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# Understanding the effect of formulation on functionality of modified chitosan films containing carvacrol nanoemulsions

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ABSTRACT

The interest towards the use of films and coatings in food preservation has been reinforced in the recent years by the development of biopolymeric matrices incorporating essential oil as antimicrobial barriers in food as an alternative to synthetic additives.

This work has therefore been addressed to investigate the effect of composition parameters on antimicrobial activity and properties of films based on modified chitosan containing different types of carvacrol nanoemulsions. Specifically, response surface methodology, applied for the concentration of biopolymer and carvacrol nanoemulsion in the film forming dispersions to maximize the antimicrobial activity against two model microorganisms, *Escherichia coli* and *Listeria innocua*, as well as surface hydrophobicity, was used to determine the optimum conditions for comparison of most promising systems.

Results showed that emulsion formulations had a significant effect on the intrinsic antimicrobial activity, but also that their interaction with the modified chitosan matrix affected film properties and resulting bactericidal action. The two most active emulsion formulations, based on a combination of polysorbate 20 and glycerol monooleate, and on whey protein isolates, respectively, when incorporated in modified chitosan films significantly increased the inhibition zone against *E. coli* and *L. innocua* from 7.2–7.4 mm (modified chitosan alone) to 13.4–16.1 mm, while still ensuring surface hydrophobicity of the film. In comparison to the use of pure carvacrol, the encapsulation into nanoemulsions also fostered the production of more homogeneous films with better appearance.

This work hence contributes to promoting, through the advancement of the knowledge in the field, the incorporation of nanoemulsions of essential oils in edible films and coatings for integrated food preservation strategies.



## 1. Introduction

Incorporation of essential oils (EOs) in biopolymeric edible films or coatings has attracted considerable attention in the last decade for the preparation of antimicrobial barriers to be applied to food surfaces. Recent reviews (Rojas-Graü, Soliva-Fortuny, & Martin-Belloso, 2009; Sung et al., 2013) excellently summarized the advances in the field and possible applications and highlighted that an increasing interest is raising both from the academy and industry towards the exploitation of this approach for the development of natural preservation methods.

Most of the previous reports on edible films or coatings containing EOs are focused on the direct incorporation of EOs in the biopolymeric matrix. This approach, which on one side offers the undoubted advantage of a simple and versatile fabrication process, on the other side (a) significantly reduces the EOs loading capability in the coating, (b) increases the risk of oiling off of EOs, as well as (c) undermines the mechanical properties of the film or coating. For example, direct EOs loading has been reported to be responsible for increased surface coarseness (Norajit, Kim, & Ryu, 2010; Shojaee-Aliabadi et al., 2014), probably owing to EOs volatilization during water evaporation, resulting in a holey structure (Sanchez-Gonzalez, Chiralt, Gonzalez-Martinez, & Chafer, 2011).

Very recently, we tested the use of modified chitosan coatings containing EOs nanoemulsions, in combination with non-thermal technologies, for food preservation purposes (Donsì et al., 2015;

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Severino et al., 2014b, 2015, 2014a). The EOs were selected by their ability to develop antimicrobial synergies against *Listeria monocytogenes* or *Listeria innocua* with modified chitosan, which is characterized by intrinsic antimicrobial properties, as well as with different non-thermal treatments. The nanoemulsions were used to stabilize the EOs in the modified chitosan matrix, without altering its film forming properties.

Despite the several advantages of the use of nanoemulsions in reducing the adverse effects associated with direct EOs inclusion, to date, no systematic studies have been conducted on the optimization of the films and coatings composition, and in particular on the effect of EOs nanoemulsion loading on the film forming properties of the biopolymer.

Also, the role played by nanoemulsion formulation and properties (in particular mean droplet size and surface charge) on the resulting antimicrobial activity of encapsulated EOs calls for more detailed investigations. Recently, several investigations were addressed to clarify the effects of different nanoemulsion formulations to encapsulate essential oils on the *in vitro* antimicrobial activity against various microorganisms (Donsì, Annunziata, Sessa, & Ferrari, 2011; Donsì, Annunziata, Vincensi, & Ferrari, 2012; Liang et al., 2012; Salvia-Trujillo, Rojas-Grau, Soliva-Fortuny, & Martin-Belloso, 2013; Ziani, Chang, McLandsborough, & McClements, 2011). However, this body of data is of difficult correlation, due to the different experimental procedures used (including test microorganism strains), as well as to the variety of EOs and food matrices tested.

Starting from these different considerations, this work was addressed to investigate the effect of the composition of modified chitosan films containing carvacrol nanoemulsions, on their antimicrobial activity, as well as on selected film properties, such as color (transparency) and hydrophobicity. Specifically, the effect of different nanoemulsion formulations on carvacrol activity was preliminary addressed in the selection of most promising antimicrobial films. Carvacrol was selected as model EO component not only because of its well-known antimicrobial activity but also because its incorporation in modified chitosan has already been reported to be a challenging task, owing to the development of an antagonistic antimicrobial effect in broccoli florets decontamination from *Listeria monocytogenes* (Severino et al., 2014b).

## 2. Materials and methods

### 2.1. Bacterial cultures

The bacterial suspensions were prepared by dispersing 100 μL of a pre-culture of *Escherichia coli* (ATCC 26) or *Listeria innocua* (ATCC 51742) in 100 mL of tryptic soy broth (TSB, Oxoid, UK) and brain heart infusion broth (BHIB, Oxoid, UK), respectively. After 24 h of incubation at 30 °C in a Function Line 7000 incubator (Heraeus Instruments, Germany), the broth reached a microbial concentration of approximately $10^8$ CFU/mL for both bacterial strains. Subsequently, it was diluted with buffered peptone water (BPW Oxoid, UK) to reach a final concentration of the working dispersions of $10^6$ CFU/mL.

### 2.2. Carvacrol nanoemulsions

#### 2.2.1. Preparation

Oil-in-water (O/W) nanoemulsions were prepared by adding different proportions of carvacrol essential oil with a purity >98% (Sigma-Aldrich s.r.l., Italy), used as antimicrobial agent, into sunflower oil (Sagra, Italy), in order to prevent Ostwald ripening (Donsì, Cuomo, Marchese, & Ferrari, 2014) and to regulate its equilibrium concentration into the aqueous phase (Donsì et al., 2012).

A combination of glycerol monooleate and Tween 20 (both from Sigma-Aldrich s.r.l., Italy) at 1:1 weight ratio, as defined in previous studies (Donsì et al., 2012, 2014; Sessa et al., 2013; Spigno et al., 2013), or whey protein isolates (Volactive UltraWhey 90, a kind gift of Volac International Limited, UK) were used as emulsifying agents. According to manufacturer specifications, whey protein isolates consisted of β-lactoglobulin (50–60% w/w), glycomacropeptide (15–20% w/w), α-lactalbumin (15–20% w/w), bovine serum albumin (1.0–2.0% w/w), immunoglobulin G (1.0–2.0% w/w), immunoglobulin A (0.1–1.0% w/w), and lactoferrin (0.1–0.5% w/w).

The ingredients were mixed and dispersed in bidistilled water by high shear mixing (HSM) with an Ultra Turrax T25 (IKA Labortechnik, Jahnke und Kunkel, Germany) at 24,000 rpm for 5 min, maintaining the samples in an ice bath. Subsequently, the samples were further processed by high pressure homogenization (HPH) to produce nanometric size emulsions, using an in-house developed system, equipped with an 80 μm diameter orifice valve (model WS1973, Maximator JET GmbH, Schweinfurt, Germany) and an air-driven Haskel pump model DXHF-683 (EGAR S.r.l., Milano, Italy). The HPH treatment consisted of three passes at 200 MPa. After each pass, the emulsions were cooled at 5 °C in a tube-in-tube heat exchanger, mounted immediately downstream of the orifice valve.

For comparison, a blank nanoemulsion was also prepared, where carvacrol EO was substituted by sunflower oil.

Nanoemulsions were characterized by mean droplet size (z-diameter), polydispersity index (PDI) and zeta potential, using a Zetasizer Nano ZS (Malvern Instruments, Alfatest, Italy). Mean droplet size (expressed as hydraulic diameter $d_H$) and polydispersity index (PDI) were determined by measuring the back-scattered light (173°) in polystyrene cuvettes. Zeta potential was measured by calculating the electrophoretic mobility of the nanoemulsion droplets resulting from their surface charge in disposable folded capillary cells.

All measurements were carried out 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 twice, on three independently prepared samples, with the means and the standard deviations being calculated.

#### 2.2.2. Antimicrobial activity: minimum inhibitory concentration

The minimum inhibitory concentrations (MIC) of carvacrol nanoemulsions, in comparison with pure carvacrol as well as with a blank nanoemulsion, were determined using an optical density method. Briefly, 10 mL of broth (TSB or BHI for *E. coli* or *L. innocua*, respectively) were first inoculated with 100 μL of bacterial working suspension, and then added with pure carvacrol or nanoemulsions, with final carvacrol concentrations ranging from 0.01 to 0.5% w/w. The optical density was expressed as absorbance of the samples measured at 580 nm with a V-650 UV–vis spectrophotometer (Jasco Instruments, USA), immediately after preparation and after incubation at 30 °C for 24 h. Plain broth (either TSB or BHI) was used as blank.

Considering that, in case of microbial growth, the optical density of the samples is increased after 24 h of incubation, the MIC value was defined as lowest carvacrol concentration (either pure or in nanoemulsion), at which the optical density of the sample immediately after preparation was equal to (or higher than) the sample after 24 h of incubation. MIC values were determined by plotting the optical densities of non-incubated and incubated samples as a function of carvacrol concentration.

MIC tests were conducted in triplicate on independently prepared samples.