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Effect of ripening inhibitor type on formation, stability, and antimicrobial activity of thyme oil nanoemulsion

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ABSTRACT

The objective of this research was to study the impact of ripening inhibitor level and type on the formation, stability, and activity of antimicrobial thyme oil nanoemulsions formed by spontaneous emulsification. Oil-in-water antimicrobial nanoemulsions (10 wt%) were formed by titrating a mixture of essential oil, ripening inhibitor, and surfactant (Tween 80) into 5 mM sodium citrate buffer (pH 3.5). Stable nanoemulsions containing small droplets ($d < 70$ nm) were formed. The antimicrobial activity of the nanoemulsions decreased with increasing ripening inhibitor concentration which was attributed to a reduction in the amount of hydrophobic antimicrobial constituents transferred to the separated hydrophobic domain, mimicking bacterial cell membranes, by using dialysis and chromatography. The antimicrobial activity of the nanoemulsions also depended on the nature of the ripening inhibitor used: palm ≈ corn > canola > coconut which also depended on their ability to transfer hydrophobic antimicrobial constituents to the separated hydrophobic domain.

1. Introduction

Essential oils consist of a complex mixture of different volatile and non-volatile compounds that are normally extracted from different parts of plants (Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Dorman & Deans, 2000). Many of the compounds in essential oils have bioactive properties, e.g., antimicrobial, antiviral, antifungal, and antiseptic, that have applications in food, pharmaceutical and medical products (Burt, 2004). The fact that essential oils are natural components has led to particular interest within the food industry, since many consumers are interested in purchasing foods that do not contain synthetic ingredients. *In vitro* and *in vivo* studies have shown that many essential oils have strong potency against pathogenic bacteria and fungi (Dorman & Deans, 2000; Gutierrez, Barry-Ryan, & Bourke, 2009; Sienkiewicz, Lysakowska, Denys, & Kowalczyk, 2012). Essential oils isolated from the herb thyme have relatively strong antimicrobial activity, and are suitable for incorporation into food products (Burt, 2004). Nevertheless, it has been reported that thyme contains different proportions of active components depending on the season, location, extraction method, and the parts of the plant from which it was extracted (Hedhili, Romdhane, Abderrabba, Planche, & Cherif, 2002; McGimpsey, Douglas, Van Klink, Beauregard, & Perry, 1994). The current study focuses on using thyme oil extracted from *Thymus vulgaris* as an antimicrobial agent in nanoemulsion-based delivery systems. The major components in this thyme oil have been reported to be thymol, carvacrol, β-caryophyllens, γ-terpinene, and ρ-cymene (Bakkali et al., 2008; Borugă et al., 2014; Burt, 2004; Hedhili et al., 2002; Sienkiewicz et al., 2012). The mechanism of antimicrobial activity of essential oils (such as oregano oil) against *Pseudomonas aeruginosa* and *Staphylococcus aureus* was attributed to the presence of carvacrol and thymol constituents, which increased the permeability of the bacterial membranes thereby leading to the leakage of inorganic ions, ATP, and amino acids (Lambert, Skandamis, Coote, & Nychas, 2001; Ultee, Bennik, & Moezelaar, 2002). This mechanism was supported by another study using flow cytometry and specific fluorescent dyes to detect the leakage of specific components from cells exposed to carvacrol and thymol (Xu, Zhou, Ji, Pei, & Xu, 2008).

Despite their potential as natural antimicrobial agents, there are a number of factors that currently limit the widespread utilization of essential oils in foods. Firstly, essential oils are typically hydrophobic substances with a relatively low water solubility, which means that they have to be introduced into foods using appropriate delivery systems (such as in organic solvents, microemulsions, or emulsions). Second, the antimicrobial activity of essential oils is often compromised when they are introduced into complex food matrices because of their tendency to interact with other components in the systems. Third, many essential oils have a strong flavor (aroma and taste), which restricts the type of food products that they can be successfully incorporated into. Many of

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these challenges can be overcome using well-designed colloidal delivery systems.

Numerous studies have shown that essential oils can be successfully encapsulated within nanoemulsion-based delivery systems (Chang, McLandsborough, & McClements, 2012, 2013; Donsi, Annunziata, Vincensi, & Ferrari, 2012; Liang et al., 2012). These delivery systems consist of small ($d < 200$ nm) oil droplets dispersed within an aqueous medium (McClements, 2010, 2014). This type of delivery system is particularly useful for incorporating essential oils into a wide range of aqueous-based food products. In particular, antimicrobial nanoemulsions can be designed to be optically transparent, physically stable, and suitable for rapidly delivering essential oils to bacteria surfaces. Conventionally, nanoemulsions are produced using high-energy methods that involve mechanically applying intense disruptive forces to a mixture of oil and water to break up the oil phase into tiny droplets, *e.g.,* high pressure homogenizers, microfluidizers, and sonicators (McClements & Rao, 2011). However, nanoemulsions can also be formed using low-energy methods, such as "spontaneous emulsification", which simply involves titrating a mixture of oil and surfactant into water (Komaiko & McClements, 2014). This method is highly advantageous for certain commercial applications because it is simple to implement and does not require the use of expensive or sophisticated manufacturing equipment, such as that needed for high-energy homogenization. A number of studies have shown that essential oil nanoemulsions can be formed by spontaneous emulsification, and that they have good antimicrobial activity (Chang et al., 2013; Landry, Chang, McClements, & McLandsborough, 2014; Landry, Micheli, McClements, & McLandsborough, 2015; Tian, Lei, Zhang, & Li, 2016). For instance, carvacrol nanoemulsions were shown to be highly effective at inhibiting the growth of *Salmonella* Enteritidis or *E. coli* O157:H7 in contaminated mung bean, alfalfa seed, broccoli, radish seeds (Landry et al., 2014, 2015). Indeed, the antimicrobial nanoemulsions were reported to be more effective than conventional chlorination treatments for this application.

One of the major challenges that must be overcome during the development of essential oil nanoemulsions is the tendency for the oil droplets to grow during storage due to Ostwald ripening (OR). Droplet growth occurs through this mechanism as a result of the diffusion of oil molecules from the smaller droplets to the larger droplets through the intervening aqueous phase due to differences in the chemical potential of the oil inside droplets with different dimensions (McClements, 2014; Taylor, 1998). OR leads to an increase in the mean particle size over time, which eventually causes emulsion instability due to creaming and phase separation. Essential oil nanoemulsions are particularly prone to OR because even though the oil phase is predominantly hydrophobic it still has a significant solubility in the aqueous phase. Droplet growth due to OR can be retarded by incorporating water-insoluble oils, known as ripening inhibitors, into the oil phase prior to nanoemulsion formation (Wooster, Golding, & Sanguansri, 2008). Nevertheless, essential oil nanoemulsions must still be carefully formulated since the presence of the ripening inhibitor can reduce the efficiency of nanoemulsion formation, as well as reducing the antimicrobial efficacy of the essential oils. For example, a previous study has shown that the minimum inhibitory concentration (MIC) of thyme oil nanoemulsions depends on the type and amount of ripening inhibitor they contained (Chang et al., 2012). Even so, the effect of different ripening inhibitor types on nanoemulsion performance has not previously been established, and the impact of ripening inhibitor type on the mechanism of action of essential oils has not been investigated. The purpose of the current research is therefore to examine the impact of ripening inhibitor type on the formation, stability and antimicrobial activity of antimicrobial thyme oil nanoemulsions, and to provide some insight into the potential physicochemical mechanisms involved.

2. Materials and methods

2.1. Materials

The materials used to form the oil phase of the antimicrobial nanoemulsion were thyme oil (Sigma-Aldrich W306509 – 1KG – K) and five different ripening inhibitors. The main ripening inhibitor used was medium chain triglyceride (MCT) oil (Miglyol 812), which was purchased from Sassol Germany GmbH, Witten, Germany. The manufacturer reported that the fatty acid composition of this oil was 58.1% caprylic acid (C8:0) and 41% capric acid (C10:0). The other ripening inhibitors used were corn oil (Stop and Shop, Foodhold U.S.A., LLC Landover, MD), canola oil (Stop and Shop, Foodhold U.S.A., LLC Landover, MD), palm oil (Deganim, Equador), and coconut oil (Carrington Farms, Closter, NJ). The coconut oil was reported to contain 93% w/w saturated fatty acids comprising mainly of lauric acid (C12:0, 30.7%w/w), caprylic acid (C8:0, 30% w/w), and capric acid (C10:0, 24.3% w/w) (Carrington Farms, Closter, NJ). Consequently, the MCT and coconut oil can be considered to be mainly medium chain triglycerides, whereas the corn, canola, and palm oils can be considered to be long chain triglycerides (LCT). Tween 80 (T80) (Sigma-Aldrich P1754-500G) was used as a non-ionic surfactant to facilitate nanoemulsion formation and stability. For analysis of partitioning behavior of components in nanoemulsion by HPLC, three components of thyme oil in analytical standards, thymol (Sigma-Aldrich 72477), carvacrol (Sigma-Aldrich 42632), ρ-cymene (Sigma-Aldrich 30039) were purchased.

2.2. Formation of antimicrobial nanoemulsion

The antimicrobial nanoemulsions were prepared according to the optimized formulation and fabrication method described by (Chang et al., 2013). The oil phase was prepared by mixing essential oil and ripening inhibitor together. Control emulsions without essential oil were prepared with MCT alone. This oil phase was then mixed with an equal mass of Tween 80 to obtain a surfactant-to-oil ratio (SOR) of 1:1. Then, the surfactant-oil mixture was titrated at a rate of 2 mL/min into the water phase (5 mM sodium citrate buffer), which was being continually stirred at 600 rpm using a magnetic stir bar throughout the titration and for an additional 15 min. The resulting nanoemulsion was sterilized by filtering it through a 0.45 μm syringe filter (Cole-Palmer Cat# 02915–22) and was then stored in separate sterile containers within 4 and 20 °C incubators. For each oil phase composition, three replicates were prepared.

2.3. Bacterial culture conditions

Three strains of *Salmonella enterica* subspecies *enterica* (*Salmonella* sp.), representing serovars Enteritidis (BAA-1045), Gaminara (BAA-711), and Michigan (BAA-709) were obtained from the American Type Culture Collection (Manassas, VA). Long term storage of each culture was stored at −80 °C in a mixture of tryptic soy broth (TSB; BD Diagnostic Systems, Cat# DF0064-07-6) containing 25% v/v glycerol. Monthly, working stock of each culture were prepared by streaking from frozen stocks onto tryptic soy agar (TSA; BD Diagnostic Systems). After incubation at 37 °C, working stocks were stored at 4 °C for a month. For experiments, colonies were selected from working stock plates, and inoculated individually into TSB and incubated at 37 °C for 18 h on a 125 rpm shaker.

2.4. Determination of minimal inhibitory concentration

The antimicrobial efficacy of the essential oil nanoemulsions was obtained by measuring their minimal inhibitory concentration (MIC) against a cocktail of three strains of *Salmonella* sp. prepared from 18 h growth of each culture. To prepare the cocktail, each culture was