Effect of Carrier Oil and Co-Solvent on The Formation of Clove Oil Nanoemulsion by Phase Inversion Technique

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Abstract. Development of nanoemulsion is gaining considerable attention for use in delivering hydrophobic constituents such as clove oil in foods and agriculture system. The small size of the oil droplets in the nanoemulsion system offers many advantages such as high stability, optical clarity, and improved water solubility and bioactivity. This research was aimed at investigating the effect of incorporation of carrier oil and co-solvent on the formation of clove nanoemulsion. Clove oil-loaded nanoemulsions were prepared by a low energy – phase inversion technique involving a carrier oil (medium chain triglyceride/MCT), at different ratios to the clove oil (1:2, 1:1, 2:1), a co-solvent (glycerol) at ratios of 0 and 1:1 to the mixture of clove oil and MCT and a non-ionic surfactant (Tween 80) at a ratio of 1:1, with two concentration levels of mixture of clove oil and MCT (5% and 10%). The formation and characteristics of nanoemulsion were evaluated including particle size, polydispersity index, zeta potential, and freeze-thaw stability, as well as their possible mechanisms of destabilization. Particle sizes ranged from 45.98 to 220 nm with narrow ranges of polydispersity index (0.072 – 0.286) and zeta potentials (-12.8 and -22.6 mV). Incorporation of carrier oil at low proportions gave smaller size of oil droplets, and the presence co-solvent enhanced nanoemulsion stabilization. Creaming accompanied by oiling-off was found upon destabilization of nanoemulsion with different rate and appearance as influenced by nanoemulsion composition. This study provides important information about stabilization of nanoemulsion by incorporating carrier oil and co-solvent suitable for foods and agrochemical formulation.

1. Introduction
The use of essential oils in the development of natural food ingredients has gained considerable interest due to their aromatic properties as well as other functionalities such as antimicrobials [1–5] and antioxidant [3,6]. Most of the essential oils are insoluble in water and are difficult to incorporate them into water-based food formulation. Transforming the oil into a colloidal delivery system such as oil-in-water (o/w) nanoemulsion is an interesting approach to overcome their water insolubility problems.

In nanoemulsion system, oil droplets are sized down to below 100 nm [7], 20 – 200 nm [8] to ranges below 300 nm [9] so that the tiny size of droplets generates unique properties providing potential advantages such as high physical stability, enhanced bioactivity and transparent appearance [10,11]. Fabricating nanoemulsion using low energy approaches has attracted extensive interest because of their practical application with low cost and high energy efficiency [12]. Many studies have been done to produce stable nanoemulsion using low energy approaches, but only a few uses oil model suitable for
the food system. In this study, clove oil was used as an essential oil model for the formation of nanoemulsion using phase inversion point technique. Clove oil was shown to have the largest inhibition against microbiota mainly found in food [13] clove oil nanoemulsions have been formulated using low energy approaches incorporating non-ionic surfactant with different physical and stability properties [13,14].

As a non-equilibrium system, nanoemulsion tends to undergo separation over time with many mechanisms such as Ostwald ripening, creaming/sedimentation, flocculation and coalescence [15,16]. Many efforts have been done to produce stable nanoemulsion by incorporating co-solvent and carrier oil. Incorporation of the carrier oil facilitates the small droplet formation as well as improve the stability. Co-solvents are used because they can modify the bulk properties of aqueous solutions (such as viscosity, density, refractive index, interfacial tension, and solubility) and also the structural properties of surfactant solutions (such as optimum curvature, critical micelle concentration, and phase behaviour). In general, nanoemulsion formation can be impacted by differences in oil phase viscosity, interfacial tension, and phase behaviour, while nanoemulsion stability can be influenced by differences in polarity and water-solubility of the oil molecules.

Although small initial particle diameters can be achieved using carrier oil and cosolvents, the resulting nanoemulsions are often highly unstable to droplet growth during storage. It is important that the combination of surfactant and oil components used is able to form a microemulsion at an appropriate ratio that will break down and produce fine oil droplets. This present study aimed at investigating the effect of the addition of carrier oil and co-solvent on the formation of clove nanoemulsion and determining their formulation with surfactant and oil composition that provide stable nanoemulsion.

2. Materials and Methods

2.1. Materials

Clove oil was extracted by hydro-distillation of the leaves of *Syzygium aromaticum* obtained from Manoko Experimental Station, West Jawa. The oil was used as received without further process. Non-ionic surfactant polyoxyethylene sorbitan oleate (Tween 80) and glycerol were of analytical grade (Merck, Germany). Medium chain triglyceride (Miglyol 812N) was purchased from Cremer Oleo GmbH Co. KG (Hamburg, Germany). Water used for emulsion preparation and particle size analysis was purified by Direct-Q® 3 UV-R Water Purification System (Merck, Germany).

2.2. Methods

2.2.1. Preparation of Nanoemulsion

| Table 1. Compositions of clove oil nanoemulsion |
|---------------------------------------------|
| **Formula** | **Clove Oil (%)** | **MCT (%)** | **Ratio to Clove Oil** | **Glycerol (%)** | **Ratio to Clove Oil +MCT (%)** | **Tween 80 (%)** | **Ratio to Clove Oil +MCT (%)** |
|----------------|------------------|------------|------------------------|-----------------|-------------------------------|-----------------|-------------------------------|
| C1             | 1.7              | 3.3        | 2:1                    | 0.0             | 0                             | 5.0             | 1:1                           |
| C2             | 1.7              | 3.3        | 2:1                    | 5.0             | 1:1                           | 5.0             | 1:1                           |
| C3             | 3.3              | 6.7        | 2:1                    | 0.0             | 0                             | 10.0            | 1:1                           |
| C4             | 3.3              | 6.7        | 2:1                    | 10.0            | 1:1                           | 10.0            | 1:1                           |
| C5             | 2.5              | 2.5        | 1:1                    | 0.0             | 0                             | 5.0             | 1:1                           |
| C6             | 2.5              | 2.5        | 1:1                    | 5.0             | 1:1                           | 5.0             | 1:1                           |
| C7             | 5.0              | 5.0        | 1:1                    | 0.0             | 0                             | 10.0            | 1:1                           |
| C8             | 5.0              | 5.0        | 1:1                    | 10.0            | 1:1                           | 10.0            | 1:1                           |
| C9             | 3.3              | 1.7        | 1:2                    | 0.0             | 0                             | 5.0             | 1:1                           |
| C10            | 3.3              | 1.7        | 1:2                    | 5.0             | 1:1                           | 5.0             | 1:1                           |
| C11            | 6.6              | 3.3        | 1:2                    | 0.0             | 0                             | 10.0            | 1:1                           |
| C12            | 6.7              | 3.3        | 1:2                    | 10.0            | 1:1                           | 10.0            | 1:1                           |
Nanoemulsion was prepared using a low energy phase inversion technique. This technique involves the formation of the water-in-oil emulsion and the subsequent phase inversion into the oil-in-water emulsion. Clove oil was placed into a beaker and mixed with MCT and Tween 80 under magnetic stirring at 750 rpm for 30 min to form an organic phase. Separately, the water phase was prepared by mixing water and glycerol under the same stirring condition as the organic phase preparation. Nanoemulsion was then formed by adding the water phase into the organic phase upon stirring for 60 min to reach the final weight of the emulsion system of 50 g. The nanoemulsion formed was placed into sample bottles for further use of analysis. The compositions of nanoemulsion were described in Table 1. Experiments were done in two replications.

2.2.2. Particle Size and Zeta Potential Analysis

Emulsion droplet size was measured using a Malvern ZetaSizer (Nano-ZS, Malvern Instruments, Malvern, UK). Emulsion sample (2 drops) was placed into a cuvette and diluted in water (2 ml) for particle size measurement. Sample for zeta potential measurement (25 μl) was placed into a capillary cell and diluted in water (2 ml). The particle size was measured as Z-average applying Stokes-Einstein relation with its corresponding polydispersity index (PDI). Measurement of particle size and zeta potential of each sample was taken three times.

2.2.3. Freeze-Thaw Stability

The nanoemulsion was placed into reaction tubes and tested for their stability against freeze and thaw cycles by keeping it at -18°C for 20 hours and 40°C for 2 hours. The stability was determined by evaluating the phase separation upon freeze-thaw cycles.

3. Results and Discussion

3.1. Oil Droplet Size

Oil droplet size varied with the differences in the concentration of the mixture of oil and MCT, the oil to MCT ratio, the concentration of Tween 80 and the concentration of glycerol, ranging from 46 to 220.2 nm. The smallest droplet sizes were observed in the use of the lowest proportion of MCT (oil to MCT ratio of 2:1) (Figure 1). With the presence of glycerol, the use lower concentration of Tween 80 and mixtures of oil and MCT resulted in larger particle size. Conversely, in the absence of glycerol, the lower concentration of Tween 80 and mixtures of oil and MCT produced smaller particle sizes were obtained in.

In the clove oil-loaded nanoemulsion system which the density of the oil higher than water (1.06 g/cm³), the presence of MCT with the density of 0.94 g/cm³ acts as a lighting agent that reduces the density difference [17] and prevent gravitational separation. This might facilitate the formation of ultrafine droplet sizes under the certain surfactant-to-oil ratio. Addition of MCT in large proportions (oil to MCT ratio of 1:1 and 1:2) produced nanoemulsions with larger droplet sizes. In the higher MCT proportions, the larger density differences between the oil and water phase might result in the formation of larger oil droplet sizes. Similar results were also reported for a mixture of MCT with capsanthin and vitamin E [18] that used relatively low ratios of MCT to produce nanoemulsion with small droplet size.

The presence of MCT might also modify the oil viscosity and solubility that determine the formation of small droplets in the nanoemulsion [16,19]. The addition of MCT with a viscosity of 27 – 33 mPa.s could increase the watery viscosity of clove oil. At the lowest proportion of MCT (Clove oil to MCT ratio of 2:1), the minimum increase in oil phase viscosity possibly enabled the effective mass transport of Tween 80 through the oil and into the aqueous phase [20] and rapid droplet disruption during nanoemulsion preparation.

The incorporation of glycerol into the aqueous phase modifies many physicochemical and molecular properties such as density, viscosity and refractive index that may affect the droplet size [18]. It has been reported that the addition of glycerol at high level decreased oil-water interfacial tension,
increased the critical micelle concentration and decreased the HLB number thus facilitated the formation of small droplets. In this present work, glycerol was added at a relatively low level (a maximum of 10%) and resulted in a slight decrease in droplet size. Lower levels of glycerol addition (5%) were found to increase the droplet size. Similar results were also observed in the use of low concentration of glycerol in the preparation of vitamin E nanoemulsion [21]. The explanation can be found in the phenomenon of the formation of highly viscous crystalline liquid that complicates the breakup of the oil-water interface for the formation of small droplets.

The concentration of oil (a mixture of clove oil and MCT) and Tween 80 played also roles in determining the oil droplet size. The use of high concentration of oil produced nanoemulsion with larger oil droplet size, particularly at oil to MCT ratio of 1:2 and 2:1. Although the use of high concentration of oil was also accompanied by the addition of high concentration of Tween 80, larger droplet sizes were observed. These results suggest that the physicochemical properties of the bulk phase together with the phase behaviour of specific surfactant-oil-water system are important in the formation of the nanoemulsion.

**Figure 1.** Oil droplet size of nanoemulsion as influenced by the oil to MCT ratio and the organic phase and glycerol concentration.

### 3.2. Polydispersity

Polydispersity index (PDI) varied between 0.045 and 0.285, and had different trends with that of the droplet size. The lowest PDI was found in the use of oil and MCT at the same ratio (1:1) (Figure 2). Incorporation of lower or higher proportion of MCT (oil to MCT ratio of 1:2 and 2:1, respectively) showed higher PDI. The presence of glycerol exhibited also the increases in PDI, both at low and high concentration. These could be associated with the physicochemical changes of the bulk phase with the variation of composition which altered the oil-water interface properties.

Larger PDI observed in the presence of MCT at a high proportion (oil to MCT ratio 1:2) might be attributed to the higher viscosity of the oil and the expected slow droplet disruption during nanoemulsion preparation as discussed in the previous section. Furthermore, under the specific composition of oil and surfactant, destabilization process such as coalescence or Ostwald ripening might occur. The broad size distribution can be as a result of destabilization through the coalescence mechanism, while the narrow distribution can be attributed to the Ostwald ripening [22,23]. Coalescence takes place when the surfactant film covered the droplet surface ruptured upon contact each other [23]. In the Ostwald ripening, the larger droplets grow at the expense of smaller droplets [16,24–26]. The presence of glycerol showed increases in PDI, both at low and high concentration. The high PDI at the addition of glycerol can be explained by the interactive effect of surfactant concentration.

It was interesting to note that the small PDI observed was not associated with the small corresponding droplet size (Figure 3). This might be related to the rate of droplet disruption during mixing and the rate of destabilization after the droplet formation. Often, ultrafine droplets produced at the beginning grow rapidly during storage, which may be due to the occurrence of coalescence or Ostwald ripening.
3.3. Zeta Potential
Zeta potential of clove oil nanoemulsion varied with different composition, ranging from -12.80 to -22.60 mV. The addition of glycerol at high level showed low values of zeta potential for the whole combination of oil concentration, Tween 80 and oil to MCT ratio, while the incorporation of glycerol at low concentration gave higher values of zeta potential at the certain oil to MCT ratio (1:2 and 1:1). The presence of MCT at the same proportion with clove oil together with the addition of glycerol and the oil mixture at low concentration produced droplets with the highest value of zeta potential.

Although non-ionic surfactant was used in this experiment, negative values of zeta potential were observed in all samples. This phenomenon was also found in the use of non-ionic surfactant in the preparation of some nanoemulsions [28] which might be associated with the interaction of oxyethylene group of Tween 80 and water molecules in the presence of hydroxyl ion at the oil-water interface.

The addition of glycerol was reported to alter the solubility of surfactant monomer and partially dehydrate the hydrophilic head-group. These might also change the oxyethylene group of Tween 80 and water molecules that altered electrostatic forces surrounding the particles. It was observed that zeta potential decreased with the incorporation of glycerol at high concentration. This effect was more pronounced at the use of MCT at medium to high concentration.

All samples had relatively low absolute of zeta potential (a maximum of 22.60 mV). Generally, the absolute values of zeta potential lower than 30 mV indicate low stability against aggregation due to electrostatic attractive forces between droplet molecules. However, a stable nanoemulsion against freeze-thaw test was produced within the specific formulation, indicating the role of other stabilization mechanisms than repulsive forces.

Figure 2. Polydispersity index of nanoemulsion as influenced by the oil to MCT ratio and the organic phase and glycerol concentration.

Figure 3. Zeta potential of nanoemulsion as influenced by the oil to MCT ratio and the organic phase and glycerol concentration.
3.4. Freeze-thaw Stability
Freeze-thaw test provides an explanation about the instability mechanism of nanoemulsion upon chilling and cold storage. During freezing and thawing, some physicochemical changes take place including fat crystallization, ice formation, interfacial phase transition, interfacial layer conformation, and chemical or electrostatic interaction which can cause instability. Nanoemulsion samples had different freeze-thaw stability, varied between 1 cycles and 6 cycles. Samples containing a high concentration of oil tended to undergo separation at the first or second cycle. Large phase separations were observed in nanoemulsion with high oil to MCT ratio. Only one sample showed separation at cycle 5 and finally, only one sample was stable against separation after 6 cycles. The most stable nanoemulsion was observed in the use of a high ratio of MCT with low concentrations of oil, Tween 80 and glycerol.

Upon freezing, the formation of ice pushes the oil droplets to move into close proximity. When the repulsive forces on the surface of the droplets are not strong enough, the droplets contact each other and the coalescence occurs. The use of small molecule surfactant such as Tween 80 forms a thin layer of membrane on the surface oil droplets so it gives less protection against coalescence during freezing (16). However, since the concentration of oil droplets used in this study was low (5%), they were less prone to coalescence during freezing. Droplet concentration is an important factor determining freeze-thaw stability as the droplet concentration might increase upon freezing resulting in coalescence instability.

4. Conclusion
Nanoemulsion of clove oil can be produced by low energy using phase inversion technique. The concentration of total oil (clove oil and MCT) and the incorporation of MCT as a carrier oil and glycerol as a co-solvent modified the characteristics of the nanoemulsion. The use of carrier oil at low concentration with or without glycerol produced small droplet size but susceptible to destabilization by the freeze-thaw test. The use of MCT at the same ratio with the clove oil both with and without glycerol gave the lowest polydispersity index. The use of low concentration of total oil together with the presence of MCT at high concentration and the addition of glycerol at low concentration produced larger droplet size but stable against 6 cycles of freeze-thaw test, indicating the role of MCT and glycerol in inhibiting destabilization. Further research is required to evaluate the possible mechanisms of destabilization.

5. References
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