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Physicochemical Properties and Antimicrobial Efficacy of Carvacrol Nanoemulsions Formed by Spontaneous Emulsification

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ABSTRACT: A simple cost-effective method (spontaneous emulsification) for fabricating physically stable antimicrobial nanoemulsions from essential oils is described. These nanoemulsions (10 wt % total oil phase) were formed by titration of a mixture of essential oil (carvacrol), carrier oil (medium chain triglyceride, MCT), and nonionic surfactant (Tween) into an aqueous solution with continuous stirring. Oil phase composition (carvacrol-to-MCT mass ratio) had a major impact on initial droplet diameter, with the smallest droplets ($d \approx 55$ nm) being formed at 2.5 wt % carvacrol and 7.5 wt % MCT. Surfactant type also had an appreciable impact on mean droplet diameter, with Tween 80 giving the smallest droplets ($d \approx 55$ nm) from a group of food-grade nonionic surfactants (Tween 20, 40, 60, 80, and 85). The droplet size also decreased (from >5000 to <25 nm) as the total surfactant concentration was increased (from 5 to 20 wt %). The long-term stability and antimicrobial efficacy of selected nanoemulsions was examined at ambient temperature. The stability of the nanoemulsions to droplet growth during storage decreased as the carvacrol concentration in the oil phase increased. Conversely, the antimicrobial efficacy of the nanoemulsions increased as the carvacrol concentration increased. These results have important implications for the design and utilization of nanoemulsions as antimicrobial delivery systems in the food and other industries. They suggest that the carrier oil concentration must be carefully controlled to obtain good physical stability and antimicrobial efficacy.

KEYWORDS: *nanoemulsions, emulsions, spontaneous emulsification, encapsulation, delivery, low energy, essential oil, carvacrol, antimicrobial*

INTRODUCTION

Essential oils are natural compounds produced by aromatic plants as secondary metabolites that have antioxidant, antiradical, and antimicrobial properties and therefore have been widely used as functional ingredients in food, cosmetic, and pharmaceutical applications.[1,2] The major constituents in commercial essential oils can be classified into three classes: phenols, terpenes, and aldehydes.[1−3] Some essential oils have been shown to exert strong antibacterial activities against food-borne pathogens,[1,4,5] leading to their broad application as natural antimicrobial additives to extend the shelf life of food and beverage products. The fact that essential oils are considered to be "natural" components makes them highly desirable for use in many commercial applications due to growing consumer demand for natural rather than synthetic additives.[6−8] However, the utilization of essential oils is often limited owing to their relatively low water solubility. A simple way to solve this problem is to encapsulate essential oils in oil-in-water (O/W) emulsions or nanoemulsions.[9,10] These emulsion-based delivery systems have previously been used to encapsulate various kinds of lipophilic bioactive components, including antitumor agents,[5,11] anti-inflammatory agents,[11] vitamins,[12,13] and antimicrobials.[5,9,10,14−17] After encapsulation, the lipophilic components can be easily incorporated into aqueous-based foods and beverages due to their improved water dispersibility.

Emulsion-based delivery systems can be classified as conventional macroemulsions (radius >100 nm) or nanoemulsions (radius <100 nm) depending on the dimensions of the droplets they contain.[18−21] In many respects nanoemulsions have similar properties to conventional emulsions in terms of their compositions, structures, and thermodynamic properties.[18−20] Nevertheless, differences in droplet size mean that nanoemulsions and macroemulsions often have very different functional properties.[18−20,22] The size of the droplets in nanoemulsions is often much smaller than the wavelength of light ($d \ll \lambda$), and so they do not scatter light strongly, making them transparent or only slightly turbid. They can therefore be used to incorporate lipophilic bioactive compounds into transparent aqueous-based products, such as clear beverages, sauces, and syrups. The small size of the droplets in nanoemulsions also means that they typically have much better stability to gravitational separation, flocculation, and coalescence than macroemulsions.[18−20,22] Finally, the activity of encapsulated compounds may increase as the droplet size in emulsions decreases.[11,23,24]

Nanoemulsions can be fabricated by a number of different approaches, which are usually categorized as either high-energy or low-energy methods.[20] High-energy methods utilize mechanical devices that are capable of disrupting and intermingling the oil and aqueous phases into tiny oil droplets dispersed in water. In the food industry, emulsions and nanoemulsions are usually produced using high-energy methods, such as high-pressure homogenization, microfluidization, and sonication.[25] Low-energy methods mainly rely on the

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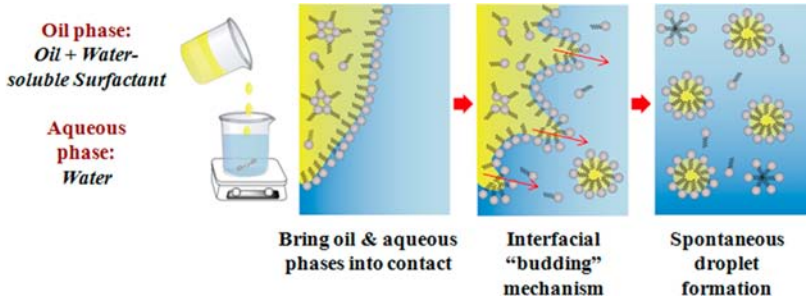

Figure 1. Schematic representation of spontaneous emulsification: fine oil droplets are spontaneously formed when an organic phase containing a surfactant is mixed with an aqueous phase. The surfactant moves from the organic phase to the water phase (red arrows), leading to interfacial turbulence and spontaneous oil droplet formation. Adapted from McClements and Rao.[20]

spontaneous formation of droplets at the boundary between oil and water phases and depend strongly on the nature of any surface active molecules present, e.g., their solubility and molecular geometry.[25] A number of different low-energy approaches have been developed to form nanoemulsions, including spontaneous emulsification, phase inversion temperature (PIT), and phase inversion composition (PIC) methods.[25−28] These methods are not widely used in the food industry at present, and where they are used there is still a relatively poor understanding of the factors affecting their performance.[25,29] Low energy approaches may have advantages over high-energy approaches for certain applications: they are often more effective at producing very fine droplets, they have lower equipment and energy costs, and they are simpler to implement. On the other hand, there are also some potential disadvantages of low-energy systems, including limitations on the types of oils and surfactants that can be used to form stable nanoemulsions and the fact that relatively high surfactant-to-oil ratios (SOR) are typically needed to produce them.[27,28,30]

In the current study, we examine the potential of using the low-energy *spontaneous emulsification* method for producing essential oil nanoemulsions suitable for utilization in foods and beverages. Carvacrol was used as a model essential oil in this study, but other essential oils may also be used (such as thymol or eugenol); however, they may exhibit differences in the formation, stability, and performance of antimicrobial nanoemulsions due to their different physicochemical and biological properties. In general, this method involves pouring an organic phase (containing oil and surfactant) into an aqueous phase, which leads to the spontaneous formation of fine droplets due to rapid diffusion of the surfactant from the oil phase into the aqueous phase.[26,29,31] The movement of the hydrophilic surfactant from the oil phase to the aqueous phase after mixing leads to the spontaneous formation of fine oil droplets at the oil−water boundary (Figure 1). This method allows nanoemulsions to be fabricated at room temperature using simple stirring rather than expensive homogenization equipment. This approach may therefore be quite suitable for utilization in the food industry. To our knowledge, the formation of essential oil nanoemulsions by spontaneous emulsification has not been reported previously. Nevertheless, a number of other studies have shown that food-grade antimicrobial nanoemulsions can be produced using high-energy methods that are effective against a range of different microorganisms.[5,10,32−36]

Previous studies using the spontaneous emulsification method have shown that the size of the droplets generated depends on many factors, including interfacial tension, interfacial and bulk rheology, surfactant solubility characteristics, surfactant phase behavior, surfactant structure, and system composition.[31,37,38] The purpose of the present study was to investigate the major factors influencing the formation of nanoemulsions containing fine droplets. In particular, we were interested in developing antimicrobial essential oil delivery systems that have both high physical stability and strong antimicrobial efficacy. The results of this study have important implications for the design and utilization of nanoemulsions as antimicrobial delivery systems in the food and other industries.

MATERIALS AND METHODS

Materials. Carvacrol and nonionic surfactants (Tween 20, 40, 60, 80, and 85) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Tween surfactants consist of a polyoxyethylene head group and a fatty acid tail group of various lengths with the two moieties being linked together via a sorbitol molecule. Medium chain triglyceride (MCT) oil (Miglyol 812) was purchased from Sassol Germany GmbH (Witten, Germany). The manufacturer reported that the MCT used contained 50−65% caprylic acid (C8:0) and 30−45% capric acid (C10:0) in terms of its fatty acid composition. The aqueous phase used to prepare the emulsions was citrate buffer solution (5 mM; pH 3.5). Double distilled water was used in the preparation of all solutions and emulsions. A pH of 3.5 was used in this study to mimic the aqueous phase of many commercial food and beverage products, such as soft drinks and salad dressings.

Nanoemulsion Preparation. Nanoemulsions formation was carried out using a method based on a spontaneous emulsification procedure described previously[29] with some minor modifications. In brief, spontaneous emulsification was performed by addition of an organic phase (containing different amounts of carvacrol, MCT, and nonionic surfactant) to an aqueous phase (5 mM citrate buffer, pH 3.5) while magnetically stirring (500 rpm) the system at ambient temperature (~25 °C). Unless otherwise stated, the experiments were carried out using standardized conditions: 10 wt % oil (carvacrol + MCT), 10 wt % surfactant, and 80 wt % aqueous phase In these samples, the oil (10 g) and surfactant (10 g) were first mixed together, and then the mixture was slowly titrated into 80 g of aqueous phase at a rate of 2 mL/min. In some experiments, nanoemulsions with different surfactant-to-oil ratios (SOR) were prepared by varying the amount of surfactant and water in the system. For example, to prepare a system with SOR = 2, 20 g of surfactant and 10 g of oil were mixed together and then titrated into 70 g of aqueous phase.

Particle Size Measurements. The particle size distributions and mean particle diameters (*Z*-averages) of nanoemulsions were measured using a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments, Malvern, U.K.). This instrument determines the particle size from intensity−time fluctuations of a laser beam (633 nm) scattered from a sample at an angle of 173°. Each individual measurement was an average of 13 runs. To avoid multiple scattering effects, samples were diluted appropriately before the particle size measurements using citrate solution (5 mM, pH 3.5).

Yeast Strains. Four acid-resistant spoilage yeast strains were used as target microorganisms to examine the antimicrobial effects of the nanoemulsions: *Zygosaccharomyces bailii* (ZB), *Saccharomyces cerevisiae* (SC), *Brettanomyces bruxellensis* (BB), and *Brettanomyces naardenensis* (BN). These strains were obtained from the Pepsico R&D Culture Collection (Valhalla, NY). Yeast stock cultures were kept frozen at −70 °C in 25% glycerol. The yeast strain was refreshed on malt extract agar plates (Becton Dickinson, Sparks, MD), and a single yeast colony from the plate was then inoculated into 10 mL of malt extract broth (MEB) media (Becton Dickinson, Sparks, MD), which was previously adjusted to pH 3.5 by citrate buffer with a final strength of 5 mM. The culture was incubated at 32 °C under mild agitation (150 rpm in a rotary shaker) for 2−3 days until the optical density (turbidity) at 600 nm (OD_{600}) was around 1.0 (1-cm path length). As a guideline, an OD_{600} of 1.0 corresponds to approximately 5×10^6 CFU/mL for cultures of yeast strains. The culture was then diluted to about 10^6 CFU/mL using fresh MEB (pH 3.5) to conduct the following antimicrobial assay.

Determination of Antimicrobial Activity. All of the nanoemulsions subject to antimicrobial assay were filtered sterilized using 0.45 μm polyethersulfone membrane filters (F2500-14, Thermo Scientific, Germany). The particle sizes distributions of the nanoemulsions did not change after the filter sterilization (data not shown), indicating that all the droplets were able to pass through the filter pores. A certain amount of the sterile nanoemulsions was then mixed with appropriate amounts of double strength MEB and single strength MEB media, to achieve 10 mL of single strength MEB media (pH 3.5, 5 mM citrate buffer) containing a final concentration of 10,000 μg/mL (= 1 wt %) carvacrol. Five milliliters of the above media was then diluted with the same amount of single strength MEB media, and this procedure was continued so as to make successive 2-fold dilutions until the carvacrol concentration dropped to 156 μg/mL. The final concentrations of carvacrol in each MEB media were therefore 10,000, 5,000, 2,500, 1,250, 625, 312, and 156 μg/mL, respectively. Prior to exposure to antimicrobial treatments, the target yeast strains were freshly subcultured and diluted to about 10^6 CFU/mL as stated previously, and were then 1/100 inoculated into MEB media containing varying levels of carvacrol, to achieve initial cell levels around 10^4 CFU/mL. The surviving cell numbers were monitored after 120 h of incubation at 25 °C. Enumeration was carried out by using a spiral plater (Spiral Biotech, Norwood, Massachusetts). The MIC (minimum inhibitory concentration) was used to compare the antimicrobial efficacy of different carvacrol nanoemulsions. MICs were determined as the lowest concentration of pure carvacrol (not nanoemulsion) that inhibited growth after 5 days of incubation (at least 0.5 log reduction compared to the initial inoculation level). The antimicrobial experiments were conducted with duplicate samples of each treatment, and the entire study was carried out in triplicate.

Statistical Analysis. All experiments were carried out two or three times using freshly prepared samples, and the results are reported as the mean and standard deviation of these measurements. Statistical analysis was performed through subjection of data to analysis of variance (ANOVA) using statistical software (SPSS, Inc., Chicago, IL). Means were subjected to Duncan's test, and a *P*-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSIONS

Effect of Oil Phase Composition on Nanoemulsion Formation. Initially, we examined the influence of oil phase composition on the initial size of the oil droplets formed in nanoemulsions produced using spontaneous emulsification. Oil phase composition was varied by combining different mass ratios of essential oil (carvacrol) and carrier oil (MCT) prior to emulsification. Otherwise, the system composition was standardized: 10 wt % total oil (carvacrol + MCT), 10 wt % surfactant (TWEEN 80), and 80 wt % aqueous phase.

The initial lipid phase composition had a major influence on the formation and stability of the essential oil nanoemulsions. As the carvacrol concentration in the lipid phase increased, the mean droplet diameter initially decreased until it reached a minimum value and then increased (Figure 2). The smallest

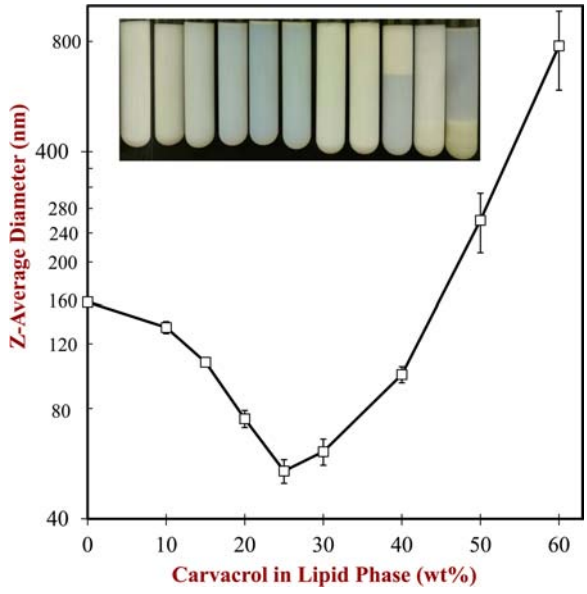

Figure 2. Effect of oil phase composition (wt % carvacrol in oil phase) on mean particle diameter of emulsions and nanoemulsions produced by spontaneous emulsification. Emulsions and nanoemulsions were prepared using 10 wt % oil (carvacrol + MCT), 10 wt % surfactant (TWEEN 80), and 80 wt % water (pH 3.5 citrate buffer solution) at a stirring speed of 500 rpm at ambient temperature (~25 °C). *Note*: data for emulsions prepared using 80 or 100 wt % carvacrol in the lipid phase were not reported because these systems were highly unstable so that reliable measurements could not be made. From left to right, the photograph shows carvacrol concentrations in the lipid phase of 0, 10, 15, 20, 25, 30, 40, 50, 60, 80, 100 wt %, respectively.

droplets ($d \approx 55$ nm) were obtained when the lipid phase contained 25 wt % carvacrol and 75 wt % MCT (i.e., 2.5 wt % carvacrol + 7.5 wt % MCT in the system). Systems containing more than 60 wt % carvacrol in the lipid phase were highly unstable to droplet aggregation and underwent rapid phase separation, and so reliable particle size measurements could not be made. Systems containing 50% carvacrol in the lipid phase had relatively small initial droplet diameters ($d \approx 800$ nm) and were stable to visible phase separation over a few hours (Figure 2); however, creaming and oiling off were observed in these samples after a few days of storage (data not shown). Systems containing ≤40 wt % carvacrol in the lipid phase formed nanoemulsions (i.e., $d < 100$ nm) that were relatively stable to visible creaming during storage over a few days. As mentioned above, the initial size of the oil droplets produced in the nanoemulsions depended on the composition of the lipid phase, with the smallest droplets being formed around 25 wt % carvacrol. There are a number of possible reasons for the observed minimum in mean droplet size at an intermediate essential oil concentration. Oil phase composition will influence the formation of small oil droplets during spontaneous emulsification due to its impact on oil phase properties (e.g., viscosity, interfacial tension, and polarity) and surfactant properties (e.g., solubility, partitioning, and optimum curvature). Oil phase composition will also influence the subsequent stability of the oil droplets to rapid growth through Ostwald ripening and coalescence mechanisms (see below). It is

therefore possible that increasing the amount of carvacrol present in the lipid phase decreased the initial size of the droplets formed but also decreased their subsequent stability to rapid growth. This phenomenon would account for the fact that there was a minimum droplet size produced at a particular lipid phase composition (Figure 2). At a particular carvacrol concentration small droplets could be produced by spontaneous emulsification that were also stable to droplet growth.

It should be noted that the type of carrier oil used to stabilize the antimicrobial nanoemulsions was also important. We found that stable carvacrol nanoemulsions could not be formed at any lipid phase composition when long chain triglycerides (LCT, i.e., corn oil and canola oil) were used as carrier oils (data not shown). The reason that LCT oils were unable to form stable carvacrol nanoemulsions, whereas MCT oils were, is currently unknown. Differences in the molecular characteristics (chain length and unsaturation) of the MCT and LCT may have led to differences in physicochemical properties that impact spontaneous emulsification, such as viscosity, interfacial tension, phase behavior, or optimum curvature. In general, the physicochemical mechanisms governing the size of the droplets produced by spontaneous emulsification are still not clearly understood, and further research is clearly needed in this area.[28]

Effect of Surfactant Type on Nanoemulsion Formation. The impact of nonionic surfactant type on the formation and stability of carvacrol nanoemulsions was investigated. A series of antimicrobial nanoemulsions with fixed lipid phase composition (25% carvacrol + 75% MCT) were prepared using different surfactant types (TWEEN 20, 40, 60, 80, and 85). The type of nonionic surfactant had a pronounced effect on the mean particle diameter of the colloidal dispersions formed (Figure 3). The smallest droplets were formed in the systems prepared using TWEEN 80 ($d \approx 55$ nm), whereas the largest droplets were formed in the systems prepared using TWEEN 85 ($d \approx 1{,}300$ nm). The hydrophilic−lipophilic balance (HLB) of the surfactants appeared to play a role in their ability to form small droplets. Surfactants with a high HLB number (TWEEN 20, HLB = 16.7, $d \approx 610$ nm) or a low HLB number (TWEEN 85, HLB = 11, $d \approx 1{,}300$ nm) were unable to form carvacrol nanoemulsions with small droplets. In contrast, surfactants with intermediate HLB numbers (TWEEN 40, 60, and 80; HLB = 15.6, 14.9, and 15.0, respectively) were all able to form nanoemulsions with small particle sizes ($d < 100$ nm). However, there was not a strong correlation between the particle size produced and the HLB numbers of these surfactants. For example, TWEEN 60 and 80 both had HLB numbers around 15 but gave appreciably different particle sizes (d = 84 nm and 55 nm, respectively). The HLB number is often used as a rough guide for selecting surfactants to stabilize emulsions;[39,40] however, there are other factors that need to be considered. In particular, the molecular geometry of a surfactant is known to play a major role in determining the formation and stability of emulsions and nanoemulsions.[41] TWEEN 60 and 80 both have similar polar head groups and 18-carbon-atom nonpolar tail groups, which accounts for the fact that they have fairly similar HLB numbers of ∼15. However, the nonpolar tails in TWEEN 60 are saturated and therefore fairly linear, whereas those in TWEEN 80 are unsaturated and therefore more kinked. This difference will affect the packing of the surfactant molecules at the oil−water interface, which may impact the tendency for ultrafine droplets to be spontaneously produced when the organic phase is mixed with the aqueous phase. Previous studies have also reported that the presence of double bonds in the nonpolar chains of nonionic surfactants favors the formation of nanoemulsions with smaller droplet sizes.[39,42] In the following experiments, we used TWEEN 80 as a surfactant since it produced emulsions with the smallest initial droplet sizes.

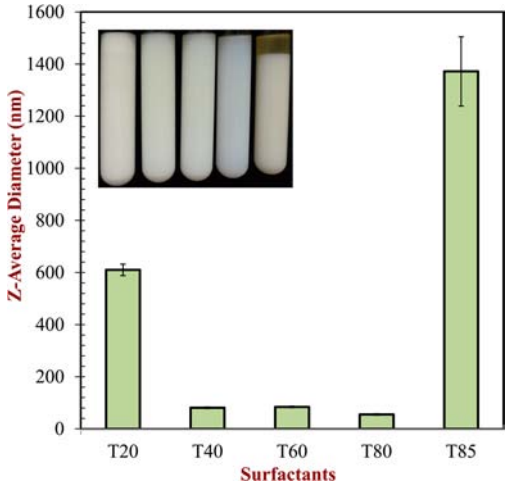

Figure 3. Effect of surfactant type on mean particle diameter and physical stability of emulsions produced by spontaneous emulsification. Oil phase = 2.5% carvacrol + 7.5% MCT. Surfactant was 10 wt % in the system. Surfactants were Tween 20, Tween 40, Tween 60, Tween 80, and Tween 85 as indicated. From left to right, the photograph shows surfactant types from Tween 20 to Tween 85.

Effect of Surfactant Concentration on Particle Size. The effect of surfactant concentration on particle size was investigated by preparing a series of 10 wt % oil-in-water systems with a fixed lipid phase composition (25% carvacrol + 75% MCT) and surfactant type (TWEEN 80) but different surfactant concentrations. The amount of surfactant present in the systems had a major impact on the initial droplet size produced, with smaller droplets being formed at higher surfactant concentrations (Figure 4). For example, the mean droplet diameter was relatively large ($d \approx 6{,}000$ nm), and phase separation rapidly occurred when only 2.5 wt % TWEEN 80 was used. However, the mean droplet size decreased and the creaming stability increased when increasing amounts of surfactant were used to form the nanoemulsions: $d \approx 168$, 55, 34, and 25 nm at 5, 10, 15, and 20 wt % TWEEN 80, respectively. This finding is in agreement with previous studies of nanoemulsion formation using spontaneous emulsification, where smaller droplets were reported at higher surfactant concentrations.[29] A number of physicochemical mechanisms have been proposed to account for the decrease in droplet size with increasing surfactant concentration.[43] First, the amount of surfactant present will influence the interfacial tension and mobility of the oil−water boundary where the oil droplets are spontaneously formed. Second, higher surfactant concentrations mean that a larger number of surfactant molecules diffuse from the organic phase into the aqueous phase when they come into contact, which may promote the formation of finer oil droplets at the oil−water boundary. Third, higher surfactant concentrations will favor different structural organizations of the surfactant, oil, and water molecules in the system, and some of these arrangements are known to be more advantageous for nanoemulsion formation.

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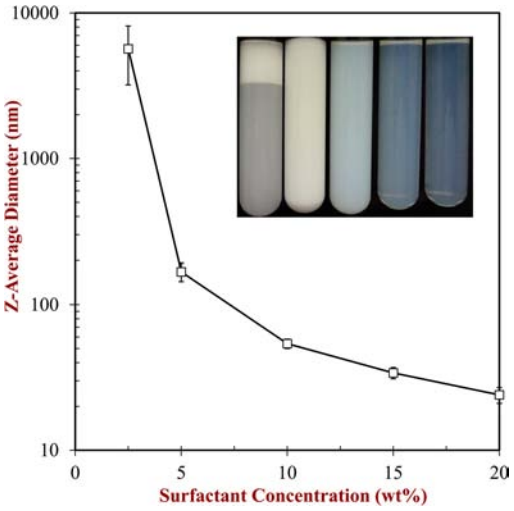

Figure 4. Effect of surfactant concentration on mean particle diameter and physical stability of emulsions produced by spontaneous emulsification. Surfactant (Tween 80) concentration in emulsion varied from 2.5 to 20 wt % as indicated. Oil phase = 2.5% carvacrol + 7.5% MCT. From left to right, the photograph shows surfactant concentrations in the system of 2.5, 5, 10, 15, and 20 wt %, respectively.

It should be noted that we also observed appreciable differences between the short-term stabilities of the carvacrol nanoemulsions formed at different surfactant concentrations. Although very fine droplets were initially formed in the nanoemulsions prepared using 15 or 20 wt % TWEEN 80, these systems were highly unstable to droplet growth, with the mean droplet diameter increasing rapidly over a few days (data not shown). This result suggests that an intermediate level of surfactant may be more suitable for preparation of physically stable antimicrobial nanoemulsions. Additionally, high surfactant levels increase ingredient costs and lead to adverse sensory (taste) issues in commercial applications. For these reasons we used 10 wt % TWEEN 80 in the remainder of the experiments. The physicochemical origin of droplet growth at high surfactant concentration is currently unknown and may have been due to droplet coalescence and/or Ostwald ripening at elevated surfactant levels.

Storage Stability of Carvacrol Nanoemulsions. For most commercial applications it is important that nanoemulsions remain physically stable throughout their shelf life, i.e., there is little or no change in their particle size. We therefore examined the influence of storage time on the stability of a series of nanoemulsions that were found to be stable to visible creaming over short periods (a few days). These systems consisted of 10 wt % oil (0 to 40% carvacrol), 10 wt % surfactant (TWEEN 80), and 80 wt % aqueous phase (pH 3.5 buffer). Initially, these systems had different mean diameters due to the influence of oil phase composition on the efficiency of nanoemulsion formation: 133, 107, 75, 55, 60, and 99 nm for 10%, 15%, 20%, 25%, 30%, and 40% carvacrol in oil phase. For this reason, we calculated the relative increase in droplet size during storage: $\Delta d = 100 \times (d_t - d_0)/d_0$, where d_t and d_0 are the mean droplet diameters at time t and 0, respectively. Experiments were carried out on undiluted samples (10% total oil phase) and on samples that were diluted (×5) with buffer solution (2% total oil phase).

The increase in droplet size in the undiluted nanoemulsions after 30 days of storage ranged from 0% to 132% depending on the initial carvacrol concentration in the lipid phase (Figure 5a).

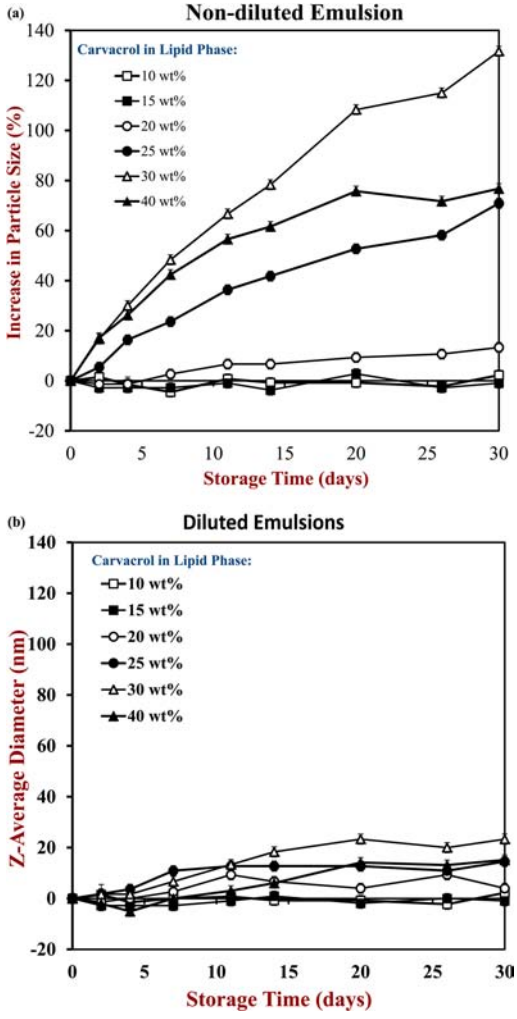

Figure 5. Percentage increase in mean particle diameter of selected nanoemulsions during 30 days of storage at room temperature. Nanoemulsions were prepared using 10 wt % oil (carvacrol + MCT of varying ratios), 10 wt % surfactant (TWEEN 80), and 80 wt % water (pH 3.5 citrate buffer solution) at a stirring speed of 500 rpm at ambient temperature (~25 °C). The nanoemulsions were either undiluted (a) or diluted 5 times by citrate buffer (b).

In general, the increase in droplet size after storage was higher as the amount of carvacrol in the lipid phase increased: $\Delta d \approx 0$ for ≤15 wt % carvacrol, $\Delta d \approx 13\%$ for 20 wt % carvacrol, and $\Delta d > 70\%$ for ≥25 wt % carvacrol. Nevertheless, when the same nanoemulsions were diluted 5 times in buffer solution (5 mM citrate buffer, pH 3.5) prior to storage, the rate of their size increase was greatly reduced (Figure 5b). This observation has important consequences for the practical application of antimicrobial nanoemulsions. If a nanoemulsion is going to be stored in an undiluted form, then the carvacrol concentration in the lipid phase should be kept relatively low (≤15 wt %). On the other hand, it if is going to be stored (or used) in a diluted form then it may be possible to incorporate higher levels of essential oil into the lipid phase. In practice, antimicrobial nanoemulsions are often used in a highly diluted form in commercial products (such as beverages), and therefore

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the rate of droplet growth may be relatively slow after dilution has been carried out. An alternative approach to enhancing the long-term stability of the system would be to store the antimicrobial as an organic phase containing essential oil and carrier oil (carvacrol and MCT) and then add this organic phase to an aqueous product when needed.

The growth of the droplets in the nanoemulsions during storage may have occurred due to coalescence or Ostwald ripening. Coalescence is the process whereby two or more droplets merge together when they collide.[44,45] Droplet coalescence may have been accelerated at high essential oil concentrations because of the relatively high polarity and low interfacial tension of this kind of oil.[46] Indeed, the rate of droplet coalescence in emulsions containing more polar oils often occurs more rapidly than those containing more nonpolar oils because of the influence of the properties of the oil molecules on the optimum curvature.[47,48] The addition of nonpolar triglyceride oils (such as MCT) may therefore have decreased the coalescence rate by decreasing the polarity and increasing the interfacial tension. Ostwald ripening is the process whereby large droplets grow at the expense of smaller ones due to the mass transport of dispersed phase through the intervening continuous phase.[49,50] Carvacrol has a finite solubility in water (~0.83 g/L at ambient temperature),[51] and so nanoemulsions made from it are susceptible to Ostwald ripening.[19,20,52] As shown previously, Ostwald ripening can be inhibited by incorporating a highly water-insoluble oil into a relatively water-soluble oil prior to homogenization due to an entropy of mixing effect.[9,10,33,53] In this study, MCT was beneficial not only for the spontaneous formation of carvacrol nanoemulsions (Figure 2) but also for ensuring their long-term stability, presumably by acting as an Ostwald ripening and/or coalescence inhibitor.

Our results also showed that the stabilities of the nanoemulsions were greatly improved after dilution with water. There are a number of possible reasons for this phenomenon. First, the droplet−droplet collision frequency decreases with decreasing droplet concentration, which would reduce the droplet aggregation rate.[44] Second, the optimum curvature of the surfactant monolayer at the oil−water boundary may have been changed, which would alter the coalescence stability.[47] Third, high levels of surfactant in the aqueous phase may increase Ostwald ripening rates because surfactant micelles can solubilize and transfer oil molecules between droplets.[54] Fourth, high levels of surfactant may promote droplet aggregation through a depletion mechanism.[55] Consequently, dilution of the nanoemulsions may have improved their stability by reducing Ostwald ripening and/or coalescence rates in the system.

Antimicrobial Efficacies of the Spontaneously Emulsified Carvacrol Nanoemulsions. Finally, we determined the antimicrobial efficacy of a series of carvacrol nanoemulsions with different lipid phase compositions. The antimicrobial efficacy was established by measuring the minimum inhibitory concentration (MIC) of carvacrol in a nutrient MEB medium (5 mM citrate buffer, pH 3.5) against four representative acid-resistant yeast strains: *Zygosaccharomyces bailii* (ZB), *Saccharomyces cerevisiae* (SC), *Brettanomyces bruxellensis* (BB), and *Brettanomyces naardenensis* (BN). Initially, the nanoemulsions were filter sterilized to avoid the influence of any endogenous microorganisms, and then different amounts of these sterile nanoemulsions were added to a broth containing yeast cell levels of ~10^4 CFU/mL. Growth curves were obtained by monitoring the cell numbers throughout a total incubation period of 5 days at room temperature. MIC was determined as the lowest concentration of pure carvacrol (not nanoemulsion) that inhibited growth after 5 days incubation.

The antimicrobial efficacy of the nanoemulsions increased as the carvacrol concentration in the oil phase increased, i.e., lower amounts of carvacrol were needed to completely inhibit yeast growth if the nanoemulsion contained higher carvacrol levels in its lipid phase (Table 1). For example, when the carvacrol concentration in the lipid phase was ≤20 wt %, the nanoemulsions could not inhibit growth of any of the yeasts even with carvacrol concentration as high as 10,000 μg/mL (= 1 wt %), but at 40 wt % they could inhibit growth in all the yeasts with carvacrol concentration at only 625 μg/mL (Table 1). At the highest carvacrol level studied (40 wt %), we could not detect any viable cells (<1 CFU/mL) for any of the four yeast strains after 5 days of incubation, which indicated that there was at least a 4-log reduction in microbial numbers (data not shown). The efficacy of the antimicrobial nanoemulsions also depended on the strain of yeast tested, with the effectiveness of the nanoemulsions decreasing in the following order: BN > BB > SC > ZB. This difference may be due to differences in the structural architecture of the yeast cells walls or other biochemical differences between them.

Table 1. Dependence of the Minimal Inhibitory Concentration (MIC) of Carvacrol in Nanoemulsions with Varying Carvacrol Concentration in the Lipid Phase[a]

carvacrol levels in lipid phase (wt %)	MIC (μg/mL)			
	ZB	SC	BB	BN
10	>10,000	>10,000	>10,000	>10,000
15	>10,000	>10,000	>10,000	>10,000
20	>10,000	>10,000	>10,000	>10,000
25	>10,000	>10,000	1250	625
30	10,000	1250	625	625
40	625	625	312	158

[a]The determination of MICs was performed in a nutrient MEB medium (pH 3.5) against four acid -resistant yeasts as indicated: *Zygosaccharomyces bailii* (ZB), *Saccharomyces cerevisiae* (SC), *Brettanomyces bruxellensis* (BB), and *Brettanomyces naardenensis* (BN).

The results of this study are in agreement with those of a recent study on the effects of water-insoluble oils on the formation, stability, and antimicrobial efficacy of essential oil (thyme oil) nanoemulsions produced using a high energy method, i.e., microfluidization.[9] In this previous study, we also found that increasing the level of water-insoluble oils (corn oil or MCT) in the lipid phase of antimicrobial nanoemulsions improved their storage stability but reduced their antimicrobial efficacy. We postulated that this reduction in efficacy was caused by a decrease in the concentration of antimicrobial molecules within the microorganisms due to partitioning into the oil phase of the nanoemulsions. A lipophilic antimicrobial will partition between oil phases, aqueous phases, and microorganism cell walls depending on their relative concentrations and oil−water partition coefficients.[35] As the total amount of MCT in a nanoemulsion increases, more antimicrobial agent (carvacrol) will partition into it, and therefore there will be less available to partition into the cell walls of the microorganisms.

Overall, these results suggest that the lipid phase composition of antimicrobial nanoemulsions must be carefully controlled to ensure they have small droplets, good physical stability, and high antimicrobial efficacy. In addition, the type and amount of surfactant used to formulate the antimicrobial nanoemulsions must also be optimized. The current study suggests that carvacrol nanoemulsions with small droplet sizes and good antimicrobial efficacy can be produced using the following composition: 4 wt % carvacrol, 6 wt % MCT, 10 wt % Tween 80, and 80 wt % water (pH 3.5). These systems were unstable to droplet growth during long-term storage (Figure 5a), but their stability could be greatly improved by dilution with water prior to storage (Figure 5b). In practice, antimicrobial nanoemulsions would be highly diluted when incorporated into commercial products. For example, to obtain a carvacrol concentration in a final product that was effective against all four strains of yeast studied (i.e., 625 μg/mL carvacrol), one would need to dilute the concentrated nanoemulsions described above over 64 times, which would be more than sufficient to reduce droplet growth.

Conclusions. Low-energy nanoemulsions are particularly suitable for developing delivery systems for antimicrobial essential oils. One of the main drawbacks of the low-energy emulsification method is the high amount of surfactant required to form nanoemulsions; however, these systems would be suitable for applications where the nanoemulsion is highly diluted in the final product (e.g., in soft drinks). The spontaneous emulsification method allows antimicrobial nanoemulsions to be produced in a simple and cost-effective manner: titration of an organic phase into an aqueous phase with stirring. In this article, we examined the influence of system composition (carrier oil and surfactant) on the formation, stability, and antimicrobial efficacy of antimicrobial nanoemulsions. Oil phase composition (carvacrol to MCT mass ratio) had a major effect on initial droplet size, with the smallest droplets being formed at 25 wt % carvacrol and 75 wt % MCT in the lipid phase. Surfactant type also had an appreciable impact on particle size, with TWEEN 80 giving the smallest droplets from a group of food-grade nonionic surfactants (TWEEN 20, 40, 60, 80, and 85). The initial size of the oil droplets produced in the nanoemulsions decreased with increasing surfactant concentration, but the instability of the nanoemulsions to droplet growth also increased. The rate of droplet growth also increased with increasing amounts of essential oil in the lipid phase, but this effect could be reduced by diluting the nanoemulsions prior to storage. On the other hand, the antimicrobial efficacy of the nanoemulsions increased as the amount of carvacrol in the lipid phase increased. It is therefore necessary to optimize the oil phase composition to ensure good physical stability and high antimicrobial efficacy. In summary, the results reported in this study have important implications for the design and utilization of nanoemulsions as antimicrobial delivery systems in the food and other industries. In future studies it would be informative to examine the influence of essential oil type on the formation, stability, and activity of antimicrobial nanoemulsions.

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