions, using an in-house developed system, equipped with an 80 μm diameter orifice valve (model WS1973, Maximator JET GmbH, Germany), previously described (Ferrari et al., 2017). The HPH treatment consisted of 3 passes at 200 MPa, with intermediate cooling at 5°C in a tube-in-tube heat exchanger.

The total oil concentration was set at 2.67 wt% because preliminary experiments showed that higher oil fractions had negative effects on vine leaves. The carvacrol:ripening inhibitor ratio was investigated as formulation parameters. The emulsifier:total oil ratio was set to 1:2 w/w, which is in the lower range of values reported for the preparation of essential oil nanoemulsions (Donsi and Ferrari, 2016), to reduce the use of the emulsifier, which might have a negative impact on vine leaves. The stabilizer concentration was set at 0.67 wt% of total formulation because preliminary experiments showed that lower concentrations had negligible stabilizing effects, while higher concentrations caused depletion flocculation, as previously discussed (Donsi et al., 2017).

The nanoemulsions stabilized with sodium alginate were prepared by dissolving the sodium alginate in water (magnetic stirring for 2 h at 70°C), prior to the spontaneous emulsification process. Zein-stabilized nanoemulsions were prepared by dissolving zein in an alcoholic solution (80 wt% ethanol in water) upon magnetic stirring overnight and then adding dropwise the alcoholic solution to the nanoemulsions up to the desired amount. Subsequently, ethanol was removed by rotavapor evaporation (Donsi et al., 2017).

The nanoemulsions were characterized by mean droplet diameter (d_H), polydispersity index (PDI) and zeta potential, using a Zetasizer Nano ZS (Malvern Instruments, Alfatest, Italy), at 25°C, on undiluted samples for size, and in samples diluted 1:100 with bidistilled water for zeta potential, to avoid multiple scattering effects. Each measurement was replicated on three independently prepared samples, with the means and the standard deviations being calculated.

Carvacrol release from the nanoemulsions was determined using Franz static diffusion cells, which consist of two compartments, the donor and the receptor compartments, separated by a cellulose membrane (dialysis tubing cellulose membrane, MWCO = 14 kDa, from Sigma- Aldrich, Germany). The receptor compartment, agitated with a magnetic stirrer, is equipped with a sampling tube through which periodic withdrawals are carried out and is thermostated at 37 °C with an external jacket. The donor chamber was filled with the carvacrol nanoemulsions (1 mL), while the receptor chamber was filled with a phosphate buffer solution (pH = 7.4). When sampling, 0.2 mL of the solution from the receptor compartment is withdrawn, and replaced with 0.2 mL of fresh solution. The reported results are the mean values from 4 replicated measurements.

Carvacrol concentration in the receptor compartment was measured through the FRAP (Ferric Reducing Antioxidant Power) assay method, which resulted to be well correlated to the carvacrol concentration in PBS,

although depending on nanoemulsion formulation. Briefly, 0.5 mL of the sample was added to the FRAP solution (300 mM sodium acetate/acetic acid buffer solution at pH = 3.6, 20 mM FeCl₃·6H₂O solution, 10 mM of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution at 10:1:1 volume ratio), and, after 10 min storage in the dark, the absorbance values were measured at 593 nm by a UV-Vis spectrophotometer. Different calibration curves were obtained for the different carvacrol nanoemulsion formulations tested.

3. Results and Discussion

One of the main requisites for a delivery system for antifungal agents of commercial interest is sufficient stability over time. In the case of essential oils, their low but non-negligible solubility in water triggers a specific coalescence phenomenon, called Ostwald ripening, causing the growth of larger droplets at the expense of smaller ones. Preventing Ostwald ripening is one of the main difficulties associated with the formulation of physically stable essential oil nanoemulsions (Chang and McClements, 2014).

Long-chain triglycerides (i.e. corn, sunflower, sesame, or peanut oils), which exhibit a negligible aqueous phase solubility, are less prone to Ostwald ripening, and therefore their addition to the essential oils is an effective strategy to reduce this phenomenon (Rao and McClements, 2012). Carvacrol is known for causing Ostwald ripening-associated instability phenomena in nanoemulsions (Donsi et al., 2014). Therefore, different nanoemulsion formulations were tested for carvacrol delivery. Initially, the effect of two different ripening inhibitor oils, MCT and PO, was investigated, by measuring the hydraulic diameter d_H of the nanoemulsions, as a function of carvacrol concentration in the oil phase. The results are shown in Figure 1.

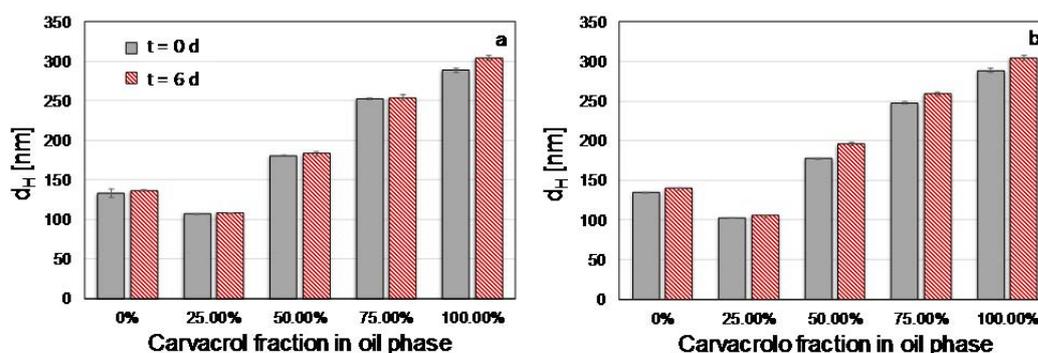


Figure 1: Mean droplet diameter (d_H) of the nanoemulsions formulated with different concentration of carvacrol in different ripening inhibitor oils, (a) MCT or (b) Peanut oil, at constant oil phase fraction (2.67 wt%) and using Tween 80 as emulsifier (1.33 wt%) for two storage times $t = 0$ and $t = 6$ days.

For both ripening inhibitors, the nanoemulsions with smaller droplet sizes were those containing 25 wt% of carvacrol in the oil phase (0.67 wt% of carvacrol and 2.00 wt% of oil over the total formulation), with d_H values in the range of 100-110 nm. The increase of the fraction of carvacrol caused a measurable increase in d_H , and some incipient instability, observable after 6 days. Remarkably, the nanoemulsions without carvacrol (0 wt% in Figure 1) exhibited a d_H value larger than those at 25 wt% carvacrol, because, likely, carvacrol decreased the viscosity of the oil phase, promoting the break-up phenomena during high-pressure homogenization. The d_H values increased again for a carvacrol concentration ≥ 50 wt%, because of the Ostwald ripening occurring due to the non-negligible carvacrol solubility, of about 0.3 g/L (Donsi et al., 2012). Therefore, it was decided to set the carvacrol:ripening inhibitor ratio = 1:3 w/w (25 wt% carvacrol in the total oil phase, with 0.67 wt% of carvacrol and 2.00 wt% of ripening inhibitors over the total formulation).

Table 1: Formulation and characterization of carvacrol nanoemulsions

| Sample | Ripening inhibitor | Emulsifier | Stabilizer | d_H [nm] | PDI [-] | ζ -potential [mV] |
|----------|--------------------|---------------|------------|------------|---------|-------------------------|
| MCT-T80 | MCT | Tween 80 | - | 110.1±0.9 | 0.2 | +19.6±0.7 |
| PO-T80 | Sunflower oil | Tween 80 | - | 104.2±0.6 | 0.2 | +21.1±2.0 |
| PO-T80-A | Sunflower oil | Tween 80 | Alginate | 375.7±4.5 | 0.3 | +17.6±0.5 |
| PO-T80-Z | Sunflower oil | Tween 80 | Zein | 145.7±6.0 | 0.6 | +16.7±0.4 |
| PO-LEC | Sunflower oil | Lecithin | - | 179.7±2.9 | 0.2 | +33.8±2.5 |
| PO-WP | Sunflower oil | Whey proteins | - | 341.7±6.5 | 0.5 | +28.8±1.0 |

The prepared carvacrol nanoemulsions were characterized in terms of mean droplet diameter d_H , polydispersity index PDI and ζ -potential. The results show that the effect of the ripening inhibitor is very marginal (as already seen for d_H in Figure 1) also on PDI and ζ -potential. In contrast, when changing the type of emulsifier, a significant effect was observed on all the parameters characterized. The use of lecithin (PO-LEC) enabled the formation of nanoemulsions with a d_H below 200 nm, a low degree of polydispersity ($PDI \leq 0.2$) and a fairly high ζ -potential, which might trigger the droplet stabilization by electrostatic repulsion. When whey proteins were used as an emulsifier (PO-WP), however, a worse emulsification process was achieved, with d_H of 375 nm, a high degree of polydispersity ($PDI = 0.5$), but still a fairly high ζ -potential. Finally, the addition of a stabilizer to the Tween 80-based nanoemulsions, probably due to depletion attraction, caused a significant increase in mean droplet diameter (especially for alginate), or in polydispersity (especially for zein), without any remarkable advantage on ζ -potential.

The nanoemulsions of Table 1 were then tested in the Franz cells to calculate the effective diffusivity through the cellulose membranes. The diffusion coefficient was estimated using the following equation:

$$D = \frac{1}{Bt} \ln \left[\frac{C_{1,0} - C_{2,0}}{C_1 - C_2} \right] \quad (1)$$

where D represents the diffusion coefficient in cm^2/s , t the duration of the experiment expressed in s, $C_{1,0}$ the initial concentration of the compound of interest in the donor chamber, $C_{2,0}$ the initial concentration in the receptor chamber, C_1 and C_2 the respective concentrations of the donor and receptor chambers at time t .

The term B represents the cell constant, which could be determined through the initial calibration of the cell, using a compound of known diffusivity, such as KCl in a 0.1 M solution (Lobo et al., 1998). By rearranging eq. 1 as follows:

$$A = Bt = \frac{1}{D} \ln \left[\frac{C_{1,0} - C_{2,0}}{C_1 - C_2} \right] \quad (2)$$

and plotting A as a function of time t , from the slope of the regression line ($R^2 = 0.985$) it is possible to determine the cell constant $B = 1.043 \text{ cm}^2$, as shown in Figure 2.

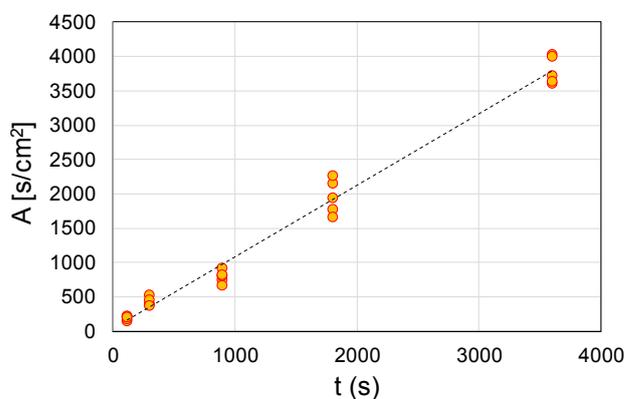


Figure 2: Values of A (eq. 2) as a function of time t for a 0.1 M KCl solution tested in the Franz cell apparatus.

Subsequently, the different nanoemulsions were loaded in the Franz cells, with the results of the carvacrol diffusion through the cellulose membrane reported in Figures 3 and 4, in terms of carvacrol concentration in the receptor compartment. Figure 3 clearly shows that the ripening inhibitor had a small but significant effect on the diffusion of carvacrol through the cellulose membrane, with higher values measured for the MCT than for PO. However, it must be remarked that the observed differences are small when compared to the effect measured when changing the emulsifier, as shown in Figure 4. In particular, lecithin and whey protein-based nanoemulsions significantly promoted the diffusion of carvacrol through the membrane, likely inducing an emulsifier micelle-mediated mass transfer, which does not occur for Tween 80. This hypothesis is confirmed by the observation that all the Tween 80-based systems (MCT-T80, PO-T80, PO-T80-A, and PO-T80-Z) all exhibited a significantly slower diffusion of carvacrol through the cellulose membrane and consequently lower concentration values in the receptor compartment.

The diffusivity of carvacrol through the membrane is calculated using the following formula (Fernández et al., 2017), through the regression of the data of Figures 3 and 4 and the determination of the slope of the regression line.

$$Y = \frac{1}{B} \ln \frac{(C_{10} - C_{20})}{(C_1 - C_2)} = Dt \quad (3)$$

The regression of the data with the linearized formula of eq. 3 resulted in a good fitting for MCT-T80, PO-T80, and PO-LEC, whereas the fitting was less accurate, but still reasonable, for the nanoemulsions comprising large macromolecules, such as in the case of PO-T80-A, PO-T80-Z and PO-WP.

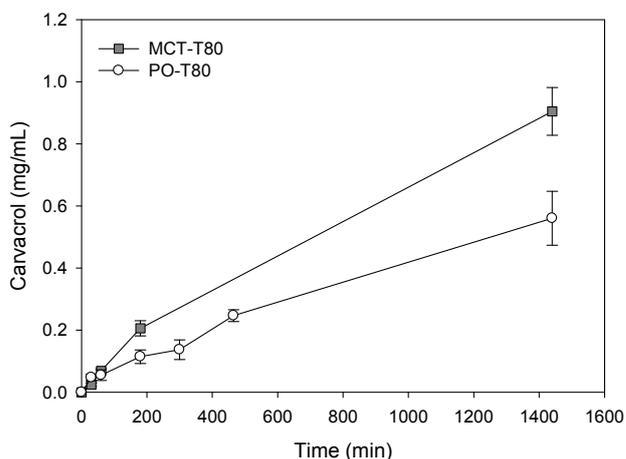


Figure 3: Carvacrol concentration in the receptor compartment of the Franz cells as a function of time, for the nanoemulsions prepared with Tween 80 and different ripening inhibitors (see Table 1).

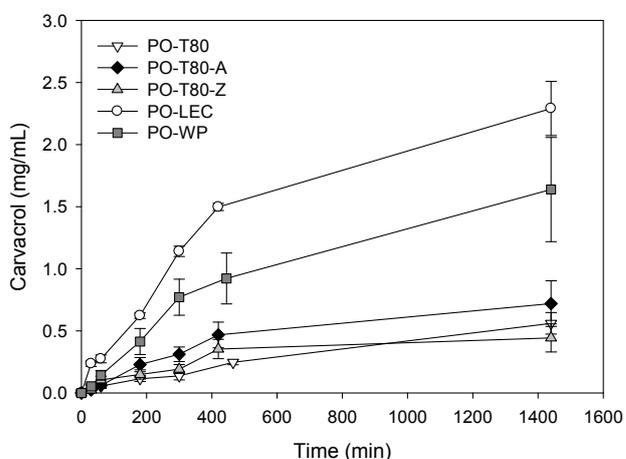


Figure 4: Carvacrol concentration in the receptor compartment of the Franz cells as a function of time, for the different nanoemulsions prepared with peanut oil (see Table 1).

Table 2: Diffusivity values of carvacrol, encapsulated in different nanoemulsions, through a cellulose membrane. The R^2 value of the regression of the release data is also reported.

| Sample | Diffusivity [cm^2/s] | R^2 |
|----------|--|-------|
| MCT-T80 | $3.976 \cdot 10^{-10}$ | 0.986 |
| PO-T80 | $1.227 \cdot 10^{-10}$ | 0.932 |
| PO-T80-A | $3.976 \cdot 10^{-10}$ | 0.737 |
| PO-T80-Z | $7.854 \cdot 10^{-11}$ | 0.894 |
| PO-LEC | $1.964 \cdot 10^{-9}$ | 0.945 |
| PO-WP | $1.964 \cdot 10^{-9}$ | 0.794 |

The results of Table 2 show that the type of emulsifier appears to be crucial in influencing the diffusivity of carvacrol through the cellulose membrane. Notably, when considering the value of the carvacrol diffusivities, the different nanoemulsions tested can be ranked as follows:

$$\text{PO-LEC} \approx \text{PO-WP} > \text{MCT-T80} \approx \text{PO-T80} > \text{PO-T80-A} > \text{PO-T80-Z.}$$

More specifically, Tween 80-based systems exhibit a scarce capacity to promote the diffusion of carvacrol through the cellulose membranes, whereas lecithin and whey proteins are significantly more effective. The addition of a macromolecular stabilizer, such as sodium alginate or zein, significantly reduces the effective diffusivity of carvacrol, probably due to its interaction with the macromolecules.

4. Conclusion

This work investigated the effect of nanoemulsion formulations on the stabilization of carvacrol in the aqueous phase and its diffusion through a cellulose membrane, with the final goal of contributing to support its application as an antifungal agent. The results have shown that the carvacrol nanoemulsions are efficiently stabilized by mixing carvacrol with a ripening inhibitor oil, at an optimal concentration of 25 wt% of carvacrol in the oil, enabling the formation of fine nanoemulsions, with droplet sizes < 150 nm. Remarkably, the composition of the oil phase did not significantly affect carvacrol diffusion, which was, instead, influenced by the type of emulsifier. In particular, despite comparable droplet sizes (< 150 nm), the nanoemulsions prepared with lecithin or whey proteins enabled a higher carvacrol diffusion, at least one order of magnitude higher than the Tween 80-based systems.

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