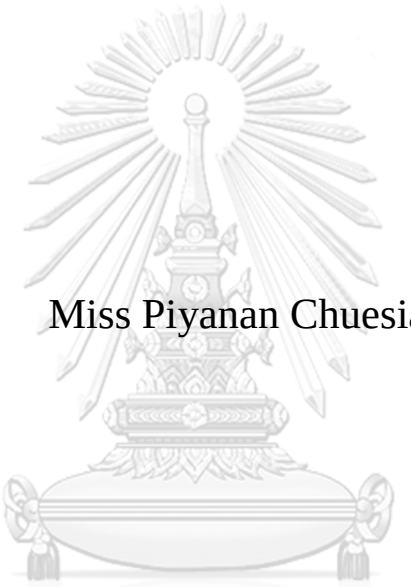
CINNAMON OIL NANOEMULSIONS FOR INHIBITION OF CONTAMINATED PATHOGENS ON ASIAN SEABASS FILLET

Miss Piyanan Chuesiang



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นาโนอิมัลชันน้ำมันอบเชยเพื่อยับยั้งจุลินทรีย์ก่อโรคที่ปนเปื้อนบนปลากะพงแล่

น.ส.ปิยะนันท์ ชื่อเสียง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	CINNAMON OIL NANOEMULSIONS FOR INHIBITION OF CONTAMINATED PATHOGENS ON ASIAN SEABASS FILLET
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Field of Study	Food Technology
Thesis Advisor	Associate Professor UBONRATANA SIRIPATRAWAN
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Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

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ปิยะนันท์ ชื่อเสียง : นาโนอิมัลชันน้ำมันอบเชยเพื่อยับยั้งจุลินทรีย์ก่อโรคที่ปนเปื้อนบนปลากะพงแล่. (CINNAMON OIL NANOEMULSIONS FOR INHIBITION OF CONTAMINATED PATHOGENS ON ASIAN SEABASS FILLET) อ.ที่ปรึกษาหลัก : รศ. ดร.อุบลรัตน์ สิริภัทราวรรณ, อ.ที่ปรึกษาร่วม : ผศ. ดร.รมณี สงวนดีกุล,- -

น้ำมันอบเชยเป็นสารตามธรรมชาติที่มีประสิทธิภาพในการต้านจุลินทรีย์ แต่การใช้น้ำมันอบเชยในอาหารยังคงถูกจำกัดเนื่องจากสมบัติความไม่ชอบน้ำของน้ำมันอบเชย งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อเตรียมนาโนอิมัลชันจากน้ำมันอบเชยโดยใช้วิธีการเปลี่ยนเฟสด้วยอุณหภูมิ เริ่มต้นจากการให้ความร้อนแก่ของผสมระหว่างเฟสน้ำมัน เฟสน้ำ และสารลดแรงตึงผิว จนกระทั่งถึงอุณหภูมิที่ทำให้เกิดการเปลี่ยนเฟส จากนั้นทำการลดอุณหภูมิของระบบลงอย่างรวดเร็วเพื่อให้เกิดหยดน้ำมันขนาดอนุภาคนาโน การศึกษาผลขององค์ประกอบของเฟสน้ำมัน คืออัตราส่วนของน้ำมันอบเชยต่อสารต้านการเกิด Ostwald ripening หรือ ไดรกลีเซอไรด์สายกลาง ต่อสมบัติทางเคมีฟิสิกส์ สมบัติต้านจุลินทรีย์ รวมถึงสเถียรภาพของนาโนอิมัลชัน พบว่า การใช้น้ำมันอบเชยในปริมาณ 30-40 เปอร์เซนต์ของเฟสน้ำมันส่งผลให้เกิดนาโนอิมัลชันที่มีหยดน้ำมันขนาดเล็ก (ประมาณ 100 นาโนเมตร) เป็นองค์ประกอบ ในขณะที่การใช้น้ำมันอบเชยที่ปริมาณอื่นไม่ส่งผลต่อการเตรียมนาโนอิมัลชัน นอกจากนี้ยังพบว่านาโนอิมัลชันที่เตรียมโดยการใช้น้ำมันอบเชย 40 เปอร์เซนต์ของเฟสน้ำมันมีค่าความเข้มข้นต่ำสุดในการยับยั้งการเจริญของ *E. coli*, *S.* Typhimurium, *S. aureus* และ *V. parahaemolyticus* ต่ำที่สุดเมื่อเทียบกับนาโนอิมัลชันที่เตรียมได้จากสภาวะอื่น ซึ่งเป็นผลมาจากหยดน้ำมันอบเชยขนาดอนุภาคนาโนที่เป็นองค์ประกอบในนาโนอิมัลชั่นช่วยเพิ่มอัตราส่วนพื้นที่ผิวต่อปริมาตรของน้ำมันอบเชยในการทำปฏิกิริยากับแบคทีเรียแม้ว่ามีปริมาณสารสำคัญซินนามาลดีไฮน์เป็นองค์ประกอบน้อยกว่านาโนอิมัลชันที่เตรียมได้จากสภาวะอื่น ดังนั้นจึงเลือกใช้นาโนอิมัลชันที่เตรียมโดยการใช้น้ำมันอบเชย 40 เปอร์เซนต์ของเฟสน้ำมันเพื่อศึกษาผลของความเข้มข้นของสารลดแรงตึงผิวต่อสมบัติทางเคมีฟิสิกส์ สมบัติการต้านการเจริญของจุลินทรีย์ และสเถียรภาพของนาโนอิมัลชัน ผลการทดลองพบว่า การเพิ่มความเข้มข้นของสารลดแรงตึงผิวจาก 10 เป็น 20 เปอร์เซนต์ทำให้ขนาดของหยดน้ำมันลดลงอย่างมีนัยสำคัญ ($p \leq 0.05$) และทำให้ลักษณะปรากฏของนาโนอิมัลชันเปลี่ยนแปลงจากสีขาวขุ่นเป็นสีขาวใส แต่ไม่ส่งผลต่ออุณหภูมิการเปลี่ยนเฟส เสถียรภาพของนาโนอิมัลชันที่อุณหภูมิ 4 และ 25 องศาเซลเซียส รูปร่างของหยดน้ำมัน รวมถึงค่าความเข้มข้นต่ำสุดในการยับยั้งการเจริญของจุลินทรีย์ อย่างไรก็ตามการเพิ่มความเข้มข้นของสารลดแรงตึงผิวส่งผลต่อการเพิ่มอัตราการยับยั้งการเจริญของจุลินทรีย์ และช่วยยับยั้งการเจริญของแบคทีเรียบางชนิดได้นานกว่าการใช้นาโนอิมัลชันที่เตรียมจากการใช้สารลดแรงตึงผิวที่ความเข้มข้น 10 เปอร์เซนต์ หรือการใช้น้ำมันอบเชยปกติ ความเข้มข้นของสารลดแรงตึงผิวส่งผลต่อการเปลี่ยนแปลงลักษณะทางสัณฐานวิทยาของจุลินทรีย์ ทำลายผนังเซลล์ของจุลินทรีย์ และทำให้เกิดการรั่วไหลขององค์ประกอบต่างๆภายในเซลล์ จากการศึกษาการประยุกต์ใช้นาโนอิมัลชันของน้ำมันอบเชยเพื่อควบคุมคุณภาพทางจุลินทรีย์ของเนื้อปลากะพงในระหว่างการเก็บรักษาที่อุณหภูมิ 4 ± 2 องศาเซลเซียส เป็นเวลา 8 วัน พบว่านาโนอิมัลชันของน้ำมันอบเชยช่วยลดจำนวนจุลินทรีย์โดยรวม จึงช่วยยืดอายุการเก็บรักษาเนื้อปลากะพงได้มากถึง 4 วันขึ้นกับความเข้มข้นของนาโนอิมัลชันที่เลือกใช้ ดังนั้นนาโนอิมัลชันของน้ำมันอบเชยมีศักยภาพในการใช้เป็นสารต้านจุลินทรีย์เพื่อยืดอายุการเก็บรักษาเนื้อปลากะพงได้

สาขาวิชา	เทคโนโลยีทางอาหาร	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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KEYWORD: NANOEMULSION, PHASE INVERSION TEMPERATURE, CINNAMON OIL, ANTIMICROBIAL ACTIVITY, ASIAN SEABASS FILLET

Piyanan Chuesiang : CINNAMON OIL NANOEMULSIONS FOR INHIBITION OF CONTAMINATED PATHOGENS ON ASIAN SEABASS FILLET. Advisor: Assoc. Prof. Dr. UBONRATANA SIRIPATRAWAN Co-advisor: Asst. Prof. Dr. Romanee Sanguandeekul,Prof. Lynne McLandsborough

Cinnamon oil is an effective natural antimicrobial agent, but the utilization is limited by its low water-solubility. In this study, cinnamon oil nanoemulsions were prepared using a phase inversion temperature (PIT) method, which simply involves heating a mixture of surfactant, oil, and water about the PIT and then quench cooling with stirring. The impact of oil phase composition (cinnamon to ripening inhibitor ratio) and surfactant concentration on the formation, stability, antimicrobial activity and chemical stability of the nanoemulsions were determined. Optimal mean droplet diameters with narrow size distribution were obtained at intermediate cinnamon oil levels (30-40 wt%) in the oil phase. Conversely, relatively larger droplets were generated at lower (0 to 20 wt%) and higher (60 to 100 wt%) cinnamon oil levels. In the present study, the system containing 40 wt% cinnamon oil and 60 wt% medium chain triglyceride (MCT) oil in the oil phase was selected for further studies since it gave the highest stability of antimicrobial cinnamon oil nanoemulsion with the smallest droplet size and lowest PIT. The impact of oil phase composition on the minimum inhibitory concentration (MIC) of cinnamon oil nanoemulsions against *Escherichia coli*, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Vibrio parahaemolyticus* was largely due to cinnamaldehyde, which is highly susceptible to chemical degradation. For this reason, a decrease in cinnamaldehyde content and an increase in a major reaction product (benzaldehyde) were observed in the cinnamon oil nanoemulsions during storage. The antimicrobial activity of cinnamon oil nanoemulsions increased (lower MICs) as the droplet size decreased, even though the cinnamaldehyde content was lower. Cinnamaldehyde degraded during emulsification and throughout storage. The impact of surfactant concentration (10-20 wt%) on PIT, droplet size, stability, particle morphology, the MIC, dynamic time kill, and changes in bacteria morphology were determined. Increasing non-ionic surfactant (Tween®80) concentration from 10 to 20 wt% significantly decreased droplet size of the nanoemulsions but had no effect on the PIT, stability (at 4 and 25 °C), particle morphology and MIC values of the nanoemulsions. However, dynamic time kill plots revealed that nanoemulsions with higher surfactant concentrations (15 and 20 wt%) led to faster killing or better prolonged inhibition of bacteria compared to those with lower concentration (10 wt%) or with bulk cinnamon oil. Morphological changes of the bacteria were more promoted for nanoemulsions containing higher surfactant concentrations as shown by field emission scanning electron microscopy (FE-SEM). The antimicrobial activity of the cinnamon oil nanoemulsions was attributed to their ability to disrupt bacterial cell wall structures and promote expulsion of internal cellular material. For further study, cinnamon oil nanoemulsion fabricated with 15 wt% surfactant was selected to study its antimicrobial activity on the shelf-life of Asian seabass flesh during chilled storage (4 °C). Decreasing total viable count (TVC) was observed in the samples treated with cinnamon oil nanoemulsion. Thus, the shelf-life of the cinnamon oil nanoemulsion treated samples can be extended up to 4 days, longer than untreated (control) sample. These results indicated that cinnamon oil nanoemulsion have high potential to be used as antimicrobial agent for preservation of fish fleshes.

Field of Study:	Food Technology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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Piyanan Chuesiang

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CHAPTER 1

INTRODUCTION

1.1. Overview of the dissertation

This dissertation proposed an effective method for fabrication of antimicrobial cinnamon oil nanoemulsions using the phase inversion temperature (PIT) method, which is simple to carry out and do not require any specialized equipment. The research was divided into 3 parts. In the first part of research, an impact of oil phase composition on the optimization of cinnamon oil nanoemulsions was studied. Important characteristics such as PIT temperature of the system, oil droplet size, particle size distribution, physical and chemical stability and antimicrobial activity (against foodborne pathogenic bacteria; *Escherichia coli*, *Salmonella* Typhimurium, *S. aureus* and *V. parahaemolyticus*) of the nanoemulsions were used to select the most appropriate system in order to study the effect of surfactant concentration on the physicochemical properties and antimicrobial activity of the nanoemulsions in the second part. Basic characteristics such as PIT temperature, oil droplet size, particle size distribution, morphology, physical and chemical stability and antimicrobial activity of the nanoemulsions were also studied. Moreover, dynamic time kill assay and field emission scanning electron microscopy (FE-SEM) techniques were used to provide further insights into the antimicrobial activity of the nanoemulsions. After the optimization of cinnamon oil nanoemulsions using oil phase composition and surfactant concentration, the most appropriate system was then selected again and used in the third part of research as the antimicrobial agent for extending the shelf life of Asian seabass flesh. Comparison between cinnamon oil nanoemulsions and bulk cinnamon oil were made separately on the microbiological property of the flesh.

1.2. Background and significant of the study

Cinnamon oil is documented as an antimicrobial agent against many types of foodborne pathogenic bacteria in foods and food products for centuries due to the ability of its constituents (Hilbig, Ma, Davidson, Weiss, & Zhong, 2016; Ribeiro-Santos et al., 2017; Zhang, Liu, Wang, Jiang, & Quek, 2016) which penetrate through the microbial membrane, cause the leakage of ions and cytoplasmic content, thus leading to the cellular breakdown (Gill & Holley, 2004; Siripatrawan, 2016; Zhang et al., 2016). However, incorporation of cinnamon oil into food and beverage products directly is still limited due to the interferences on food organoleptic properties caused by its hydrophobicity, high reactivity, and strong odor. The use of plant essential oil (such as cinnamon oil) combined with nanoemulsion delivery system is recently proposed as a new and effective method to control overall qualities of food and beverage (Komaiko & McClements, 2016; Ozogul et al., 2017). The nanoemulsions provide a number of advantages over the conventional emulsions due to the small droplet size (20-200 nm), resulting in the increasing of i) stability against gravitational and droplet aggregation, (ii) optical clarity; and (iii) bioactivities such as antimicrobial activity of the system (Komaiko & McClements, 2016; Ryu, McClements, Corradini, & McLandsborough, 2018). Although nanoemulsions could be formed by many methods, phase inversion temperature (PIT) method is a cost efficient method which provide kinetically-stable nanoemulsions in terms of small droplets size and narrow size distribution (Chuesiang, Siripatrawan, Sanguandeekul, McLandsborough, & Julian McClements, 2018; Komaiko & McClements, 2016). Thus, cinnamon oil nanoemulsions in the present study was fabricated using PIT method with the variation of oil phase composition and surfactant concentration. Until now, formation

of cinnamon oil nanoemulsions and its efficacy have never been studied for maintaining quality of fish fillets. The research is hypothesized that physicochemical and biological properties of cinnamon oil can be improved by its encapsulation with the nanoemulsion system. Hence, this research aims to extend the shelf life of Asian seabass flesh by using cinnamon oil nanoemulsions as an antimicrobial agent. Optimum cinnamon oil nanoemulsion is formulated using PIT method. Microbiological property of fish flesh after treatment is investigated and compared with that of untreated one.

1.3. Objectives of dissertation

1.3.1. To determine the optimal condition for fabrication of cinnamon oil nanoemulsions using the PIT method.

1.3.2. To investigate the impact of oil phase composition and surfactant concentration on physicochemical properties of cinnamon oil nanoemulsions.

1.3.3. To compare the antimicrobial activity of cinnamon oil nanoemulsions against foodborne pathogenic bacteria; *E. coli*, *S.* Typhimurium, *S. aureus* and *V. parahaemolyticus* to bulk cinnamon oil.

1.3.4. To extend the shelf-life of Asian seabass flesh by using cinnamon oil nanoemulsions as an antimicrobial agent and compare to those of bulk cinnamon oil.

1.4. Scope of dissertation

The impact of oil phase composition and surfactant concentration on the physicochemical properties including PIT temperature, oil droplet size, particle size distribution, physical stability, chemical stability, antimicrobial activity and

mechanism of antimicrobial activity of cinnamon oil nanoemulsions were studied in this research. The application of antimicrobial cinnamon oil nanoemulsions for extending the shelf life of Asian seabass flesh was also investigated.

1.5. Expected beneficial outcomes

1.5.1. An effective method which is simple and consistent to fabricate a highly stable cinnamon oil nanoemulsion.

1.5.2. An effective method to extend the shelf life of Asian seabass flesh.

CHAPTER 2

LITERATURE REVIEWS

2.1. Essential oils

Essential oils are secondary metabolites, produced by different part of plants, and exhibit a range of beneficial biological activities such as antimicrobial, antioxidant, antitumor, and anti-inflammatory activities due to their constituents, volatile and non-volatile compounds (Nabavi et al., 2015; Ribeiro-Santos et al., 2017). Biological properties of essential oils are mainly attributed to the major active compounds which can be categorized by their chemical structure; terpenes, terpenoids, aromatic and other compounds (Hassoun & Emir Çoban, 2017). Terpenes are hydrocarbon with isoprene units. Terpenoids *e.g.* thymol, carvacrol and linalool are terpenes constructed with oxygen molecules. The others are known as alcohols, aldehydes, ketones, esters or ethers. Many essential oils such as thyme oil, cinnamon oil and oregano oil are reported as a strong antimicrobial and antioxidant agent against pathogenic bacteria and fungi either *in vitro* or *in vivo* (Dorman & Deans, 2000; Gutierrez, Barry-Ryan, & Bourke, 2009; Sienkiewicz, Lysakowska, Denys, & Kowalczyk, 2012). In the present study, antimicrobial property of cinnamon oil is focused.

2.1.1. Cinnamon oil

Cinnamon oil is extracted from cinnamon (*Cinnamomum verum*) plant. It has been used as a flavoring agent, natural antioxidant, and antimicrobial agent in foods. The antimicrobial activity of cinnamon oil depends on the concentration of active ingredients it contains, depending on which part of the

plant it comes from (Nabavi et al., 2015). Cinnamaldehyde is the major constituent of cinnamon bark oil but various minor constituents including benzyl benzoate, linalool, eugenyl acetate and eugenol are also present (Friedman, Kozukue, & Harden, 2000; Nabavi et al., 2015). The antimicrobial activity of cinnamaldehyde has been demonstrated against a wide range of foodborne pathogens such as *Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus* and *Listeria monocytogenes* (Hilbig et al., 2016; Ribeiro-Santos et al., 2017; Zhang et al., 2016). Mechanisms of action are attributed to the interaction with the cell membrane, thus leading to the increasing in the permeability and inhibition of energy metabolism of the cells. The interruption of proton motive forces is also reported and resulted in the leakage of small inner cell materials (Gill & Holley, 2004; Siripatrawan, 2016; Zhang et al., 2016). Cinnamaldehyde also caused the alteration of bacterial morphology due to its ability to change the composition of fatty acid in bacterial cell membrane (Friedman, 2017). The fact that cinnamon oil exhibits strong antimicrobial activity has led to a considerable interest in its potential as a natural preservative. However, the direct incorporation of cinnamon oil into food and beverage products is still limited due to its hydrophobicity, high reactivity, and strong odor. Minimizing its negative impact on the organoleptic properties of foods, while still retaining its strong antimicrobial activity is a major challenge for the food industry (Donsi & Ferrari, 2016; Ryu et al., 2018).

2.2. Nanoemulsions

Oil-in-water nanoemulsions are one of the most promising types of colloidal delivery system suitable for encapsulating essential oils (Donsi & Ferrari, 2016; Fathi,

Mozafari, & Mohebbi, 2012; McClements, 2011). These systems consist of small emulsifier-coated oil droplets dispersed within an aqueous medium. The droplets in nanoemulsions typically have mean diameters below 200 nm, which is in contrast to conventional emulsions that have mean droplet diameters above this value (McClements & Rao, 2011). The small size of the droplets in nanoemulsions has a number of potential advantages for developing delivery systems: (i) good stability to gravitational stability and droplet aggregation; (ii) high optical clarity; and (iii) increased bioactivity (McClements & Rao, 2011).

2.2.1. Nanoemulsion formation approaches

In general, nanoemulsions can be fabricated using either high-energy or low-energy approaches (McClements, 2011). Each of these methods has its own advantages and disadvantages for specific applications and must be optimized to produce nanoemulsions with the appropriate stability, physicochemical, and functional properties (Karthik, Ezhilarasi, & Anandharamakrishnan, 2017).

2.2.1.1. High-energy approaches

High-energy approaches utilize specialized mechanical devices, referred to as homogenizers, which generate intense disruptive forces that breakup and intermingle the oil and water phases (Gupta, Eral, Hatton, & Doyle, 2016). The most commonly employed mechanical homogenizers used for this purpose are high pressure valve homogenizers (Yuan, Gao, Zhao, & Mao, 2008), sonicators (Amani, York, Chrystyn, Clark, & Do, 2008; Leong, Wooster, Kentish, &

Ashokkumar, 2009) and microfluidizers (Lee & Norton, 2013; Qian & McClements, 2011).

2.2.1.2. Low-energy approaches

Low-energy approaches rely on the spontaneous formation of small oil droplets when the composition or environmental conditions of certain surfactant-oil-water mixtures are altered in a specific way (Komaiko & McClements, 2016; Solans & Solé, 2012). The main advantages of low energy methods for food applications are that they are simple to carry out and they do not require any specialized equipment. Currently, different low energy methods including the phase inversion temperature (PIT), emulsion inversion point (EIP) and spontaneous emulsification (SE) methods (Komaiko & McClements, 2016; Solans & Solé, 2012) have been used to fabricate the nanoemulsions.

2.2.1.2.1. Phase inversion temperature (PIT) method

The PIT method is based on alterations in the hydration properties of nonionic surfactant head groups when the temperature changes (**Figure 2.1**).

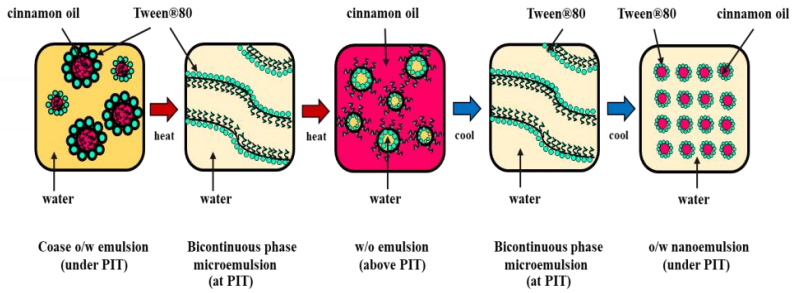

Figure 2.1 Schematic mechanism of PIT method.

The head-group is highly hydrated at relatively low temperatures but becomes progressively dehydrated as the temperature is increased (Anton & Vandamme, 2009; Chuesiang et al., 2018; Roger, Cabane, & Olsson, 2010; Roger, Cabane, & Olsson, 2011). This change in hydration alters the water-solubility of the surfactant, as well as the optimum curvature of the surfactant monolayer. At low temperatures, the surfactants are more hydrophilic and favor the formation of oil-in-water systems, but at high temperatures they are more lipophilic and favor the formation of water-in-oil systems. At intermediate temperatures (around the PIT), the system tends to exist as a bicontinuous microemulsion that is optically transparent (Chuesiang et al., 2018; Rao & McClements, 2010). If this microemulsion is rapidly cooled from temperatures around the PIT with stirring, it is possible to form nanoemulsions containing very fine oil droplets (Chuesiang et al., 2018; Roger et al., 2010).

2.2.1.2.2. Emulsion inversion point (EIP) method

The EIP method formulates nanoemulsions by causing a phase inversion when an aqueous phase is titrated into a stirring oil-surfactant mixture phase. The formation of a bicontinuous microemulsion relates to the alteration of hydration and dehydration properties of surfactant head group and surfactant curvature which depending on the volume ratio between oil and water. The mechanism of EIP method has been proposed by the production of swollen reverse micelles inside the oil droplets during the titration of an aqueous phase (Roger et al., 2010).

2.2.1.2.3. Spontaneous emulsification (SE) method

In order to formulate the nanoemulsions using the SE method, titration of an organic phase (a mixture of essential oil and hydrophilic surfactant) into an aqueous phase (water of buffer solution) is usually carried out after surfactant-oil-water ratio, temperature, stirring speed and addition rate are optimized (Komaiko & McClements, 2016). A mechanism for SE to form nanoemulsions is proposed to be similar to that of PIT method which involves in the formation of a bicontinuous microemulsion at the boundary where the organic and aqueous phases come into contact. The generation of small oil droplets is then occurred when the bicontinuous microemulsion phase breaks up (López-Montilla, Herrera-Morales, Pandey, & Shah, 2002). Although nanoemulsions can be form utilizing SE

method without an energy input, some additional mixing might be needed to complete the degradation of a bicontinuous microemulsion (Komaiko & McClements, 2016).

2.2.2. Factors affecting the properties of nanoemulsions fabricated using low energy approaches

There are a number of important factors related to the properties such as droplet size, particle size distribution and stability of the nanoemulsion produced by low-energy approaches. In this study, factors which are influenced on the properties of nanoemulsions fabricated using the PIT method are focused.

2.2.2.1. Type of ripening inhibitor

Ripening inhibitor or carrier oil is well known as a lipophilic substance with a very low water-solubility (Chuesiang et al., 2018). Addition of ripening inhibitor into the oil phase before homogenization is evident to increase the stability of nanoemulsions by inhibiting the process called "Ostwald ripening" (Chang, McLandsborough, & McClements, 2013). Although, ripening inhibitors, long chain triglyceride (LCT) oil and medium chain triglyceride (MCT) oil, are commonly used in the fabrication of nanoemulsions by low-energy method, smallest droplets in a nano-range can be formed when using the MCT oil. In contrast, LCT oil has been reported to increase the initial mean droplets size and thermal stability of the vitamin E acetate loaded nanoemulsion form by low-energy method, spontaneous

emulsification (Saberi, Fang, & McClements, 2013). Moreover, Chang, McLandsborough, & McClements (2013) also noted that stable carvacrol nanoemulsions could not be formed when LCT oil was used as a ripening inhibitor.

2.2.2.2. Oil phase composition

Oil phase composition means the ratio of oil (such as essential oils, flavor oils, or other oils used for encapsulation of their active ingredients into a nanoemulsion form) and ripening inhibitor in a total oil phase that influences on interfacial tension, phase behavior, viscosity, polarity and water-solubility of the oil phase. Thus, results in the formation and stability of nanoemulsions after emulsification and during storage (Davidov-Pardo & McClements, 2015; Komaiko & McClements, 2016). In PIT fabricated nanoemulsions, only certain composition of oil phase and MCT oil that facilitate the fabrication and stability of nanoemulsions by leading the formation of a bicontinuous microemulsion during heating (Chuesiang et al., 2018).

2.2.2.3. Surfactant concentration

Surfactant concentration is one of the most important factors affected the formation and stability of nanoemulsions fabricated by PIT method. This method is simply involving in the alteration of hydrophilic and hydrophobic properties of the surfactant head-group when the temperature change. Thus, the amount of surfactant should be enough to facilitate the formation of a bicontinuous microemulsion at a

certain temperature (≈ PIT temperature). The more surfactant concentration is believed to increase the surfactant molecules diffusing from an organic phase to an aqueous phase during the breakdown of a bicontinuous microemulsion occurred in cooling process. However, this is also a factor that must be optimized to control the formation of nanoemulsion since excessive surfactant concentration could increase the size of oil droplets due to the formation of other phases such as cubic phase, liquid crystalline phase or gel-like clump instead of a bicontinuous microemulsion.

2.2.2.4. Cooling rate

In the PIT method which the nanoemulsions formed by temperature alteration, the cooling process becomes important to induce the movement of surfactant molecules from an organic phase to an aqueous phase in order to fabricate the o/w nanoemulsions. Although, the formation of a bicontinuous microemulsion is critical in formulating of small oil droplets, the surfactant curvature at PIT temperature favors neither the oil phase nor aqueous phase resulting in a very low interfacial tension of the systems. These conditions increase the chance of flocculation and coalescence to be occurred in a slow cooling process. Thus, stable nanoemulsions with a narrow particle size distribution could not be formed. Rapid cooling process is suggested to utilize to decrease the temperature of the systems to around PIT-30°C to maintain the oil and water interface and to

fabricate the small oil droplets with stirring (Chuesiang et al., 2018; Komaiko & McClements, 2016).

2.2.3. Destabilization process of nanoemulsions

2.2.3.1. Flocculation and coalescence

Flocculation and coalescence are droplet aggregation process occur when the attractive forces between droplets are stronger than the repulsive forces. Flocculation is defined as a phenomenon which two or more droplets floc together to form a larger size of cluster without the fusion of the droplets. Whereas, coalescence which usually occurs beyond flocculation, is called when the droplets fuse together to become a one larger droplet size. Flocculation and coalescence can be prevented by i) steric repulsion which has been increased in the nanoemulsions due to the high ratio of steric layer compared to the size of droplets and ii) electrostatic repulsion occurs when the ionic surfactants are used to generate charges on the surface of the dispersed droplets.

2.2.3.2. Ostwald Ripening

Nanoemulsions are highly prone to destabilization through a physicochemical phenomenon called Ostwald ripening (OR), which is promoted by the diffusion of oil molecules with high solubility from small droplets to the larger ones via a surrounding medium (Taylor, 1998). OR is mainly caused by the differences in Laplace pressure between the smaller and larger oil droplets contained in the

nanoemulsions. As the smaller ones become more soluble in an aqueous phase, their chemical potential change and thus, they are susceptible to OR, which finally leads to a growth of droplet size over time. Addition of ripening inhibitor into the oil phase before homogenization is one way to prevent OR by changing the oil phase composition and decreasing the water solubility of the oil molecules (McClements & Rao, 2011). Since, ripening inhibitor could not diffuse out from the droplets fast, it can be assumed that the oil molecules with high solubility will be kept in the droplets contained in nanoemulsion incorporating with ripening inhibitor (Ryu et al., 2018; Taylor, 1988). However, adding of a ripening inhibitor may have an adverse effect on the properties and functionalities of the nanoemulsions (Chuesiang et al., 2018). For example, increasing in concentration of ripening inhibitor diminished the antimicrobial activity of thyme oil nanoemulsion due to a reduction of hydrophobic active ingredients transferred to hydrophobic bacterial membrane (Ryu at al., 2018).

2.3. Asian sea bass (Barramundi)

Asian seabass or barramundi (*Lates calcarifer*) is rapidly spread throughout Australia and the Southeast Asia due to its fast growth rate and high market demands. Within Thailand, the production of seabass was increased continuously from 16200 tons in 2011 to 17500 tons in 2015 which is mainly for domestic consumption or export to neighboring country such as Singapore, Hongkong and Malaysia (Tookwinas, Srichantulk, & Choongan, 1994). The quality of Asian seabass fillet or flesh has been reported as a valuable source of protein, essential fatty acid and

micronutrients (Manthey-Karl, Lehmann, Ostermeyer, & Schroder, 2016). However, its quality deteriorates rapidly after death due to the enzymatic and microbiological activities. Chilling or freezing are ways to extend the shelf life of Asian seabass fillets or fleshes by lowering, killing or injuring bacterial cells, thus decreasing the rate of spoilage. Preliminary processes including handling, scaling, washing, deheading, gutting, cutting away of the fin, and filleting (or slicing the fillets into smaller pieces), of the death whole fishes prior to chilling or freezing should be done immediately to control the quality and safety of the final products.

2.3.1. Quality assessments

Fish spoilage is a process implicated with microbiological, chemical and physical alteration of the fish during storage (Jones & Carton, 2015). Although, chilling or freezing are effective in extending the shelf life of the fish, microbial repairing or growth can be occurred due to either internal or external factors (Cheng, Sun, Han, & Zeng, 2013). Here in, microbiological property of the fish is discussed.

2.3.1.1. Microbiological quality

Microbiological quality of fresh fish is significantly attributed to the number of initial microflora, which can be varied depend on the environmental factors such as season, temperature, handling and processing of the fish after death. The bacterial strains that involve in the deterioration of fish are divided into 2 groups, spoilage organisms and foodborne pathogens. Spoilage organisms including *Pseudomonas* sp., *Photobacterium* sp., *Aeromonas* sp., and *Shewanella* sp., are

responsible for the quality changes during storage. They produce undesirable compounds which are related to unacceptable flavors, odors and texture of the fillet or flesh. Foodborne pathogens *e.g*. *E. coli*, *Salmonella* sp., *Staphylococcus aureus* or *Vibrio* sp. are food contaminated bacteria that cause a foodborne illness to human, thus they are responsible for safety of the foods. Foodborne pathogens could encounter to the fish naturally from water in the pond or cage or could come from cross contamination which occurred due to poor sanitary during handling or preparation steps. Although many bacteria grow less at the temperature below 10°C, their activities are relatively important in the temperature range from 0-25°C with a temperature fluctuation during chilling or freezing. Therefore, mandatory standards for enumeration of mesophilic bacteria or total viable count (TVC) are established. Fish fillets or fleshes with the initial number of TVC values range from 3.35-4.58 log CFU/g are defined to have a good quality (ICMSF, 1998), whereas, the TVC of 6 log CFU/g indicates the nearly spoilage of products and lastly, acceptability limit is set at 7 log CFU/g for human consumption of fresh water and marine species (ICMSF, 1986, 1998). In Thailand, the TVC of frozen fish fillets must not exceed 5.7 log CFU/g (≈5 x 10^5 CFU/g). The Most Probable Number (MPN) of *E. coli* and *S. aureus* shall not exceed 10/g and 100/g of product, respectively. *S.* Typhimurium and *V. cholerae* must not be found in 25 g of product (National Bureau of Agricultural

Commodity, Food Standards Ministry of Agriculture, & Cooperatives, 2015).

2.4. Application of essential oils on the fish preservation

Various essential oils can be applied in many foods due to their biological activities and their preservative effect. Applying essential oils directly to the fish/fillet/flesh is the most commonly used method (Hassoun & Emir Çoban, 2017). However, this method has its own disadvantage on the organoleptic quality of the products. Concentration of essential oils used to apply to the products is usually higher compared to that from the in *vitro* test. Therefore, the used of encapsulation and delivery technique such as a nanoemulsion technique has become a popular option due to its ability to minimize the effect of essential oils on sensory attributes of the products (Hassoun & Emir Çoban, 2017; Ozogul et al., 2017). It is believed that encapsulation of essential oils into nanoemulsions helps increase i) stability of the oils and their active ingredients by protecting them from undesirable reactions including chemical or environmental reaction and ii) their biological activities such as antimicrobial property by enhancing a passive cellular uptake (Sugumar, Ghosh, Mukherjee, & Chandrasekaran, 2016).

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

Cinnamon oil was purchased from CT Chemicals Ltd. (Bangkok, Thailand). Cinnamon oil used in this study was analyzed using gas chromatography/mass spectroscopy (GC/MS; 7890B GC, Triple Quadrupole GC/MS (GC-QQQ), Agilent, Belgium) for its major active compounds. Cinnamaldehyde (retention time = 14.24 min; 79.2% total peak area), benzyl benzoate (retention time = 26.13 min; 11.1% total peak area) and benzyl alcohol (retention time = 7.73 min; 9.6% total peak area) were observed as major components of the cinnamon oil (**Figure 3.1**). MCT oil (miglyol_812), used as ripening inhibitor, was purchased from Warner Graham Company (Sasol Germany GmbH). A food-grade non-ionic surfactant (Tween®80) was purchased from the Sigma–Aldrich Co. (St. Louis, MO). Deionized water was used in the preparation of all solutions and emulsions. For analysis of cinnamaldehyde and benzaldehyde content in nanoemulsions by high performance liquid chromatography (HPLC), analytical standards, cinnamaldehyde (Sigma–Aldrich 06536) and benzaldehyde (Sigma–Aldrich 09143) were purchased. *E. coli* (ATCC 25922), *S.* Typhimurium (ATCC 29630), *S.aureus* (ATCC 6538) and *V. Parahaemolyticus* (ATCC 17802) were obtained from the American type culture collection. Vacuumed-sealed frozen Asian seabass fleshes were purchased from 88 Foods Co., Ltd. (Bangkok, Thailand).

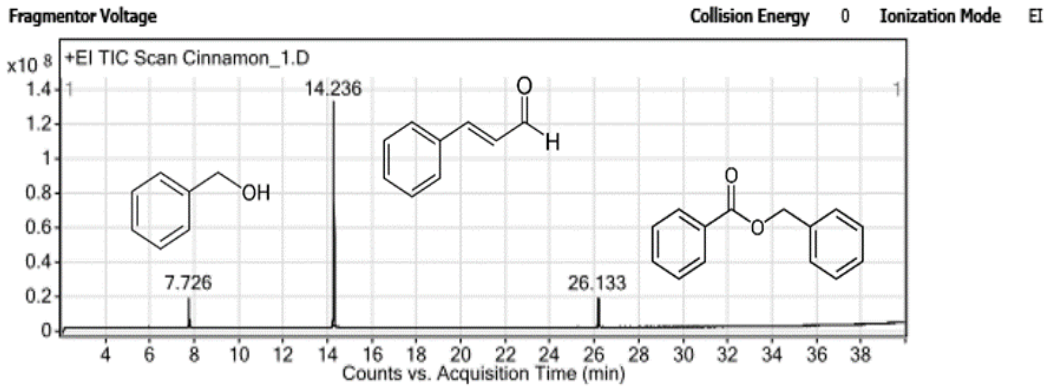

Figure 3.1 GC chromatogram of bulk cinnamon oil: cinnamaldehyde (retention time = 14.24 min; 79.23% total peak area); benzyl benzoate (retention time = 26.13 min; 11.1% total peak area); and, benzyl alcohol (retention time = 7.73 min; 9.6% total peak area).

3.2. Formulation of cinnamon oil nanoemulsions

Cinnamon oil nanoemulsions were prepared based on the method described by Chuesiang et al., (2018b). The ratio of cinnamon oil-to-MCT was varied (0 :10, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:3, 9:1 and 10:0 wt%), while keeping the total level of the oil phase (10 wt%), surfactant (10 wt% Tween® 80) and water phase (80 wt% deionized water) constant. Initially, cinnamon oil and MCT were mixed together for 3 min, and then Tween® 80 and deionized water were added. All components were mixed together for 30 min to create a coarse emulsion, each system was then heated to 15°C above the phase PIT. In this study, the PIT was defined as the temperature where the system had a low turbidity due to microemulsion formation. After that, a two-step cooling process was carried out. Firstly, the temperature was decreased to the PIT and held for 10 mins to allow a stable microemulsion to form. Secondly, a rapid cooling step was carried out by adding 250 g of cold deionized water (4°C) to

the system with continuous stirring for 3 min. The PIT was measured using the method described by Chuesiang et al. (2018b). The mean droplet diameter, particle size distribution and stability of all samples were measured using the methods described by Chang, McLandsborough & McClements (2013).

3.2.1. Determination of PIT

Turbidity measurements were used to determine the PIT in the present study. Firstly, coarse emulsions were prepared using the method described earlier. These systems were then placed in a quartz cuvette and subjected to a controlled heating/ cooling cycle using an UV/visible spectrophotometer (Ultraspec 3000 pro; Biochrom Ltd., Cambridge, UK) with a temperature control module (programmable heated cell holder, 80-2106-14; Biochrom Ltd., Cambridge, UK). The samples were heated from 25 to 90 °C at 5°C / min, held at 90 °C for 10 min, and then cooled from 90 to 25°C at 5°C /min. Turbidity (at 600 nm) *versus* temperature curves were acquired using deionized water as a reference (blank).

3.2.2. Particle size and particle size distribution measurements

The mean droplet diameter and particle size distribution of cinnamon oil nanoemulsions were measured using a dynamic light scattering instrument (Zetasizer NanoZS, Malvern Instruments Ltd, Malvern, UK) after being stored at 25°C for 0 or 31 days. Particle size information was obtained from measurements of the intensity-time fluctuations of a laser beam scattered from the samples at an angle of 173°. The samples were diluted with deionized water (1:40 v/v) prior to analysis to avoid multiple scattering effects.

3.2.3. Determination of antimicrobial activity

The antimicrobial activity of the cinnamon oil nanoemulsions was determined against Gram positive (*S. aureus*) and Gram negative (*E. coli*, *S.* Typhimurium and *V. Parahaemolyticus*) bacteria by determining the MICs value, following the method of Weerakkody, Caffin, Turner, and Dykes (2010) with slight modifications. This was achieved using the macro-broth method, which involved preparing a series of two-fold dilutions of cinnamon oil nanoemulsions, ranging from 11,429 mg/L to 179 mg/L in 1 mL of tryptic soy broth (TSB) or TSB +2 wt% NaCl (for *V. parahaemolyticus*). Before use, the bacteria cells were streaked from -80 °C glycerol stock onto each bacterium selective media: mannitol salt agar, MacConkey agar, xylose lysine deoxycholate agar and thiosulfate citrate bile salts sucrose agar for *S. aureus*, *E. coli*, *S.* Typhimurium and *V. parahaemolyticus*, respectively. A fresh single colony of each bacterial strain after incubating at 37 °C for 24 h was transferred into TSB or TSB+2wt% NaCl (for *V. parahaemolyticus*). Then the cultured cells were incubated overnight (18 h at 37 °C, stationary phase cells) and inoculated again in TSB or TSB+2wt% NaCl (for *V. parahaemolyticus*) at an initial turbidity of 0.2 at 600 nm to obtain the initial number of bacteria at approximately 8 log CFU/mL. Then 1 mL of each aliquot culture was added to the prepared nanoemulsions and mixed together using a vortex before the samples were incubated at 37°C for 24 h. MICs were determined as the lowest concentration of cinnamon oil nanoemulsions that inhibited the visible growth (turbidity) of each bacterium. In this study, MICs of the cinnamon oil nanoemulsions were compared to those of bulk cinnamon oil, prepared in the

same manner except that the concentration of bulk cinnamon oil ranged from 500,000 mg/L to 122 mg/L. The initial number of each bacterium were counted using plate counts method. Each test was performed in triplicate.

3.2.4. High Pressure Liquid Chromatography **(**HPLC**)** analysis

Cinnamaldehyde and benzaldehyde concentrations were measured in cinnamon oil nanoemulsions formulated using different oil phase compositions (0:10-10:0 wt% cinnamon oil : MCT in total lipid phase) immediately after emulsification, and after storage for 31 days using a HPLC-UV system (Gursale, Dighe, & Parekh, 2010; Kashani, Moghaddam, & Mehramizi, 2012). The HPLC (Model Prominence-i LC-2030 3D Shimadzu Corporation, Japan) was equipped with a photodiode array (PDA) detector and an auto-sampler injection valve. Wavelengths from 190 to 500 nm were selected for data acquisition. A reverse phase C_{18} column (Hypersil ODS-2,200 mm length× 4.6 mm internal diameter, Thermofisher scientific, Waltham, MA, U.S.A.) was used as an analytical column. The mixture of deionized water and acetonitrile at 50:50 %v/v was utilized as a mobile phase isothermally (25°C) at the flow rate of 1.2 ml/min. In this study, each sample was diluted in deionized water at 1:100 v/v, then l5 µl of the diluted and filtered samples were injected into the system. Cinnamaldehyde and benzaldehyde were detected at 284 nm and 242 nm, respectively as tested using analytical standards. Calculation of % normalized of benzaldehyde content were shown below. All experiments were carried out in duplicate.

$$\%\ \text{normalized of benzaldehyde content} = \left(\frac{\text{BA present}}{\text{CA present}}\right) \times 100$$

Where, $BA_{present}$ and $CA_{present}$ are the benzaldehyde concentration and cinnamaldehyde concentration from HPLC, respectively.

3.3. Impact of surfactant concentration on the nanoemulsion properties

The effect of surfactant level on the properties of selected cinnamon oil nanoemulsion was determined. These systems contained 10 wt% total oil phase, but different levels of surfactant (10, 15 and 20 wt%Tween®80), with the remainder being aqueous phase (deionized water). The PIT, mean droplet diameter, particle size distribution, particle morphology, physical stability, antimicrobial activities and chemical stability of the samples were then measured using the following methods.

3.3.1. PIT Determination

The effect of surfactant level on the PIT of cinnamon oil nanoemulsions fabricated with different level of surfactant was examined using the methods described in Section 3.2.1.

3.3.2. Particle size and particle size distribution characteristics

The change in particle size of the nanoemulsions was measured throughout storage at 4, 25 and 32 °C for 31 days. The mean droplet diameter and particle size distribution of the samples were determined using a dynamic light scattering instrument (Zetasizer NanoZS, Malvern Instruments Ltd, Malvern, UK) at 25°C as described in Section 3.2.2. However, the samples were diluted differently with deionized water: 1: 40 v/v (10 wt% Tween®80 sample); 1:20 v/v (15 wt% Tween®80 sample); and, no dilution (20 wt% Tween®80 sample). Different dilution levels were required because the samples had different turbidities (light scattering efficiencies).

3.3.3. TEM analysis

The morphology of the droplets in the cinnamon oil nanoemulsions was determined using transmission electron microscopy (JEOL 2000FX Electron Microscope, LaB6 Source, JEOL institute, USA). Cinnamon oil nanoemulsions (10 µl) were dropped on an ultra-thin TEM grid (FCF400 Cu UB 400 mesh, Electron Microscopy Science) for 10 sec and were then stained using uranyl acetate negative stain solution for 20 sec (Chuesiang et al., 2018). After that, the TEM grids were washed 3 times by touching them onto the surface of a drop of deionized water. Finally, the TEM grids were dried overnight at 25°C. The morphology of the oil droplets was then observed at 12,000x magnification at an operating acceleration voltage of 200 kV (Chuesiang et al., 2018).

3.3.4. Determination of nanoemulsion stability

The stability of the cinnamon oil nanoemulsions was studied by measuring the change in the mean droplet diameter and polydispersity index throughout 31 days storage at 4, 25, and 32 °C (Chuesiang et al., 2018).

3.3.5. Determination of antimicrobial activities

The four tested bacteria, including one Gram positive (*S. aureus*) and three Gram negative (*E. coli*, *S.* Typhimurium and *V. parahaemolyticus*) strains, were kept at -80°C in a mixture of tryptic soy broth (TSB) or TSB+2 wt% NaCl (for *V. parahaemolyticus*) containing 25 %v/v of glycerol. Before use, each strain was prepared as described in section 3.2.3. The antimicrobial activity of cinnamon oil nanoemulsions was determined by investigating their

MICs and dynamic time kill plot. In addition, bacteria morphological change after exposure to the cinnamon oil nanoemulsions was conducted using field emission scanning electron microscopy (FE-SEM).

3.3.5.1. Determination of MICs

The MICs of cinnamon oil nanoemulsions against the four foodborne pathogens were determined by adding 1 mL of each bacterium (8 log CFU/mL) into 1 mL of TSB or TSB + 2wt% NaCl containing various concentrations of cinnamon oil nanoemulsions (ranging from 11429 to 179 mg/L). Inoculated tubes were mixed with a vortex prior to incubation at 37°C for 24 h. Each experiment was carried out in triplicate.

3.3.5.2. Dynamic time kill assay

Dynamic time kill assay of cinnamon oil nanoemulsions prepared with differing concentrations of Tween®80 (10, 15 and 20 wt%) against the four foodborne pathogens were investigated at their MICs following the method of Chuesiang, Siripatrawan, Sanguandeekul, McClements, and McLandsborough (2019) with slight modifications. Briefly, 1 mL of overnight growth of each bacterium (approximately 8 log CFU/ mL) was inoculated into a test tube containing 1 mL of TSB (for *E. coli*, *S.* Typhimurium and *S. aureus*) and TSB + 2 wt% NaCl for *V. parahaemolyticus* with MIC concentration of cinnamon oil nanoemulsions. The tubes were incubated at 37°C for 0, 1, 2, 3, 4, 8, 12 and 24 h. The dilution series

were prepared and the surviving cells in all samples were enumerated after exposure to the treatments at each time point by using a spread plate technique. The dynamic time kill was determined by plotting between log N_0 – log N_T against Time (h), given that N_0 is the initial number of each bacteria and N_T is the survival cell number of bacteria after treatment at each contact time. The experiments of those bacteria treated with bulk cinnamon oil, sterile deionized water, or 20 wt% Tween®80 solution were prepared in the same manner. Each experiment was carried out in triplicate.

3.3.5.3. FE-SEM

Bacterial morphology was studied using FE-SEM (JSM-7610F, Tokyo, Japan) following the method described by Chuesiang et al. (2019). Gram negative, *E. coli*, *S.* Typhimurium and *V. parahaemolyticus* (approximately 8 log CFU/mL) were treated with cinnamon oil nanoemulsions at their MICs obtained from section 3.3.5.1. and incubated at 37°C for 3 h. Gram positive S. *aureus* was treated and incubated at 37°C for 8 h. The cell suspensions were then centrifuged at 1500 g for 10 min and fixed with 2.5% glutaraldehyde in phosphate buffer solution at 4°C for 2 h. After that, the suspension was dropped on a glass cover slip coated with poly-L-lysine for 10 min prior to wash twice using 0.1 M phosphate buffer solution pH 7 for 5 min each. All bacteria were washed again with distilled water for 5 min and dehydrated using a sequential grade ethanol at various concentration (30, 50, 70, 95 and 100%) for 5 min each. Bacteria were

then dried in a critical point dryer (Leica, EM-CPD 300, Austria) for 1 h and sputtering coated with gold under vacuum in a sputter coater (Balzers model SCD 040, Germany) for 2 min, followed by microscopic examination using a FE- SEM.

3.3.6. High Pressure Liquid Chromatography (HPLC) analysis

Cinnamaldehyde and benzaldehyde concentrations were measured in cinnamon oil nanoemulsions formulated using different surfactant concentration (10-20 wt% Tween® 80) at immediately after emulsification, and after storage for 31 days using a HPLC-UV system (Gursale et al., 2010; Kashani et al., 2012). The HPLC (Model Prominence-i LC-2030 3D Shimadzu Corporation, Japan) used in this study was in the same condition as described in section 3.2.4.

3.4. Applications of cinnamon oil nanoemulsions on frozen Asian seabass fleshes

Vacuum-sealed frozen Asian seabass fleshes (approximately 25 g per piece) were temperature-controlled (-25 °C) transported to the laboratory and were immediately thawed at 4 ± 2 °C overnight (20 h) before used. They were then tested for microbiological property. Samples were separated into 5 groups and treated with different treatments as described in **Table 3.1**.

Table 3.1 Description of treatments for Asian seabass fleshes.

Treatment	Description
CTRL	Control: fish fleshes without treatment
CON1	Samples soaked in 1429 mg/L of cinnamon oil nanoemulsion (fabricated 15wt% Tween® 80)
CON2	Samples soaked in 11429 mg/L of cinnamon oil nanoemulsion (fabricated 15wt% Tween® 80)
BCO1	Samples soaked in 488 mg/L of bulk cinnamon oil
BCO2	Samples soaked in 11429 mg/L of bulk cinnamon oil

- Concentrations of cinnamon oil nanoemulsions and bulk cinnamon oil used in this study were obtained from their MIC.

3.4.1. Proximate analysis

The proximate composition of Asian seabass fleshes was analyzed following the AOAC method (2016). The method number 950.46(b), 981.10, 948.15 and 938.08 were used for analyzing of moisture, protein, fat and ash content, respectively. Carbohydrate content was calculated by subtracting other contents from 100.

3.4.2. Sample preparation

For preparing the samples, 10 pieces of flesh were separately soaked in 300 mL of treatments including CON1, CON2, BCO1 and BCO2 for 30 min at 25°C. After that, 2 pieces of fish flesh (approximately 50 g per 2 pieces) were packed individually in a polyethylene (PE) bags and heated seal using an impulse sealer (PHS 450 10D). All samples were stored at 4 ± 2 °C.

3.4.3. Microbiological analysis

For microbiological analysis, enumeration of total viable count (TVC) in thawed and unpacked fleshes were determined. During storage, fish fleshes from each treatment were aseptically unpacked and transferred to a stomacher bag containing 450 mL of 0.1% sterile peptone water. A mixture was homogenized in a stomacher set to 230 rpm for 2 min. Then, 10-fold dilution series were created and plated on to total plate count agar and incubated at 30°C for 48 h. Enumeration of TVC was measured in every 2 days for 8 days of storage period. The experiment was carried out in triplicate.

3.5. Statistical analysis

Statistical analysis was performed using software (IBM SPSS Statistic Version 22) with one-way analysis of variance being carried out and differences between means being compared using Duncan's new multiple range test at a significant level lower than 0.05 ($p < 0.05$).

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Formation of cinnamon oil nanoemulsions using PIT method

Nanoemulsions containing 10 wt% oil phase (cinnamon oil and MCT), 10 wt% surfactant (Tween® 80) and 80 wt% aqueous phase (deionized water) were initially prepared using the PIT method. The oil phase composition was varied by altering the mass ratio of cinnamon oil to MCT. The PIT, mean droplet diameter, particle size distribution, physical stability, antimicrobial activity and chemical stability of the nanoemulsions were then measured.

4.1.1. Impact of oil phase composition on PIT

The turbidity-temperature profiles of the surfactant-oil-water mixtures were measured when they were heated and then cooled in a UV-visible spectrophotometer (**Figure 4.1-4.2**). Initially, the experimental data obtained during the heating process is discussed. For some samples, an appreciable decrease in turbidity was observed over a certain temperature range, whereas for other samples the turbidity remained high across the entire temperature range. These results suggest that only certain levels of cinnamon oil (10 - 50 wt%) in the oil phase provided conditions where a transparent bicontinuous microemulsion could be generated at intermediate temperatures (**Figure 4.1**). If the cinnamon oil level was too high or too low, then it was not possible to obtain transparent systems at any temperature (**Figure 4.2**). The phase inversion temperature was defined as the temperature where the turbidity first decreased steeply during heating. The PIT decreased from 90°C to 67°C as the

cinnamon oil level in the lipid phase increased from 10 - 40 wt%, but then increased when it was increased further. The turbidity-temperature profiles of the samples were also measured when they were cooled. A pronounced decrease in turbidity during cooling was observed in the same samples where a pronounced decrease in turbidity occurred during heating. However, the temperature range where clear systems were obtained was broader during cooling than during heating. This effect may have been because the kinetics of microemulsion formation/disruption and nanoemulsion formation/disruption were different. Alternatively, it may have occurred because the inherent lag-time where the temperature of the sample has to catch up to the set temperature was different during heating and cooling. Previous studies have shown that the formation of a bicontinuous microemulsion during cooling is usually a prerequisite for the subsequent formation of a nanoemulsion (Anton, Gayet, Benoit, & Saulnier, 2007; Anton & Vandamme, 2009; Roger et al., 2010). At the PIT, the surfactant monolayer has an optimum curvature close to unity, the interfacial tension is relatively low, and the surfactant has a similar affinity for both the oil and water phases. As a result, a bicontinuous microemulsion is formed that contains small colloidal structures that do not scatter light strongly. When the temperature is reduced below the PIT, the optimum curvature of the surfactant monolayer favors the formation of oil droplets, the interfacial tension increases, and the surfactant has a much stronger affinity for the aqueous phase. As a result, small oil droplets dispersed in water are spontaneously formed when the system is cooled below the PIT (Chuesiang et al., 2018). Consequently, only those oil phase

compositions leading to the formation of transparent bicontinuous microemulsions at intermediate temperatures would be expected to form nanoemulsions (Chuesiang et al., 2018b). However, the cooling rate is also important for generating small stable oil droplets using the PIT process (McClements & Rao, 2011). If the system is cooled too slowly, then the oil droplets formed tend to rapidly coalesce with each other in the temperature range just below the PIT due to their relatively low interfacial tensions (Kabalnov & Wennerström, 1996). Typically, it is necessary to rapidly cool the system to a temperature that is about 30 °C below the PIT (For example in the present study, the temperature was decreased from 67 °C to 29 °C after adding 250 g of cooled deionized water into the system containing 40 wt% and 60 wt% of cinnamon oil and MCT, respectively) to obtain nanoemulsions that are stable to coalescence (McClements & Rao, 2011).

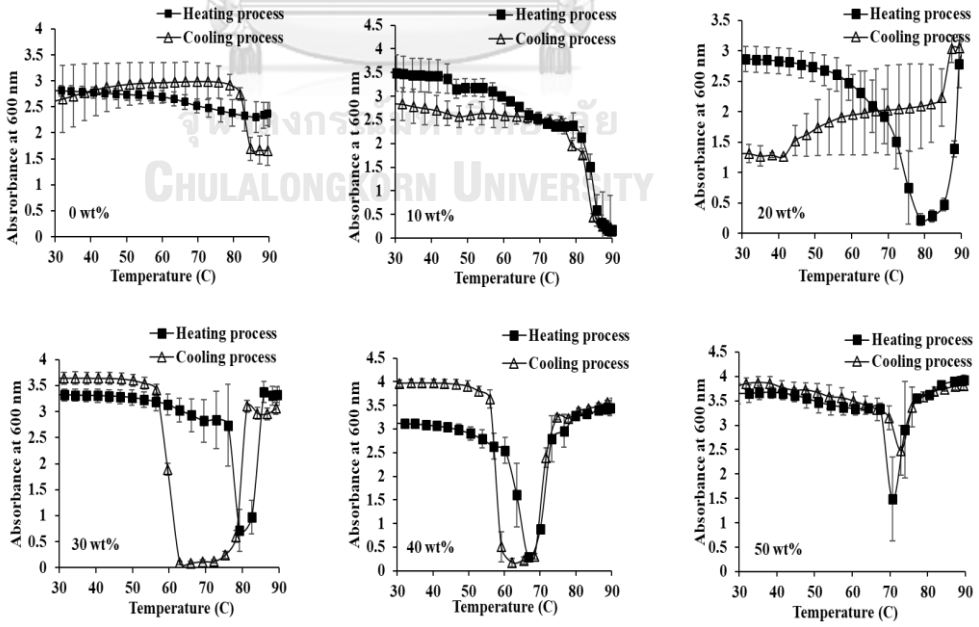

Figure 4.1 Absorbance at 600 nm of nanoemulsions using 0 - 50 wt% of cinnamon oil in total lipid phase.

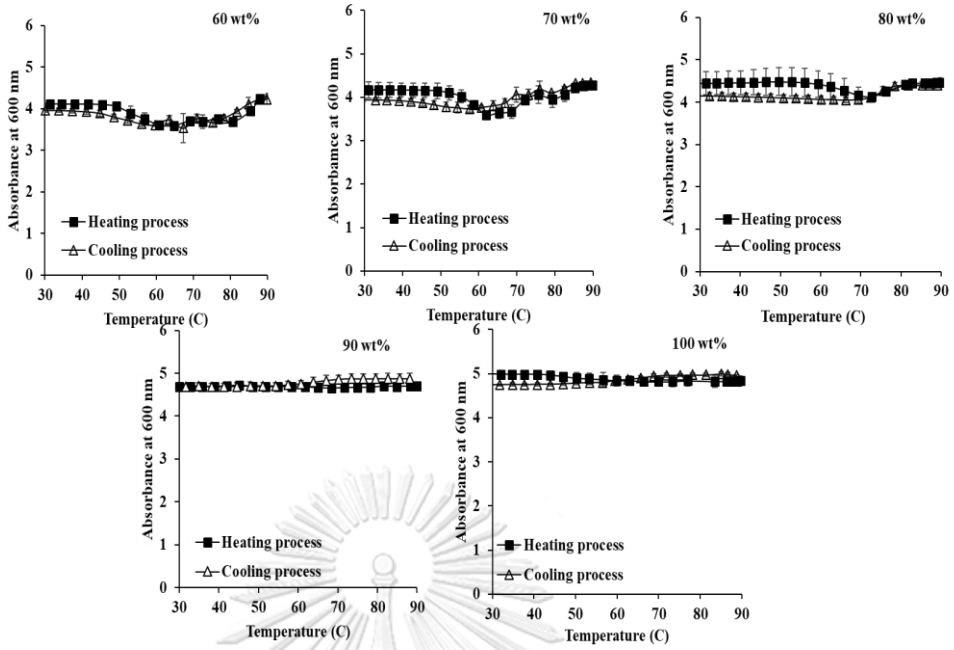

Figure 4.2 Absorbance at 600 nm of nanoemulsions using 60 - 100 wt% of cinnamon oil in total lipid phase.

4.1.2. Impact of oil phase composition on droplet size

The effect of oil phase composition on the droplet size of the nanoemulsions formed after rapid cooling is shown in **Table 4.1**. Relatively small mean droplet diameters were obtained at intermediate cinnamon oil levels in the oil phase, *e.g.*, 30 wt% (107.3 nm) and 40 wt% (100.8 nm). Conversely, relatively large droplets were generated at low (0 to 20 wt%) and high (60 to 100%) cinnamon oil levels, which can be attributed to the fact that these oil phase compositions did not lead to the formation of a bicontinuous microemulsion during heating, and so small oil droplets were not generated during the subsequent cooling process. Moreover, systems containing high levels of cinnamon oil are highly prone to instability through Ostwald ripening and coalescence, which would lead to rapid droplet growth after nanoemulsion formation. A number of these systems underwent visible phase separation after

prolonged storage, and therefore reliable particle size measurements could not be obtained. Our results are in agreement with previous studies that also reported that the smallest droplets are formed at intermediate oil phase compositions in systems containing essential oils and ripening inhibitors (Chuesiang et al., 2018; Komaiko & McClements, 2016). It should be noted that the nanoemulsions formed at intermediate cinnamon oil levels (*e.g.*, 40 wt%) had monomodal particle size distributions with a relatively small polydispersity index (0.173±0.005).Conversely, most of the other systems had broad multimodal particle size distributions (**Figure 4.3**).Thus, these experiments indicated that nanoemulsions containing small uniform droplets could be formed by rapidly cooling a system with an intermediate level (40 wt%) of cinnamon oil in the oil phase.

Table 4.1 PIT and mean droplet diameter of cinnamon oil nanoemulsions fabricated with differing oil phase compositions.

Oil phase composition (wt%)		PIT (°C)	Mean droplet diameter (nm)
Cinnamon oil	**MCT**		
0	100	>90	$447.00^{d} \pm 33.00$
10	90	90	$815.00^{g} \pm 56.00$
20	80	85	$559.00^{e} \pm 55.00$
30	70	80	$107.30^{a} \pm 5.00$
40	60	67	$100.80^{a} \pm 1.00$
50	50	71	$132.00^{ab} \pm 11.00$
60	40	>90	$162.40^{b} \pm 1.10$
70	30	>90	$174.00^{b} \pm 12.00$
80	20	>90	$232.00^{c} \pm 17.00$
90	10	>90	$572.00^{e} \pm 39.00$
100	0	>90	$620.10^{f} \pm 0.80$

** Numbers are mean ± standard error. Different superscript letters in the same column indicate mean values which significantly different ($p \leq 0.05$).

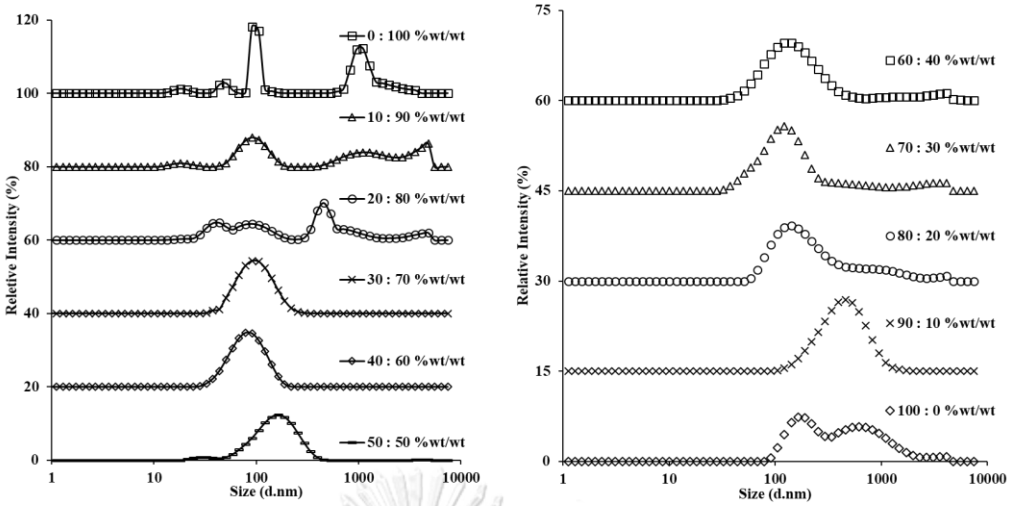

Figure 4.3 Size distribution curve of nanoemulsions with different cinnamon oil in total lipid phase (0 - 100 wt%) prepared using 10 wt% of Tween® 80 and 80 wt% deionized water by PIT method.

4.1.3. Impact of oil phase composition on antimicrobial activity

The impact of oil phase composition on the antimicrobial activity of the cinnamon oil nanoemulsions was established by measuring their MICs against four foodborne pathogens: *E. coli*, *S.* Typhimurium, *S. aureus*, and *V. parahaemolyticus*. The experiments were tested immediately after emulsification and the results for the nanoemulsions were compared to those for bulk cinnamon oil. Both bulk cinnamon oil and cinnamon oil nanoemulsions inhibited growth of all tested bacteria within 24 h of incubation, but the MIC varied depending on the level of cinnamon oil in the lipid phase and the bacterial strain. The MICs of cinnamon oil nanoemulsions against Gram negative *E. coli* and *S.* Typhimurium were higher than those against Gram positive *S. aureus*, which can be attributed to differences in their cell wall components and structures (Salton, 1953; Zhang et al., 2016).

Surprisingly, Gram negative *V. parahaemolyticus* was susceptible to cinnamon oil nanoemulsions and exhibited the lowest MIC value compared to other bacteria. This could be attributed to the fact that *V. parahaemolyticus* is a halophilic bacterium that requires salt and depends on the environmental osmolarity to grow (Xu, Ren, Wang, & Peng, 2004). Addition of an antimicrobial agent into the culture medium would not only disturb its normal osmolarity, but would also generate hostile conditions that alter its outer membrane protein (OMPs) pattern (Xu et al., 2004). Additionally, cinnamaldehyde has been reported as an effective antimicrobial agent that made *Vibrio* sp. more sensitive under stressed conditions by strongly interfering with membrane permeability and the growth of cells (Brackman et al., 2008; Burt, 2004).

The MICs of the antimicrobial nanoemulsions initially decreased with increasing cinnamon oil level, then reached a minimum level, and then increased again. The minimum MIC levels were around 40 wt% cinnamon oil for *E. coli*, *S.* Typhimurium, and *S. aureus*, and around 50 wt% cinnamon oil for *V. parahaemolyticus* (**Table 4.2**). It is likely that nanoemulsions with low concentrations of cinnamon oil in the lipid phase (10-20 wt%) had higher MICs due to partitioning of some of the cinnamon oil into the emulsion droplet, thus preventing it from interacting with the bacterial membrane. When the level of antimicrobial agent in the emulsion droplets was increased, one would expect that the MICs would decrease. However, the results in this study suggested that the MICs of the nanoemulsions tended to increase when 50-100 wt%of cinnamon oil were present in the lipid phase. These results could be

explained by two possible reasons. First, a higher cinnamon oil concentration in the lipid phase led to a higher PIT, which meant that the nanoemulsions had to be heated to a higher temperature (**Table 4.1**). Consequently, there may have been more thermal degradation of the cinnamon oil during nanoemulsion formation (Turek & Stintzing, 2013). Second, the smallest mean droplet diameter (100.8 nm) occurred when there was 40 wt% cinnamon oil in the lipid phase, which enhanced the antimicrobial activity of the encapsulated cinnamon oil by increasing its surface area to volume ratio and by improving its ability to penetrate through the cell wall of the bacteria (Moghimi, Aliahmadi, McClements, & Rafati, 2016; Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2015; Wang et al., 2008).

Table 4.2 Minimum inhibitory concentration (MIC, mg/L) of bulk cinnamon oil and cinnamon oil nanoemulsions prepared by PIT method.

Type of agent	*E. coli*	*S.* Typhimurium	*S. aureus*	*V. parahaemolyticus*
Bulk cinnamon oil	488	488	488	244
Nanoemulsions with different cinnamon oil in total lipid phase (wt%)				
0	-	-	-	-
10	-	-	-	714^{c}
20	1429^{de}	1429^{de}	714^{c}	714^{c}
30	1071^{bc}	1071^{bc}	536^{b}	268^{ab}
40	714^{a}	714^{a}	357^{a}	357^{b}
50	892^{b}	892^{b}	892^{e}	223^{a}
60	1071^{bc}	1071^{bc}	1071^{f}	268^{ab}
70	1250^{c}	1250^{c}	1250^{g}	312^{ab}
80	1429^{de}	1429^{de}	714^{c}	714^{c}
90	$1473^{ef} \pm 328$	$1473^{ef} \pm 328$	804^{d}	$603^{c} \pm 220$
100	$1636^{f} \pm 364$	$1636^{f} \pm 364$	892^{e}	$670^{c} \pm 244$

* Average initial population of *E. coli*, *S.* Typhimurium, *S. aureus* and *V. parahaemolyticus* was 8.34±0.12, 8.38±0.15, 8.14±0.02 and 8.50±0.05 log CFU/mL, respectively.

** Numbers are mean ± standard error. Different superscript letters in the same column indicate mean values which significantly different ($p \leq 0.05$).

In summary, the MICs of the cinnamon oil nanoemulsions observed in our study first decreased and then increased as the cinnamon oil level in total lipid phase increased from 10 to 100 wt%.These results are opposite to those of Chang et al. (2013) who investigated the antimicrobial activity of carvacrol nanoemulsions fabricated using the spontaneous emulsification method.

Antimicrobial activity of the carvacrol nanoemulsions increased as the carvacrol concentration in the lipid phase increased (Chang et al., 2013). Cinnamaldehyde is the major constituent of the cinnamon oil utilized in this study (**Figure 3.1**). During the PIT heating process, cinnamaldehyde could change into benzaldehyde which has been reported to have no antimicrobial activity against *E. coli, S. aureus, E. faecalis* and *P. aeruginosa* (Chang, Chen, & Chang, 2001).

4.1.4. Impact of oil phase composition on cinnamaldehyde degradation during emulsification

The impact of nanoemulsion fabrication on the chemical degradation of the cinnamon oil was determined by measuring the decrease in cinnamaldehyde and increase in benzaldehyde using HPLC analysis.There was a decrease in the cinnamaldehyde concentration in all the samples after exposure to the relatively high temperatures used in the PIT process, *e.g.,* 70 to 90°C depending on oil phase composition (**Figure 4.4**). The percentage of cinnamaldehyde remaining after heating increased when the fraction of cinnamon oil increased up to about 50 wt%, after which it decreased. This is probably because the emulsions containing 40-50 wt%cinnamon oil had the lowest PIT values and so they had to be heated to lower temperatures during emulsion fabrication (Chuesiang et al., 2018). Thus, there are likely to be greater losses of cinnamaldehyde due to volatilization and chemical degradation at higher temperatures, and so using as low temperature as possible during the nanoemulsion fabrication process would be an advantage.

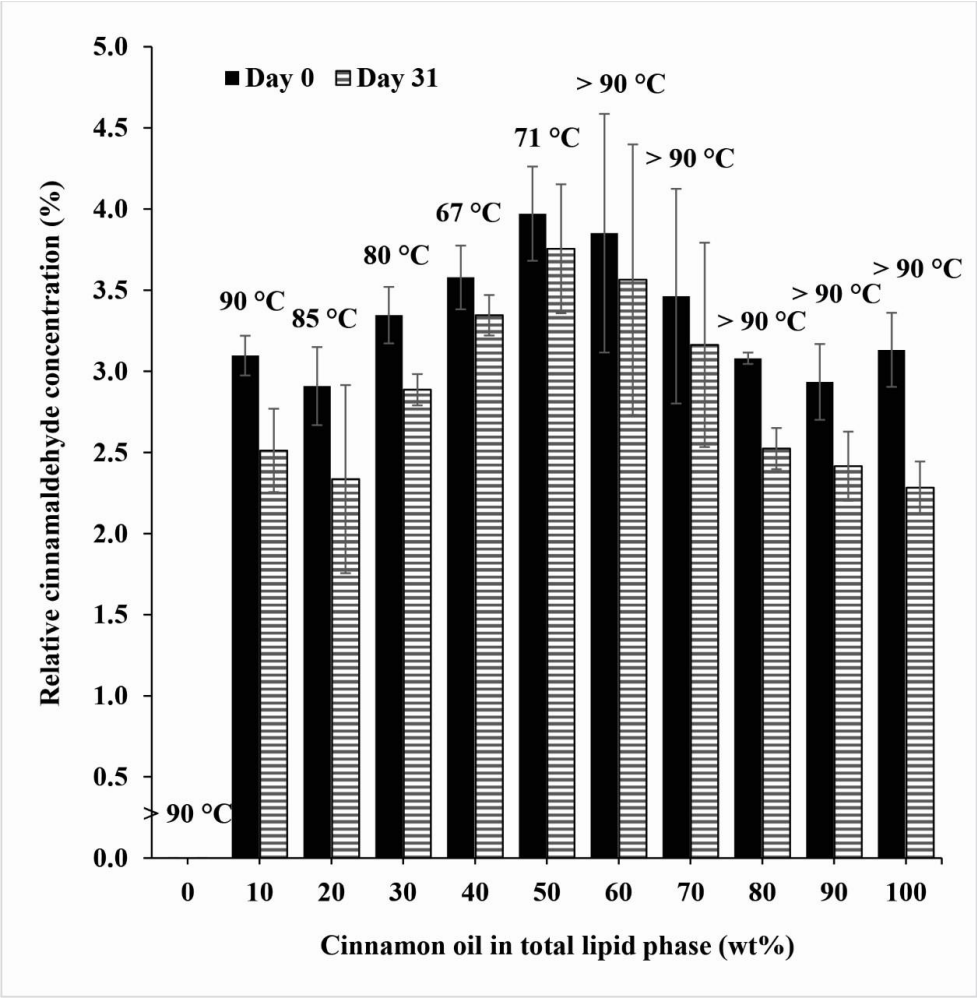

Figure 4.4 Cinnamaldehyde concentration of cinnamon oil nanoemulsions after emulsification by PIT method at day 0 and day 31 of storage time, the data labels indicate the PIT temperature of each system.

Previous studies have shown that cinnamaldehyde can be chemically degraded to other substances, such as benzaldehyde, when it is heated to relatively high temperatures (100-120°C) in the presence of water (Chen & Ji, 2011; Chen, Ji, Zhou, Xu, & Wang, 2009). HPLC analysis of the nanoemulsions after emulsion formation indicated that they all contained benzaldehyde (**Figure 4.5**), which suggests that some chemical degradation of the cinnamon oil had occurred. The normalized benzaldehyde levels in the

nanoemulsions tended to decrease with increasing cinnamaldehyde in the oil phase. This suggests that high levels of cinnamon oil inside the lipid droplets may inhibit the degradation of cinnamaldehyde, perhaps through antioxidant effect of compounds consisting in cinnamon oil. Theoretically, degradation of cinnamaldehyde to benzaldehyde is an oxidation reaction which is a temperature dependent, suggesting that more amount of benzaldehyde will be produced when cinnamaldehyde undergoes a reaction at higher temperature (Chen & Ji, 2011; Friedman et al., 2000). However, cinnamaldehyde which is containing in cinnamon oil has been reported to be stable to heat induced transformation to benzaldehyde due to the antioxidant effect of other containing compounds like eugenol, which would scavenge the oxygen and would prevent the degradation of cinnamaldehyde (Friedman et al., 2000). As reported by Friedman et al., (2000), although the reaction temperature was around 200 °C, the production of benzaldehyde was observed less when eugenol is presented. In addition, the selectivity of benzaldehyde production can be decreased as the reaction temperature increased (Chen & Ji, 2011). It is also possible that cinnamaldehyde could degrade into glyoxal; unstable reactive intermediate substance; during oxidation reaction (**Figure 4.6**). Interestingly, there was a steep decrease in the level of benzaldehyde formed after emulsification when the lipid phase composition inside the droplets changed from 30 to 40 wt%cinnamon oil. This may have been because this system was heated at a lower temperature during nanoemulsion fabrication, and so there was less chemical degradation of the cinnamaldehyde.

4.1.5. Impact of oil phase composition on cinnamaldehyde degradation during storage

The impact of oil phase composition on the chemical degradation of the encapsulated cinnamon oil was then measured after storage for 31 days (**Figures 4.4 and 4.5**). There was an appreciable decrease in the cinnamaldehyde level, and an increase in the benzaldehyde level after storage, which suggests that further chemical degradation of the cinnamaldehyde had occurred. In general, there was a trend towards less benzaldehyde being formed with increasing cinnamaldehyde level inside the droplets, with the exception of the sample containing 40 wt% cinnamon oil. This again suggests that the higher the initial level of cinnamon oil inside the lipid droplets, the lower the fraction that chemically degraded during storage. Previous studies have shown that cinnamaldehyde may be transformed to benzaldehyde and glyoxal in the presence of oxygen due to carbon-carbon bond cleavage (Friedman et al., 2000). Thus, the conversion of cinnamaldehyde to benzaldehyde during storage is probably due to this oxidation reaction (Turek & Stintzing, 2013). Moreover, benzaldehyde may also be formed by oxidation of benzyl alcohol, which is a minor constituent of the cinnamon oil used in this study (Sudareva & Chubarova, 2006).

It should be noted that the nanoemulsions fabricated from 50 to 100 wt% cinnamon oil in the lipid phase had the higher cinnamaldehyde concentrations but lower antimicrobial activity (higher MICs) than those containing 30 to 40 wt% of cinnamon oil in the lipid phase after emulsification. This effect may be due to the impact of droplet size on the

antimicrobial efficacy of the nanoemulsions. The cinnamon oil nanoemulsions fabricated from 30-40 wt% cinnamon had the lowest mean droplet diameter (100 nm) and PDI. Previous studies have shown that smaller droplets penetrate through the cell walls of bacteria more easily, which may increase their ability to inactivate bacteria (Donsi & Ferrari, 2016). These results are in agreement with the study of Xue, Michael Davidson, and Zhong (2015) who found that the encapsulation of thyme oil in nanoscale droplets (82 nm) promoted its antimicrobial activity against *E.coli*, *S. enteritidis* and *L. Monocytogenes*.

In this study, the system containing 40 wt% cinnamon oil and 60 wt% MCT in the oil phase was selected for further studies since it gave the high stability of antimicrobial cinnamon oil nanoemulsion with the smallest droplet size and lowest PIT (around 70°C).

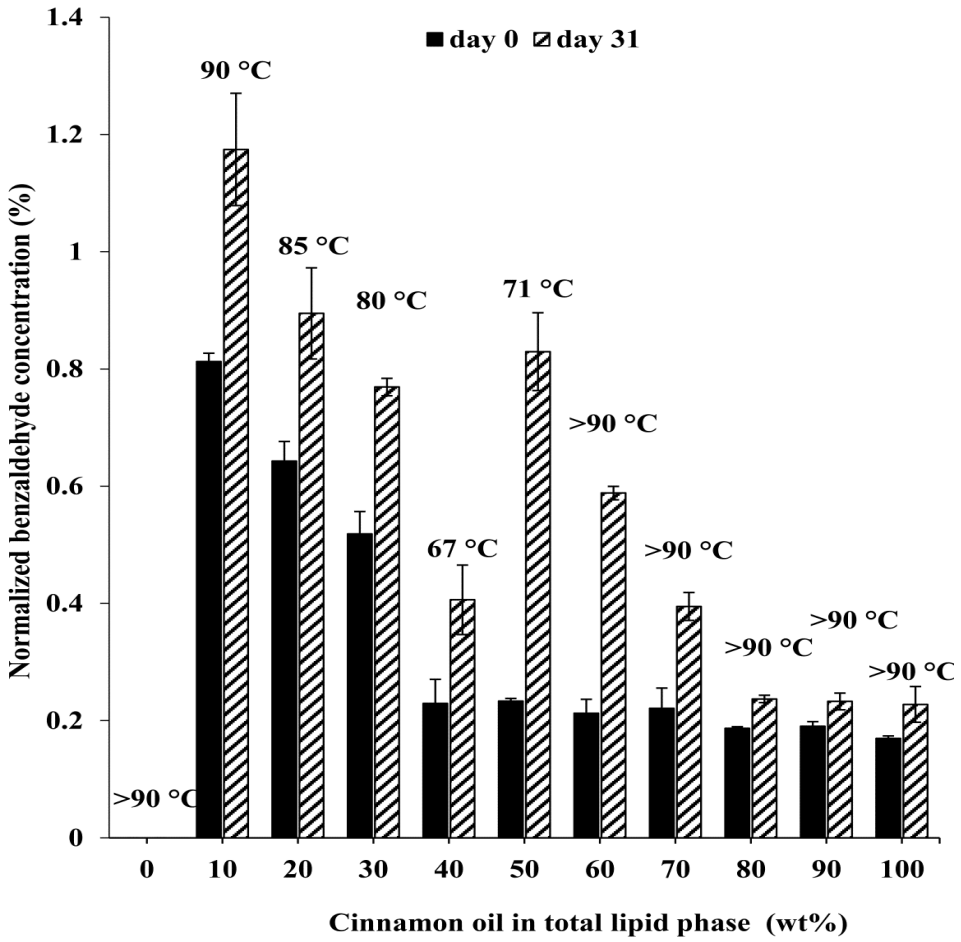

Figure 4.5 Normalized benzaldehyde concentration of cinnamon oil nanoemulsions after emulsification by PIT method at day 0 and day 31 of storage time, the data labels indicate the PIT temperature of each system.

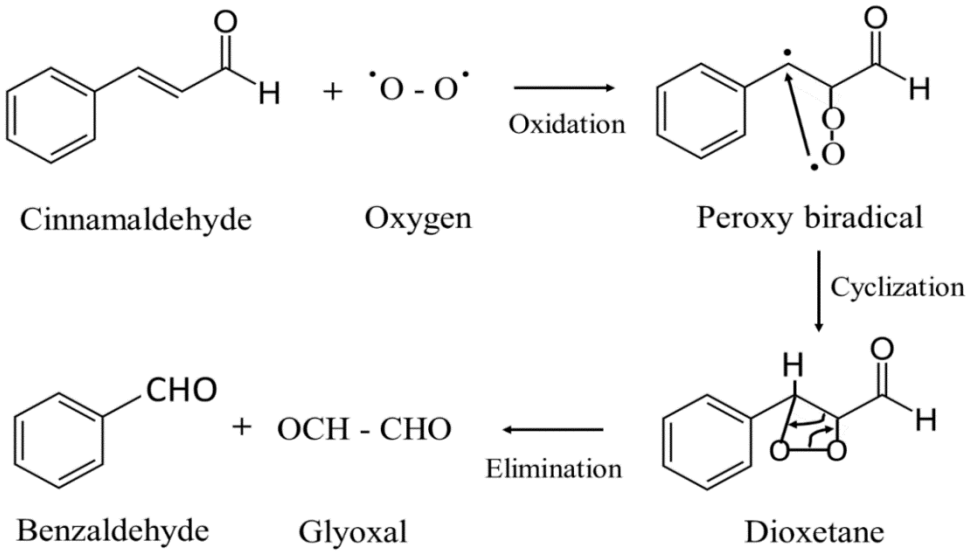

Figure 4.6 Mechanism of conversion of cinnamaldehyde to benzaldehyde and glyoxal.

4.2. Effect of surfactant level on nanoemulsion properties

In this series of experiments, the impact of surfactant level on the formation and stability of the nanoemulsions was determined. Ideally, one would like to make stable nanoemulsions at the lowest surfactant level possible, due to cost, taste, and toxicity concerns. In these experiments, the oil phase composition was fixed at 40 wt% cinnamon oil and 60 wt% MCT, while the total oil phase level was fixed at 10 wt%.

4.2.1. Impact on PIT

The influence of surfactant concentration on the turbidity-temperature profiles of the nanoemulsions during heating and cooling is shown in **Figure 4.7**. The main impact of increasing the surfactant level was to broaden the range of temperatures where they appeared transparent. This suggests that

higher surfactant levels facilitated the formation of the bicontinuous microemulsions formed at intermediate temperatures. The impact of higher surfactant levels on the appearance of the systems below, around, and above the PIT is also shown in **Figure 4.8**. These images clearly show that the microemulsions formed at the PIT are more transparent at higher surfactant concentrations. This effect can be attributed to the fact that they contained smaller oil droplets that scattered light less strongly.

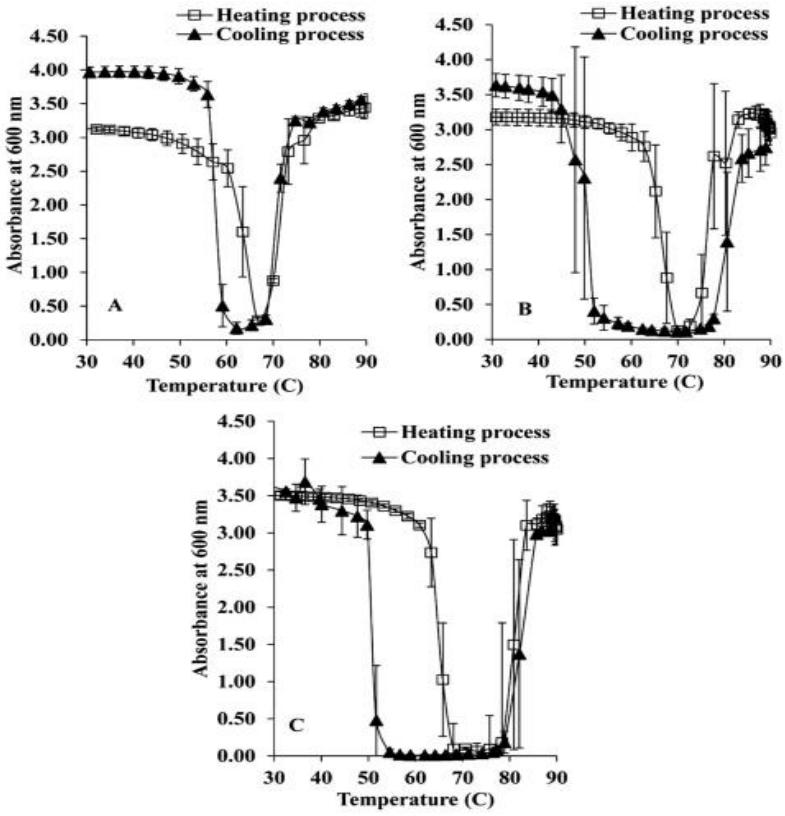

Figure 4.7 Absorbance at 600 nm of nanoemulsions using 10 wt% (a), 15 wt% (b) and 20 wt% (c) of Tween® 80 at 40 wt% of cinnamon oil in total lipid phase and their appearance at before, during and after the PIT temperature.

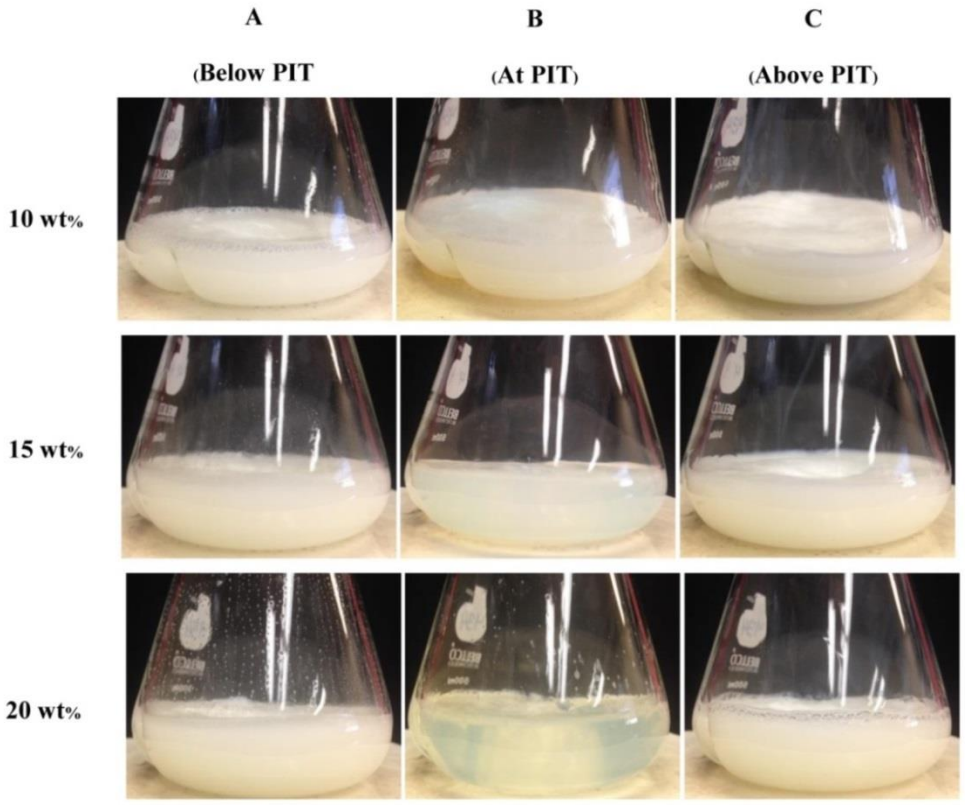

Figure 4.8 Appearance of nanoemulsions containing 40 wt% of cinnamon oil in total lipid phase with concentration of Tween®80 varied from 10 wt%, 15 wt% and 20 wt%, at below PIT temperature (column A), PIT temperature (column B) and above PIT temperature (column C).

4.2.2. Impact on droplet size

The influence of surfactant level on the droplet size of the nanoemulsions formed by the PIT method was also determined. The mean droplet diameter of the nanoemulsions decreased ($p < 0.05$) significantly with increasing surfactant level: 100.7, 50.7 and 23.5 nm at 10, 15 and 20 wt% Tween® 80, respectively (**Figure 4.9**). This effect may have occurred because smaller hydrophobic domains were formed in the bicontinuous microemulsion around the PIT at higher surfactant levels. Our results are in agreement with

previous studies that have also reported that smaller droplets are formed in essential oil nanoemulsions at higher surfactant levels (Gulotta, Saberi, Nicoli, & McClements, 2014; Saberi et al., 2013).

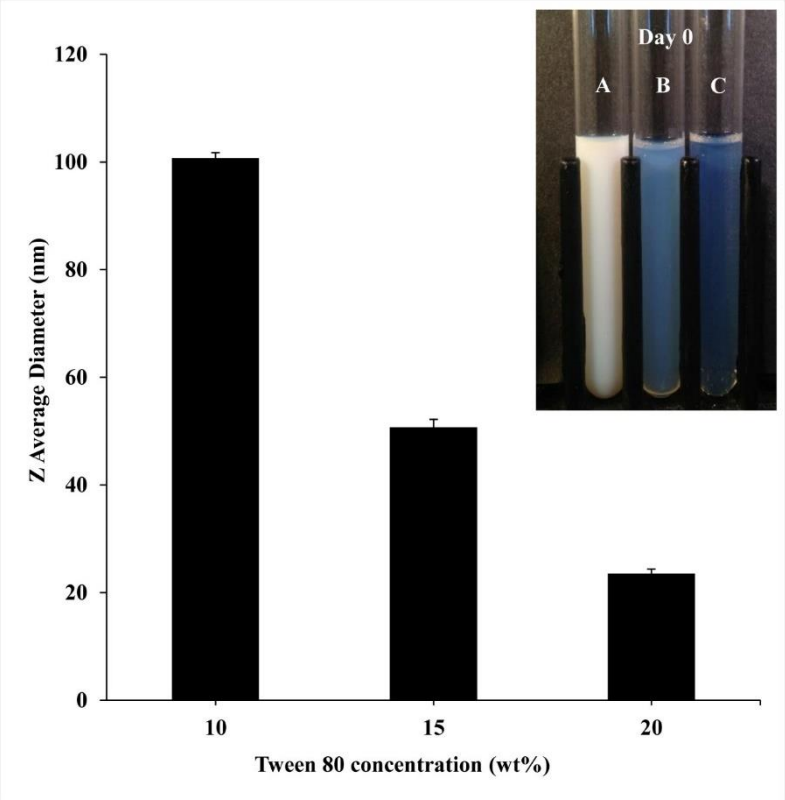

Figure 4.9 Impact of Tween® 80 (10 wt% (A), 15 wt% (B) and 20 wt% (C)) concentration on mean droplet diameter of nanoemulsions containing 40 wt% of cinnamon oil in total lipid phase.

4.2.3. Particle morphology

TEM was used to provide some information about the impact of surfactant concentration on the morphology of the particles in the nanoemulsions (**Figure 4.10**). The surfactant concentration used to prepare the nanoemulsions clearly influenced the size and morphology of the colloidal

particles present. At all surfactant concentration, spherical oil droplets were uniformly observed throughout the continuous phase, which suggests that a stable nanoemulsions were formed (**Figure 4.10A, 4.10C and 4.10E**). However, the alteration in size of nanoemulsions prepared using 15 wt% and 20 wt% of surfactant were found after 31 days of storage at 25°C (**Figure 4.10B, 4.10D and 4.10F**). At all surfactant concentrations, non-aggregated spherical droplets were observed throughout the continuous phase, which suggests that stable nanoemulsions were formed. The droplet sizes measured by TEM were similar to those measured by dynamic light scattering at the lowest surfactant level used. However, there appeared to be relatively large particles in the nanoemulsions prepared using 15 and 20 wt% of surfactant after 31 days of storage at 25°C. This suggested that the size determined by TEM was greater than that determined by dynamic light scattering. This might be due to the collapsing effect which occurs when the nanoemulsions deposited on TEM grid coated with a carbon film (Chuesiang et al., 2018).

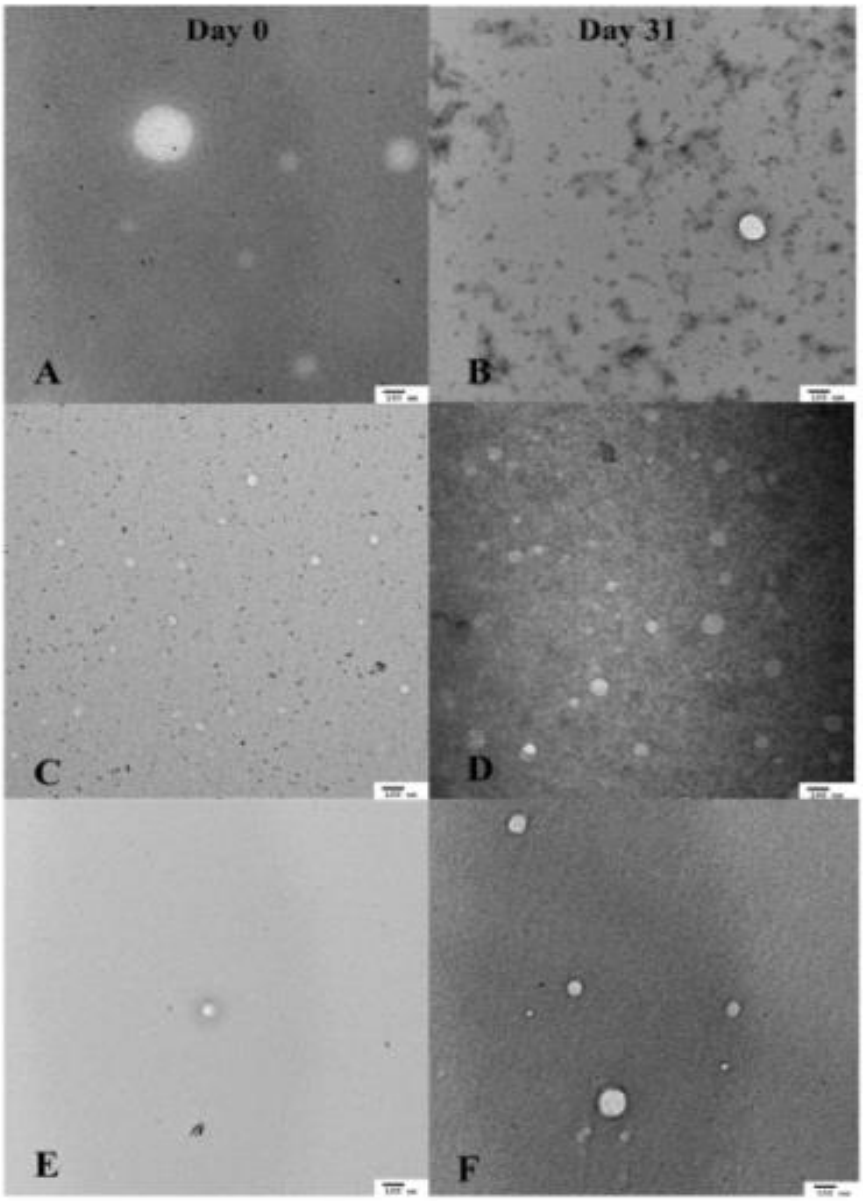

Figure 4.10 Oil droplet morphology of cinnamon oil nanoemulsions containing 40 wt% of cinnamon oil in total lipid phase with 10, 15 and 20 wt% surfactant at day 0 (A, C and E, respectively) and day 31 (B, D and F, respectively), storage at 25°C.

4.2.4. Storage stability

The impact of surfactant concentration (10, 15 or 20 wt%) on the physical stability of the nanoemulsions during storage at 4, 25 and 32 °C was

measured (**Figures 4.11**). At 4 and 25 °C, there was little change in mean droplet diameter throughout storage for the nanoemulsions containing 10 and 15 wt% surfactant, but a slight increase in droplet size for the nanoemulsions containing 20 wt% surfactant. This suggests that a higher surfactant level may have promoted droplet growth through coalescence or Ostwald ripening. High surfactant levels may accelerate these instability mechanisms through a number of physicochemical phenomena: (i) surfactant micelles may facilitate the transport of oil molecules from one droplet to another, thereby enhancing Ostwald ripening (Pena & Miller, 2006; Weiss, Canceliere, & McClements, 2000); (ii) the presence of surfactant micelles in the aqueous phase generates an attractive osmotic stress that may force droplets together and promote their coalescence (McClements, 1994); (iii) high levels of surfactant may alter the properties of the interfacial layer, thereby enhancing coalescence (Kabalnov & Wennerström, 1996). Nevertheless, the appearances of all the nanoemulsions remained fairly similar before and after storage at these lower temperatures, which suggests that the changes in particle size were not large enough to cause appreciable changes in their optical properties.

At 32 °C, there was an appreciable increase in the mean droplet diameter with time during storage, and the nanoemulsions became visibly more turbid after storage (**Figure 4.11**). This suggests that the nanoemulsions were unstable to droplet growth at the highest storage temperature used. The origin of this effect can be attributed to the fact that the rate of droplet coalescence in a nanoemulsion increases in the temperature range just below the PIT (Kabalnov & Wennerström, 1996). Accelerated droplet coalescence

occurs in this region because the surfactant head groups become partially dehydrated, which causes the optimum curvature of the surfactant monolayer to tend towards unity. As a result, the interfacial tension becomes relatively low and the monolayers become highly flexible, which promotes the merging together of oil droplets when they collide (Chuesiang et al., 2018). **Figure 4.12** showed the alteration in particle size distribution of cinnamon oil nanoemulsions fabricated with different level of surfactant at 4, 25 and 32 °C after emulsification (**Figure 4.12A**) and during storage (**Figure 4.12B-D**). After emulsification, cinnamon oil nanoemulsions fabricated with 10 wt% surfactant had a lower PDI value compared to those fabricated with higher surfactant concentration, suggesting that its droplet size was more consistent compare to those of other condition. All cinnamon oil nanoemulsions exhibited a high stability over a storage period at 4 °C (**Figure 4.12B**). However, the particle size distribution of cinnamon oil nanoemulsions became narrower during storage at 32°C (**Figure 4.12D**), meaning obviously that Ostwald ripening and coalescence occurred (Chuesiang et al., 2018).

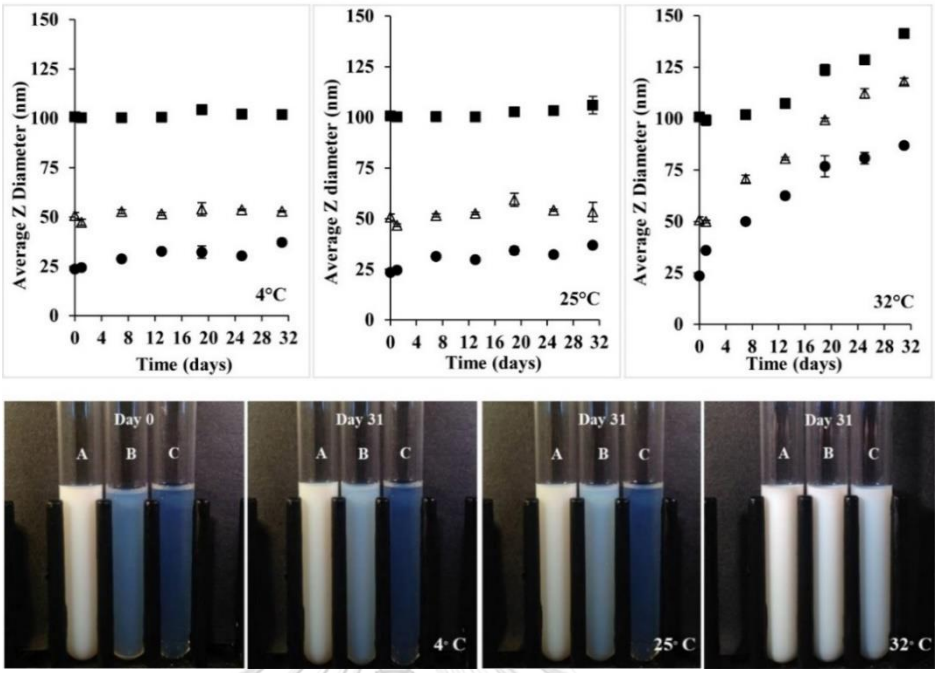

Figure 4.11 Effect of Tween® 80 (10 wt% (A,■), 15 wt% (B, Δ) and 20 wt% (C, ●)) concentration on mean droplet diameter of nanoemulsions containing 40 wt% and 60 wt% of cinnamon oil and MCT in total lipid phase and kept at 4°C, 25°C and 32°C for 31 days.

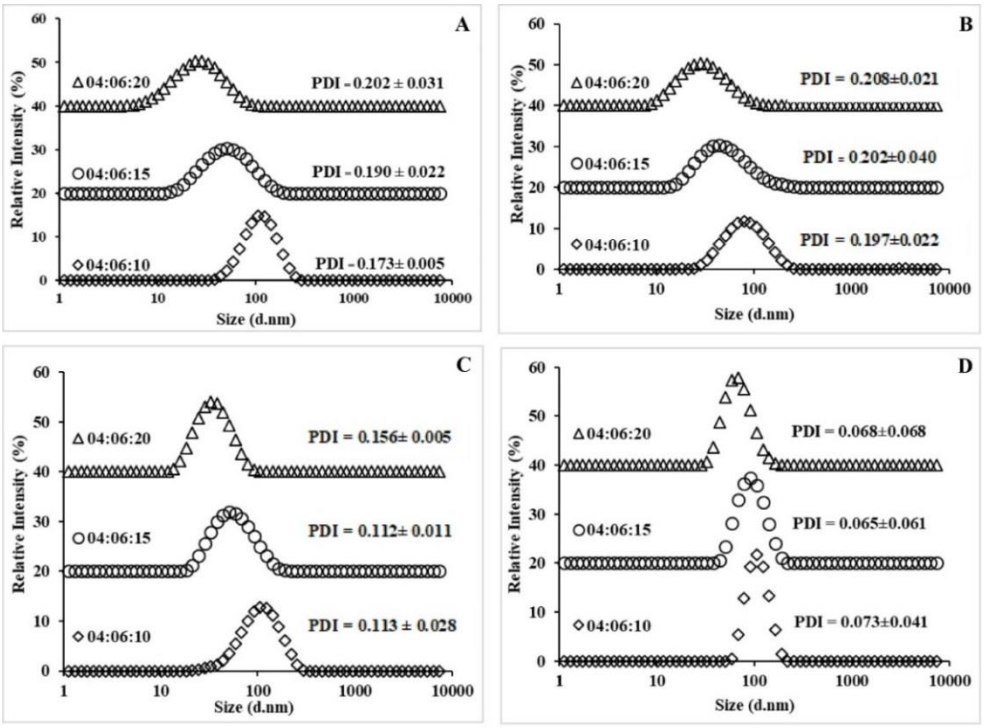

Figure 4.12 The alteration in particle size distribution of cinnamon oil nanoemulsions fabricated with different level of surfactant (10(◊), 15(○) and 20(Δ) wt%) after emulsification (**Figure 4.12A**) and during storage at 4, 25 and 32 °C (**Figure 4.12B -D,** respectively).

4.2.5. Effect of surfactant concentration on antimicrobial activity

4.2.5.1. Determination of MICs

MICs of cinnamon oil nanoemulsions containing different surfactant concentrations against four foodborne pathogens were investigated to ascertain the correlation between surfactant concentration, mean droplet diameter, and antimicrobial activity. The MICs of the nanoemulsions fabricated with 10, 15, and 20 wt% Tween®80 against *V. parahaemolyticus* were not significantly different ($p>0.05$). Whereas, increasing surfactant concentration to 15 wt% or

higher, increased the MIC against *E. coli*, *S.* Typhimurium and *S. aureus* (**Table 4.3**). These results suggest that increasing the surfactant concentration did not enhance, but instead decreased the antimicrobial activity of the cinnamon oil nanoemulsions. In a separate experiment, it was shown that Tween®80 itself had no antimicrobial activity against all tested bacteria (data not shown). Consequently, increasing the surfactant concentration may have led to more efficient encapsulation of the essential oils inside the oil droplets, thereby reducing their ability to interact with the bacterial cells (Ma, Davidson, & Zhong, 2016). These results agree with the studies of Inouye, Tsuruoka, Uchida, and Yamaguchi (2001) and Ma et al. (2016) who also found that higher amounts of Tween® 80 caused an increase in the MICs of essential oils against bacteria and fungi. The location of the antimicrobial components solubilized in the surfactant micelles has also been reported to have an impact on the antimicrobial activities of carvacrol microemulsions containing small particles (8.1 nm) (Shaaban & Edris, 2015). The greater the distance of the antimicrobial agent from the micelle surface, the lower its activity due to the decrease in its availability to interact with the bacterial cells (Terjung, Loffler, Gibis, Hinrichs, & Weiss, 2012). However, opposite effects have been reported by other researchers, which were attributed to the ability of the surfactant to increase the antimicrobial activity of thymol by disrupting the bacterial cell membranes (Li, Chang, Saenger, & Deering, 2017). Tween®80 caused around 35% of leakage of the

cytoplasmic contents from *Pseudomonas aeruginosa* after 2 h contact (J.S, Mishra, Thomas, Mukherjee, & Chandrasekaran, 2018). Previous studies have also shown that increasing the level of Tween®80 from 10-20 wt% had a pronounced effect on the antimicrobial activity of cinnamon oil nanoemulsion formed by spontaneous emulsification method against *E. coli* (Yildirim, Oztop, & Soyer, 2017), which in this case was attributed to the reduction in the mean droplet diameter of the colloidal dispersion. In fact, the true MIC could be somewhere between the lowest value that inhibits the growth of bacteria and the next lowest test value. Therefore, it is noteworthy that the obtained results could not express the exact antibacterial activity of cinnamon oil nanoemulsions (Li et al., 2015). Due to the lack of consistency in MIC data, a dynamic time kill assay was carried out to provide further insights into the antimicrobial activity of the nanoemulsions.

Table 4.3 Minimum inhibitory concentration (MIC, mg/L) of nanoemulsions containing 40 %wt of cinnamon oil with different Tween® 80 concentration, prepared by PIT method.

Tween®80 (wt%)	***E. coli***	***S.* Typhimurium**	***S. aureus***	***V. parahaemolyticus***
10	714[a]	714[a]	357[a]	357[a]
15	1429[b]	1429[b]	1429[b]	357[a]
20	1429[b]	1429[b]	1429[b]	357[a]

* Average initial population of *E. coli*, *S.* Typhimurium, *S. aureus* and *V. parahaemolyticus* was 8.34±0.12, 8.38±0.15, 8.14±0.02 and 8.50±0.05 log CFU/mL, respectively.
** Numbers are mean ± standard error (n=5). Different superscript letters in the same column indicate mean values which significantly different ($p \leq 0.05$).

4.2.5.2. Dynamic time kill assay

Antimicrobial efficacy of cinnamon oil nanoemulsions fabricated from 40 wt% cinnamon oil and 60 wt% MCT with differing amounts of Tween®80 (10, 15 and 20 wt%) was investigated. Bacteria were treated with nanoemulsions at their MICs and comparisons were made with the ones treated with bulk cinnamon oil, sterile deionized water, and a 20 wt% Tween®80 solution. Cinnamon oil nanoemulsions and bulk cinnamon oil displayed bacteriostatic and bactericidal properties against the growth of all tested bacteria since a decrease in bacterial number was observed within 24 h of treatment. In contrast, the tubes containing sterile deionized water or 20 wt% Tween®80 solution showed no antimicrobial activity, since the growth of bacteria

was detected throughout the 24 h of incubation at 37°C (results not shown). The antimicrobial activity of cinnamon oil nanoemulsions against all tested foodborne pathogens was depended on the bacterial strain type. *E. coli* was reduced to the minimum levels (indicated using a black dashed line in the figures) after contact with cinnamon oil nanoemulsions prepared using 15 and 20 wt% of Tween®80 for 8 h. In contrast, the bulk cinnamon oil required an appreciably longer contact time (12 h) to reach this low level (**Figure 4.13**).

S. Typhimurium was also reduced to the minimum levels within 8 h of contact with cinnamon oil nanoemulsions prepared using 15 and 20 wt% of Tween®80, but at a slower rate when compared to that of bulk cinnamon oil. However, the reduction number of *S.* Typhimurium decreased from 6 log CFU/mL to about 5 log CFU/mL during 12 to 24 h of treatment with bulk cinnamon oil, suggesting that the antimicrobial activity of bulk cinnamon oil against *S.* Typhimurium was reduced due to the immiscibility of oil and water (**Figure 4.14)**.

Bulk cinnamon oil and cinnamon oil nanoemulsions fabricated with 15 and 20 wt% Tween®80 exhibited similar inhibition rates against *S. aureus* at 1 h of contact time. For these systems, the number of viable *S. aureus* continued to decrease over time and they were completely inhibited within 24 h (**Figure 4.15**). However, the number of *S. aureus* maintained constant at 8 log CFU/mL after being treated with cinnamon oil nanoemulsions containing 10 wt% of Tween®80 for 4-12 h. In the present study, *S. aureus* seems to be the most resistant

bacterium against cinnamon oil, and this may be due to differences in the Gram positive cell wall structure or due to its growth in aggregates and clumps (Dastgheyb et al., 2015).

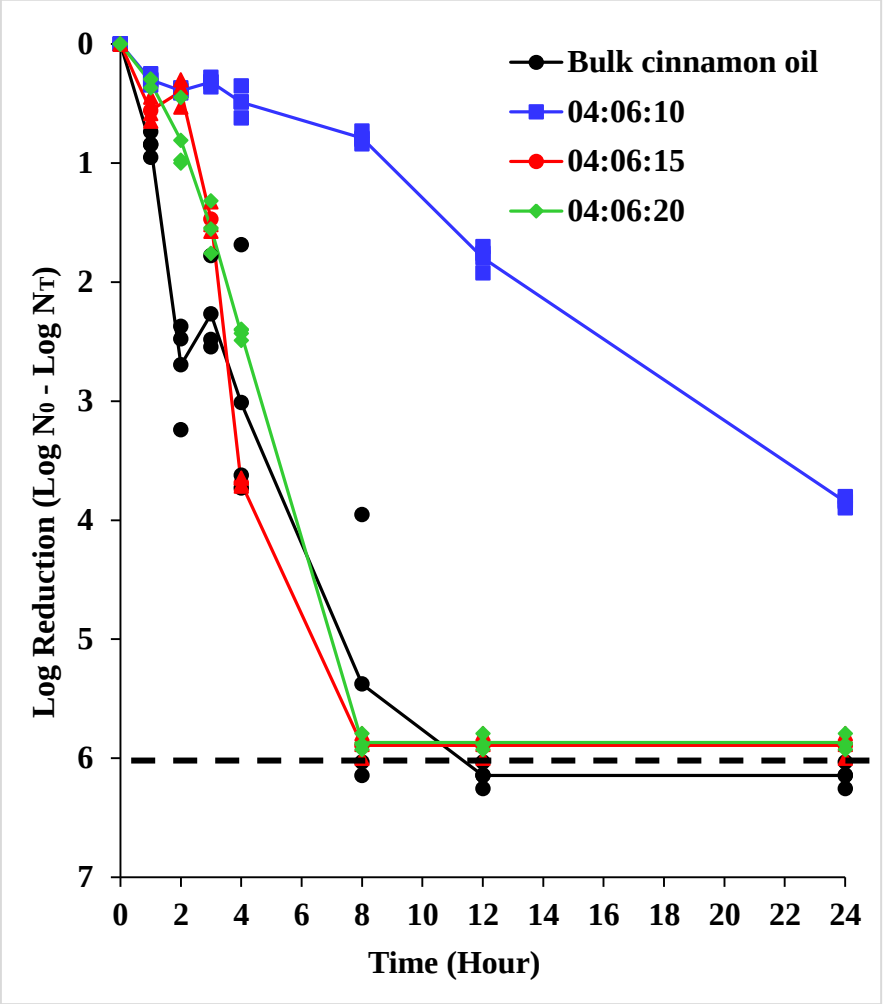

Figure 4.13 Survival curve of *E. coli* after treated with bulk cinnamon oil and cinnamon oil nanoemulsions containing 40 wt% cinnamon oil and 60 wt% MCT in total lipid phase at different surfactant concentration (10, 15 and 20 wt%), incubated at 37°C for 24 hours.

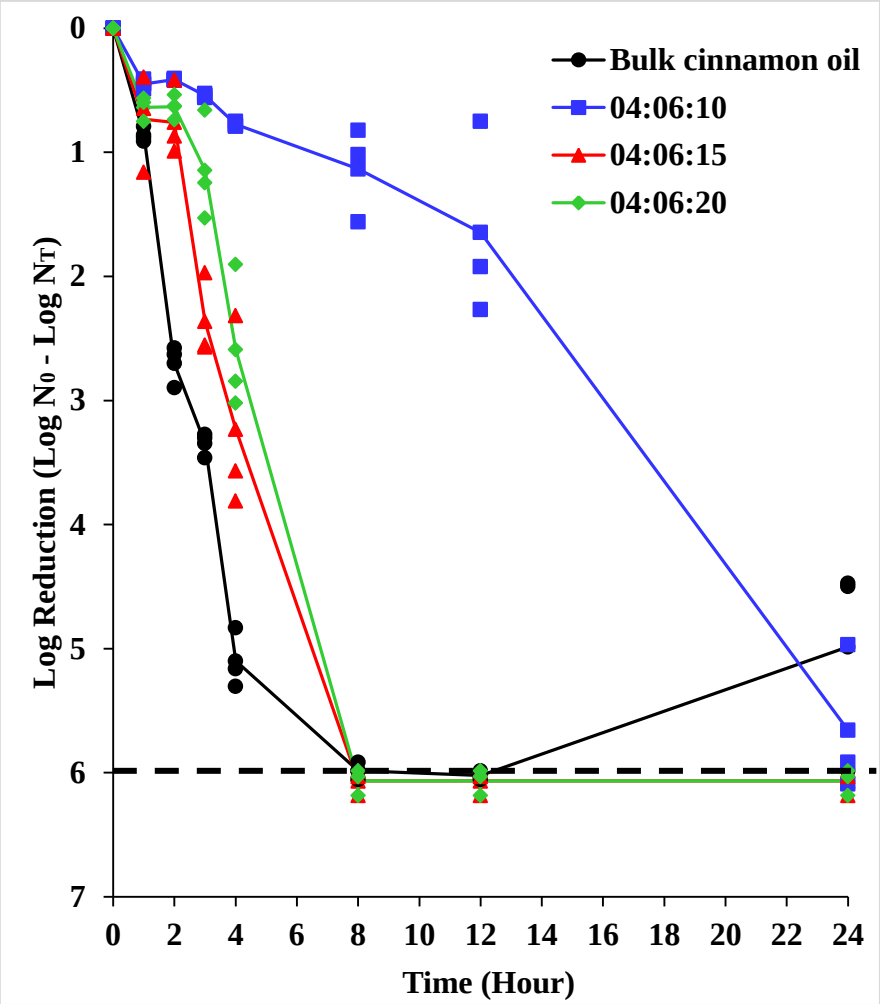

Figure 4.14 Survival curve of *S.* Typhimurium after treated with bulk cinnamon oil and cinnamon oil nanoemulsions containing 40 wt% cinnamon oil and 60 wt% MCT in total lipid phase at different surfactant concentration (10, 15 and 20 wt%), incubated at 37°C for 24 hours.

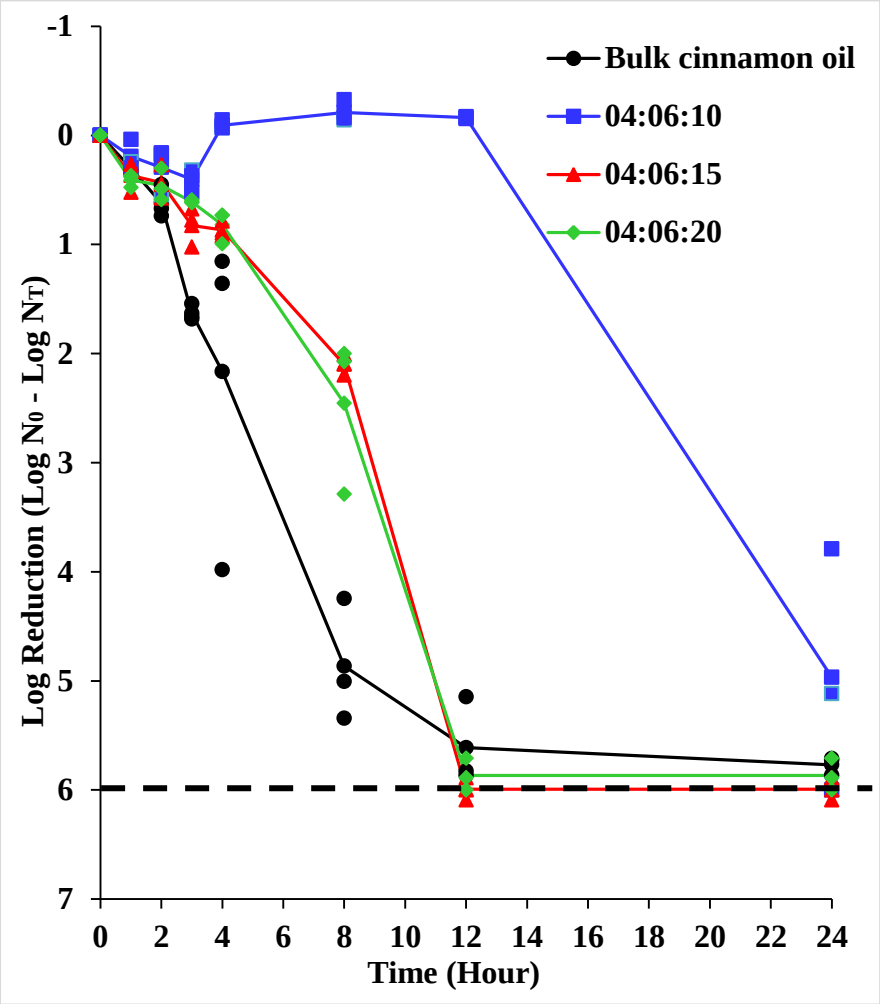

Figure 4.15 Survival curve of *S. aureus* after treated with bulk cinnamon oil and cinnamon oil nanoemulsions containing 40 wt% cinnamon oil and 60 wt% MCT in total lipid phase at different surfactant concentration (10, 15 and 20 wt%), incubated at 37°C for 24 hours.

V. Parahaemolyticus was the most sensitive bacteria to all treatments as it was rapidly inhibited at the fastest rate within 4 h and 1 h after being treated with cinnamon oil nanoemulsions and bulk cinnamon oil, respectively (**Figure 4.16**). Due to the immiscibility of

the bulk cinnamon oil and TSB, the inhibition rate of the bulk cinnamon oil against the tested bacteria had a higher standard deviation than for the nanoemulsions. The high variability in the inhibition rate of bulk cinnamon oil against the tested bacteria may therefore have been because the bulk cinnamon oil was not uniformly dispersed throughout the system. Based on the obtained results, cinnamon oil nanoemulsions fabricated with higher surfactant concentrations (15 and 20 wt%) not only exhibited faster killing rates (in the case of *E. coli* and *S. aureus*) but also showed longer term inhibition (in the case of *S.* Typhimurium) compared to bulk cinnamon oil. A possible explanation is because surfactant concentration helps encapsulate cinnamon oil in a form of small droplets or micelles and increases its water dispersibility. Thus, the small essential oil droplets are able to easily pass through the outer membrane of the bacterial cells. Previous studies have shown that the long term antimicrobial activity of cinnamon oil nanoemulsions occurred due to the slow release effect of the encapsulation system (Chuesiang et al., 2019; Donsi & Ferrari, 2016; Fu, Sarkar, Bhunia, & Yao, 2016; Li et al., 2015). Additionally, the bacterial strain is an important factor affecting susceptibility against cinnamon oil nanoemulsions, showing that all tested foodborne pathogens exhibited different survival curves. It should be noted that cinnamon oil nanoemulsions fabricated with higher surfactant concentrations (15 and 20 wt%) had a higher MIC against all tested bacteria when compared to those of cinnamon oil nanoemulsions

fabricated with 10 wt% surfactant and bulk cinnamon oil. Therefore, this might be one reason that affected antimicrobial activity of cinnamon oil nanoemulsions and bulk cinnamon oil. In addition, pathogenic bacteria including *E. coli*, *S.* Typhimurium and *V. parahaemolyticus* are known to involve in a viable. However, it should be noted that under natural stresses or bactericidal treatments a nonculturable state (VBNC) of bacteria may be in effect (Gunasekera, Sorensen, Attfield, Sorensen, & Veal, 2002; Oliver, 2005). As shown in **Figure 4.13-4.16**, instead of plotting average values with standard deviation (SD) of which the graph overlapped, all replicate data are presented in order to simultaneously facilitate data visualization and show the deviation of all replications.

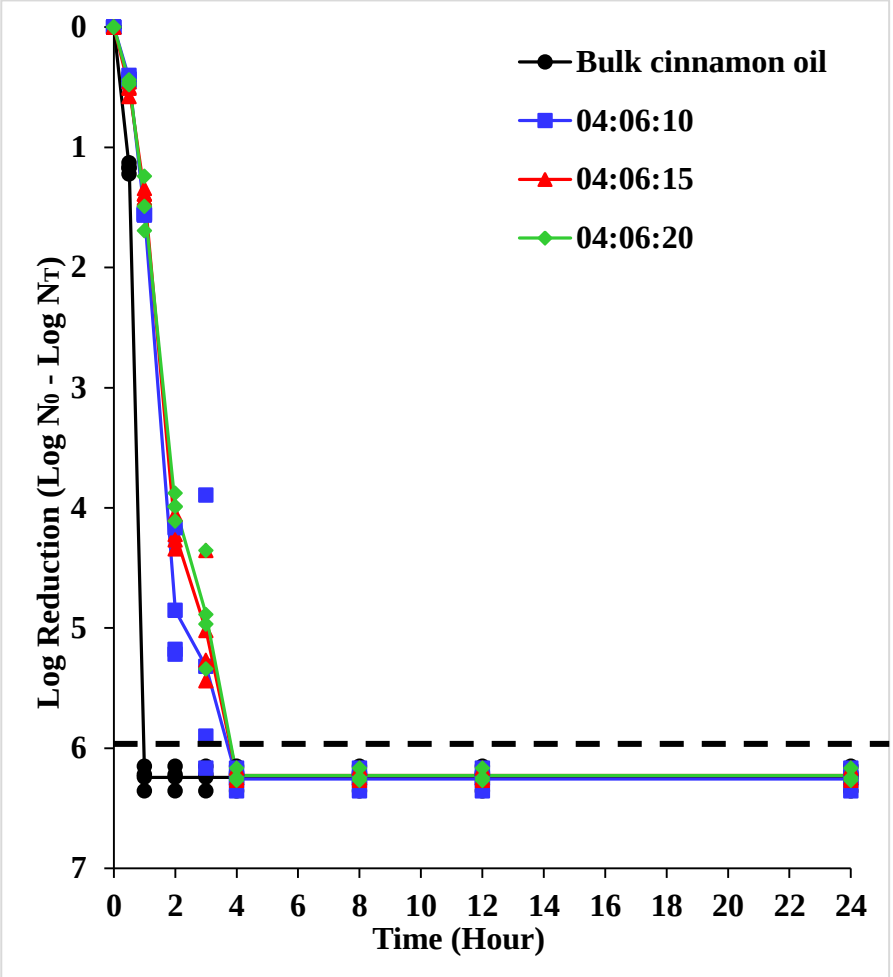

Figure 4.16 Survival curve of *V. parahaemolyticus* after treated with bulk cinnamon oil and cinnamon oil nanoemulsions containing 40 wt% cinnamon oil and 60 wt% MCT in total lipid phase at different surfactant concentration (10, 15 and 20 wt%), incubated at 37°C for 24 hours.

4.2.5.3. FE-SEM

FE-SEM analysis was employed to visualize the effect of cinnamon oil nanoemulsions containing different surfactant levels on bacterial morphology. Based on the results from the dynamic time kill assay, about 1-6 log CFU/mL of bacterial growth were inhibited at 3 h

(for *E. coli*, *S.* Typhimurium and *V. parahaemolyticus*) and 8 h (for *S. aureus*) of treatment. Therefore, the alteration of bacterial morphology was carried out at 3 h of contact time for *E. coli*, *S.* Typhimurium and *V. parahaemolyticus*, and at 8 h of contact time for *S. aureus* (incubated at 37 °C). As shown in **Figure 4.17**, cell damage was observed in all tested bacteria after being treated with cinnamon oil nanoemulsions and bulk cinnamon oil. In contrast, the bacteria had smooth, flagellate and intact cell walls indicative of cell growth after being treated with buffer solution (TSB or TSB+2 wt% NaCl). In the present study, cells treated with cinnamon oil nanoemulsions at higher level of Tween®80 had more severe morphological destruction. An impaired membrane structure and leakage of inner cell materials (shown using a red arrow in the microscopy images) were observed in bacteria treated with cinnamon oil nanoemulsions fabricated using 15 and 20 wt% Tween®80, whereas those treated with cinnamon oil nanoemulsions fabricated using 10 wt% Tween®80 and bulk cinnamon oil (for *E. coli* and *S.* Typhimurium) appeared to have only slightly deformed cell morphologies. Once again, the FE-SEM micrographs confirmed the variation in the killing efficiency of bulk cinnamon oil against the tested bacteria. These results can again be explained by the fact that the small essential oil droplets in the nanoemulsions increased the ability of the antimicrobial agents to interact with the bacterial cells (Chuesiang et al., 2019; Lu et al., 2018).

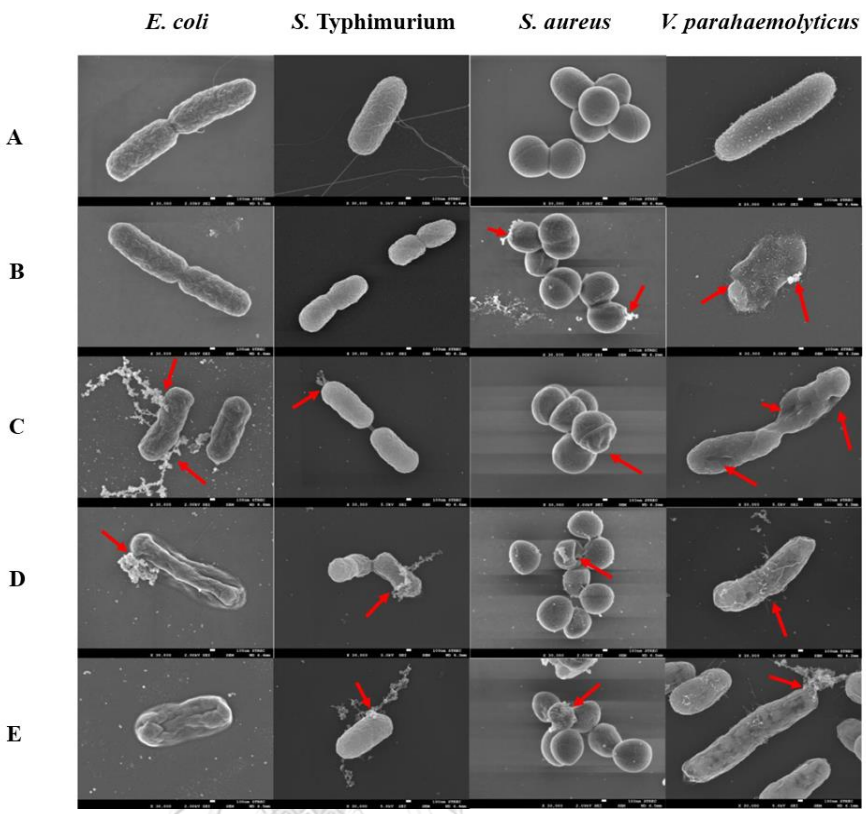

Figure 4.17 FE-SEM micrograph of *E.coli*, *S.* Typhimurium, *S. aureus* and *V. parahaemolyticus* after treated with buffer solution (TSB/TSB+2% NaCl; A), bulk cinnamon oil (B), cinnamon oil nanoemulsions with 10, 15 and 20 wt% Tween®80 (C, D and E, respectively).

4.2.6. Impact of surfactant concentration on cinnamaldehyde degradation during emulsification and storage

The effect of surfactant concentration on the degradation of cinnamaldehyde during emulsification and after storage for 31 days was also conducted in the present study. As shown in **Figure 4.18**, nanoemulsions fabricated with 10-20 wt% surfactant showed insignificantly different ($p>0.05$) in cinnamaldehyde concentration after emulsification (day 0) and after storage

(day 31), suggesting that surfactant concentration had no influence on the degradation of cinnamaldehyde contained in the nanoemulsions. The results might be attributed to the fact that increasing the surfactant level did not alter the PIT temperature of the system, thus similar cinnamaldehyde concentration was exhibited in nanoemulsions which were fabricated through the same PIT temperature.

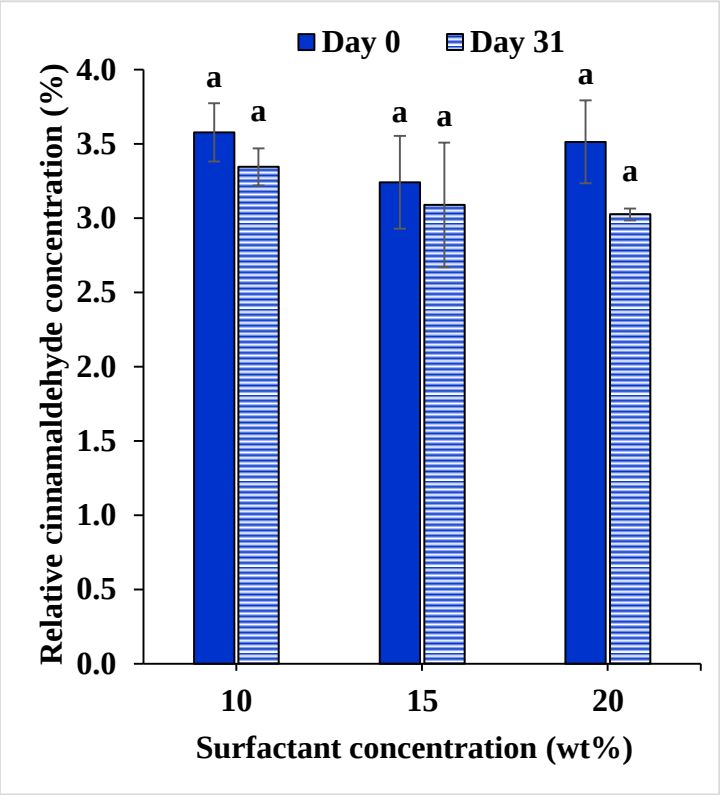

Figure 4.18 Cinnamaldehyde concentration of cinnamon oil nanoemulsions after emulsification by PIT method using different level of Tween®80, at day 0 and day 31 of storage time.

In contrast to cinnamaldehyde content, increasing in benzaldehyde content was observed in nanoemulsions fabricated with 15 and 20 wt% surfactant after emulsification. Possible explanation is about the broaden range of PIT temperature affected by surfactant concentration. However, increasing

in benzaldehyde content was found in nanoemulsion fabricated using 10 wt% surfactant during storage, suggesting that nanoemulsion with bigger droplet size was less chemically stable when compared to those with smaller droplet size.

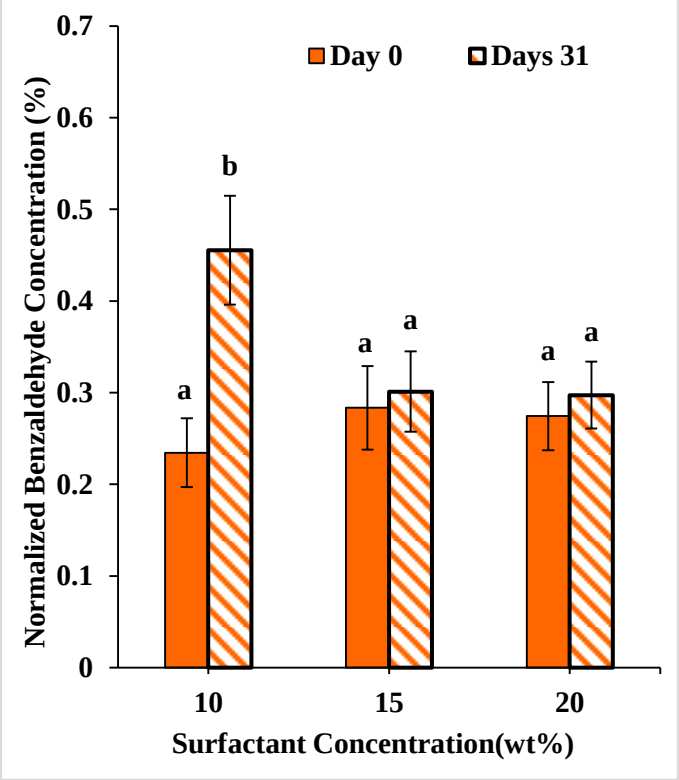

Figure 4.19 Benzaldehyde concentration of cinnamon oil nanoemulsions after emulsification by PIT method using different level of Tween®80, at day 0 and day 31 of storage time.

4.3. Application of cinnamon oil nanoemulsion on Asian seabass flesh

4.3.1. Proximate analysis

Thawed Asian seabass flesh had high moisture content (77.48%) and a higher protein content (20.14%) than total fat (2.45%) and ash (0.78%) content (**Table 4.4**). The results are in line with previously reported on chemical

composition of Asian seabass flesh, 75.70-80.20 % moisture, 18.20-20.80 % protein, 0.80-3.20 % lipid and 0.90-1.10 % ash (Manthey-Karl, Lehmann, Ostermeyer, & Schröder, 2016). Depending on their origins, species, age, harvest time and preparation technique, chemical composition of the fleshes could be varied (Cai et al., 2015; Chuesiang & Sanguandeekul, 2015; Manthey-Karl et al., 2016).

Table 4.4 Chemical composition of thawed Asian seabass flesh.

Chemical composition	%
Moisture	77.48 ± 1.22
Protein	20.14 ± 0.64
Total fat	2.45 ± 0.18
Ash	0.78 ± 0.01
Carbohydrate	0.00 ± 0.00

4.3.2. Analysis of microbiological property

Total volatile content (TVC) of Asian seabass flesh with and without cinnamon oil nanoemulsion dipping were determined during storage for 0, 2, 4, 6 and 8 days. The results were expressed as log CFU/g and are displayed in **Figure 4.20**. The initial number of TVC in the control group was 5.6 log CFU/g and those of the fleshes treated with CON1, CON2, BCO1 and BCO2 were 5.38, 4.98, 5.56 and 4.77 log CFU/g, respectively. According to the recommendation of the International Commission on Microbiological Specifications for Foods (ICMSF, 1986), the TVC of fish flesh should not exceeded 7 log CFU/g, meaning that it is dangerous for consumption due to the produced toxic substances which

can cause severe health problems (Hassoun & Emir Çoban, 2017). After 4 days storage, TVC of the flesh from control group was 7.26 log CFU/g which exceeded the upper limit for fresh fish as recommended by ICMSF (1986). Whereas, TVC of the flesh treated with CON1 or BCO1 and CON2 or BCO2 exceeded 7 log CFU/g on day 6 and day 8 of storage period, respectively. Thus, cinnamon oil nanoemulsion and bulk cinnamon oil inhibited the growth of bacteria and extended the shelf life of Asian seabass flesh for 2-4 days depending on the concentration of the cinnamon oil nanoemulsions or bulk cinnamon oil. Similar results were also reported in the study of Huang, Liu, Jia, and Luo (2017), rainbow trout fillets treated with cinnamon bark oil nanoemulsion fabricated using spontaneous emulsification method had 2 days longer in the shelf life when compared to those of untreated sample due to the antimicrobial activity of cinnamon bark oil.

It should be noted that TVC of the fleshes were immediately decreased by 0.5-1 log CFU/g after 30 min of soaking period, depending on the treatment concentration (**Figure 4.20**). Higher concentration of cinnamon oil nanoemulsion and bulk cinnamon oil (CON2 and BCO2), inhibited the growth of bacteria more quickly than lower concentration (CON1 and BCO1). To be effective for *in vivo* bacterial growth inhibition, the concentration of plant essential oils must reach their threshold. In a food system, however, the interactions between plant essential oils and food components (*e.g.* protein or fat) may lower the available essential oils' active components to interact with bacterial cells (Hassoun & Emir Çoban, 2017; Hyldgaard, Mygind, & Meyer, 2012). Therefore, the reduction of the antimicrobial efficacy of plant essential oils was observed (Hassoun & Emir

Çoban, 2017; Hyldgaard et al., 2012). The ability of nanoemulsions may decreased due to the osmotic attraction between the droplet which occurred when the concentration of polymers such as proteins are excess in the system (Landry, Micheli, McClements, & McLandsborough, 2015). However, fleshes treated with cinnamon oil nanoemulsion showed the lower TVC than those treated with bulk cinnamon oil at each time point, suggesting that fast and persistent antimicrobial activity can be achieved by the small droplets contained in a nanoemulsion delivery system (Moghimi et al., 2016; Ozogul et al., 2017).

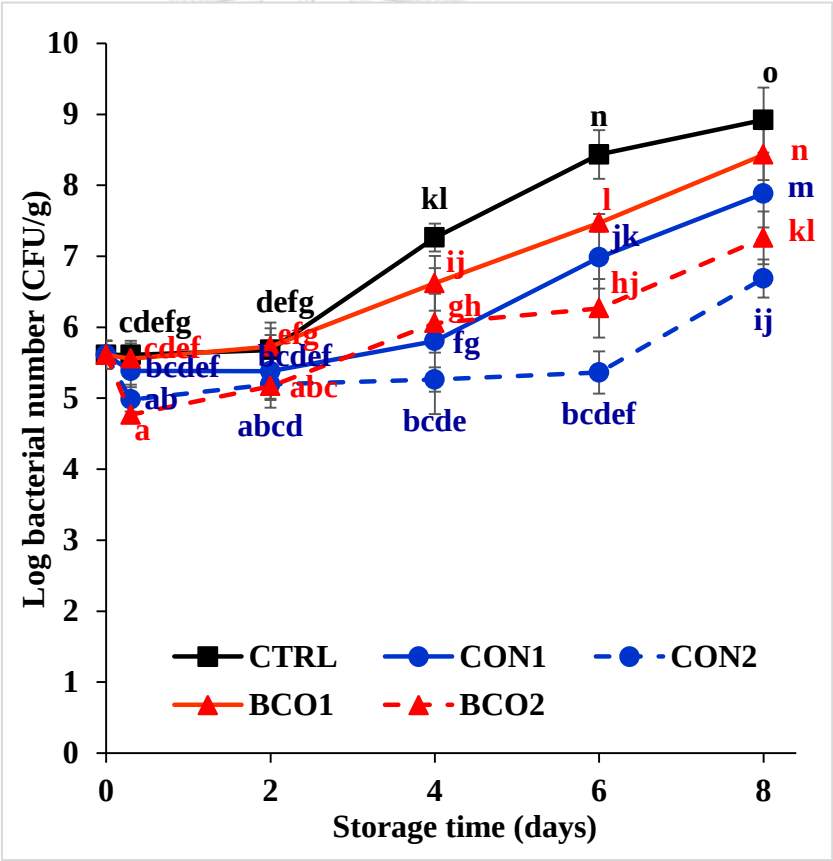

Figure 4.20 Changes in the total viable count (TVC) of Asian seabass flesh treated with/without cinnamon oil nanoemulsion during storage at 4±2 °C for 8 day.

CHAPTER 5

CONCLUSION

Phase inversion temperature method has proved to be suitable for formulating stable cinnamon oil nanoemulsions. In this study, cinnamon oil and carrier oil concentrations in total lipid phase was found to have impacts on PIT temperature, initial mean droplets size, size distribution curve, antimicrobial activity and stability of cinnamon oil nanoemulsions. Monodispersed nanoemulsion with the lowest PIT temperature, the smallest mean droplet diameter and the highest stability, were created when total lipid phase composition contained 40 wt% cinnamon oil and 60 wt% MCT.

The impact of oil phase composition on the antimicrobial activity and chemical stability of cinnamon oil nanoemulsions was investigated. The MIC of the nanoemulsions was tested against *E. coli*, *S.* Typhimurium, *S. aureus* and *V. parahaemolyticus.* The antimicrobial activity of the nanoemulsions increased with increasing cinnamon oil in the lipid phase until it reached a maximum value, which depended on bacterial strains. The optimum lipid phase composition of cinnamon oil: MCT was 40:60 wt% for *E. coli*, *S.* Typhimurium, and *S. aureus*, and 50:50 wt% for *V. parahaemolyticus.* However, when the cinnamon oil level exceeded their optimum levels, the antimicrobial activity decreased. These results may be attributed to two factors. First, the cinnamaldehyde was partially lost during the fabrication and storage of the nanoemulsions. The degree of chemical degradation during nanoemulsion fabrication increased as the holding temperature increased. However, its degradation decreased as the initial level of cinnamaldehyde inside the droplets increased, which suggests that high levels of this essential oil were able to inhibit its degradation.

Second, smaller cinnamon oil droplets are more efficiently transported through the bacterial cell walls and inhibit the growth of the cell by increasing the cell membrane permeability.

Once the optimum oil phase was found, the effect of surfactant concentration was then studied. Increasing surfactant concentration decreased the initial mean droplet diameter of cinnamon oil nanoemulsions steeply and improved their appearance from milky to transparent. However, increasing the surfactant concentration from 10 to 20 wt% did not alter the particle morphology of the cinnamon oil nanoemulsions. Cinnamon oil nanoemulsions were stable during storage at low temperature (4°C) and ambient temperature (25°C) for at least 31 days. However, the rate of droplet growth during storage increased when it was stored at higher temperature (32°C) due to Ostwald ripening.

Increasing the surfactant concentration in the nanoemulsions did not reduce their MIC values against all tested bacteria. However, it did lead to a faster killing rate and underwent prolonged inhibition against bacterial cells. The microbial strain had a major impact on the efficacy of the antimicrobial nanoemulsions, with *S. aureus* being the most resistance and *V. parahaemolyticus* being the least. Morphology studies showed that increasing the surfactant concentration reduced the size of the cinnamon oil droplets, which led to greater penetration of the cinnamaldehyde into the outer membrane of the bacterial cells resulting in more cell disruption and death. Cinnamon oil nanoemulsions fabricated in the present study exhibited better antimicrobial activities than those of bulk cinnamon oil in term of consistent ability, meaning that their killing efficiency are stable and not dependent on the location or position of bacteria in system

Effects of cinnamon oil nanoemulsion on microbiological property of Asian seabass fleshes were studied. Treating the samples with cinnamon oil nanoemulsion could decrease the number of TVC in the fleshes during chilled storage, thus extend storage life of the samples for 2-4 days as compared to those of untreated sample. Encapsulation of cinnamon oil into a nanoemulsion delivery system could increase the ability of cinnamon oil to contact with bacterial cell by serving its active ingredient in a form of small droplets, thus enhance its biological activity and extend the shelf life of the fleshes.

Overall, the results suggest that encapsulation of cinnamon oil in nanoemulsions enhances its bactericidal activity against bacteria containing in the flesh. The nanoemulsion has high potential for prolonging shelf life of fish flesh, and yet it could be a potential application in the food industry.

REFERENCES

Amani, A., York, P., Chrystyn, H., Clark, B. J., & Do, D. Q. (2008). Determination of factors controlling the particle size in nanoemulsions using Artificial Neural Networks. *Eur J Pharm Sci, 35*(1-2), 42-51.

Anton, N., Gayet, P., Benoit, J. P., & Saulnier, P. (2007). Nano-emulsions and nanocapsules by the PIT method: an investigation on the role of the temperature cycling on the emulsion phase inversion. *Int J Pharm, 344*(1-2), 44-52.

Anton, N., & Vandamme, T. F. (2009). The universality of low-energy nano-emulsification. *Int J Pharm, 377*(1-2), 142-147.

AOAC. (2016). *Official Method of Analysis*, 20th edn. Washington DC: Association of Official Analysis Chemists.

Brackman, G., Defoirdt, T., Miyamoto, C., Bossier, P., Van Calenbergh, S., Nelis, H., & Coenye, T. (2008). Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in *Vibrio* spp. by decreasing the DNA-binding activity of the quorum sensing response regulator LuxR. *BMC Microbiol, 8*, 149.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods--a review. *Int J Food Microbiol, 94*(3), 223-253.

Cai, L., Cao, A., Li, Y., Song, Z., Leng, L., & Li, J. (2015). The effects of essential oil treatment on the biogenic amines inhibition and quality preservation of red drum (*Sciaenops ocellatus*) fillets. *Food Control, 56*, 1-8.

Chang, S.-T., Chen, P.-F., & Chang, S.-C. (2001). Antibacterial activity of leaf essential oils and their constituents from *Cinnamomum osmophloeum*. *J Ethnopharmacol, 77*(1), 123-127.

Chang, Y., McLandsborough, L., & McClements, D. J. (2013). Physicochemical properties and antimicrobial efficacy of carvacrol nanoemulsions formed by spontaneous emulsification. *J Agric Food Chem, 61*(37), 8906-8913.

Chen, H., & Ji, H. (2011). β-cyclodextrin promoted oxidation of cinnamaldehyde to natural benzaldehyde in water. *Chinese J Chem Eng, 19*(6), 972-977.

Chen, H., Ji, H., Zhou, X., Xu, J., & Wang, L. (2009). Aerobic oxidative cleavage of cinnamaldehyde to benzaldehyde catalyzed by metalloporphyrins under mild conditions. *Catal Commun, 10*(6), 828-832.

Cheng, J.-H., Sun, D.-W., Han, Z., & Zeng, X.-A. (2013). Texture and structure measurements and analyses for evaluation of fish and fillet freshness quality: a review. *Compr Rev Food Sci Food Saf, 13*(1), 52-61.

Chuesiang, P., & Sanguandeekul, R. (2015). Protein hydrolysate from tilapia frame: antioxidant and angiotensin I converting enzyme inhibitor properties. *Int J Food Sci Technol, 50*(6), 1436-1444.

Chuesiang, P., Siripatrawan, U., Sanguandeekul, R., McClements, D. J., & McLandsborough, L. (2019). Antimicrobial activity of PIT-fabricated cinnamon oil nanoemulsions: effect of surfactant concentration on morphology of foodborne pathogens. *Food Control, 98*, 405-411.

Chuesiang, P., Siripatrawan, U., Sanguandeekul, R., McLandsborough, L., & Julian McClements, D. (2018). Optimization of cinnamon oil nanoemulsions using phase inversion temperature method: Impact of oil phase composition and surfactant concentration. *J Colloid Interface Sci, 514*, 208-216.

Dastgheyb, S. S., Hammoud, S., Ketonis, C., Liu, A. Y., Fitzgerald, K., Parvizi, J., . . . Hickok, N. J. (2015). Staphylococcal persistence due to biofilm formation in synovial fluid containing prophylactic cefazolin. *Antimicrob Agents Chemother, 59*(4), 2122-2128.

Davidov-Pardo, G., & McClements, D. J. (2015). Nutraceutical delivery systems: resveratrol encapsulation in grape seed oil nanoemulsions formed by spontaneous emulsification. *Food Chem, 167*, 205-212.

Donsi, F., & Ferrari, G. (2016). Essential oil nanoemulsions as antimicrobial agents in food. *J Biotechnol, 233*, 106-120.

Dorman, H. J., & Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J Appl Microbiol, 88*(2), 308-316.

Fathi, M., Mozafari, M. R., & Mohebbi, M. (2012). Nanoencapsulation of food ingredients using lipid based delivery systems. *Trends Food Sci Technol, 23*(1), 13-27.

Friedman, M. (2017). Chemistry, antimicrobial mechanisms, and antibiotic activities of cinnamaldehyde against pathogenic bacteria in animal feeds and human foods. *J Agric Food Chem, 65*(48), 10406-10423.

Friedman, M., Kozukue, N., & Harden, L. A. (2000). Cinnamaldehyde content in foods determined

by gas chromatography-mass spectrometry. *J Agric Food Chem, 48*, 5702−5709.

Fu, Y., Sarkar, P., Bhunia, A. K., & Yao, Y. (2016). Delivery systems of antimicrobial compounds to food. *Trends Food Sci Technol, 57*, 165-177.

Gill, A. O., & Holley, R. A. (2004). Mechanisms of bactericidal action of cinnamaldehyde against Listeria monocytogenes and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*. *Appl Environ Microbiol, 70*(10), 5750-5755.

Gulotta, A., Saberi, A. H., Nicoli, M. C., & McClements, D. J. (2014). Nanoemulsion-based delivery systems for polyunsaturated (omega-3) oils: formation using a spontaneous emulsification method. *J Agric Food Chem, 62*(7), 1720-1725.

Gunasekera, T. S., Sorensen, A., Attfield, P. V., Sorensen, S. J., & Veal, D. A. (2002). Inducible gene expression by nonculturable bacteria in milk after pasteurization. *Appl Environ Microbiol, 68*(4), 1988-1993.

Gupta, A., Eral, H. B., Hatton, T. A., & Doyle, P. S. (2016). Nanoemulsions: formation, properties and applications. *Soft Matter, 12*(11), 2826-2841.

Gursale, A., Dighe, V., & Parekh, G. (2010). Simultaneous quantitative determination of cinnamaldehyde and methyl eugenol from stem bark of *Cinnamomum zeylanicum* Blume using RP-HPLC. *J Chromatogr Sci, 48*(1), 59-62.

Gutierrez, J., Barry-Ryan, C., & Bourke, P. (2009). Antimicrobial activity of plant essential oils using food model media: efficacy, synergistic potential and interactions with food components. *Food Microbiol, 26*(2), 142-150.

Hassoun, A., & Emir Çoban, Ö. (2017). Essential oils for antimicrobial and antioxidant applications in fish and other seafood products. *Trends Food Sci Technol, 68*, 26-36.

Hilbig, J., Ma, Q., Davidson, P. M., Weiss, J., & Zhong, Q. (2016). Physical and antimicrobial properties of cinnamon bark oil co-nanoemulsified by lauric arginate and Tween 80. *Int J Food Microbiol, 233*, 52-59.

Huang, Z., Liu, X., Jia, S., & Luo, Y. (2017). Antimicrobial effects of cinnamon bark oil on microbial composition and quality of grass carp (*Ctenopharyngodon idellus*) fillets during chilled storage. *Food Control, 82*, 316-324.

Hyldgaard, M., Mygind, T., & Meyer, R. L. (2012). Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. *Front Microbiol, 3*, 12.

ICMSF. (1986). *Microorganisms in foods, 2: Sampling For Microbiological Analysis: Principles and Specific Applications*. Toronto, Canada: University of Toronto Press.

ICMSF. (1998). *Microorganisms in foods, 6 : Microbial ecology of food commodities*: Blackie Academic, Professional.

Inouye, S., Tsuruoka, T., Uchida, K., & Yamaguchi, H. (2001). Effect of sealing and Tween 80 on the antifungal susceptibility testing of essential oils. *Microbiol Immunol, 45*(3), 201-208.

J.S, S., Mishra, P., Thomas, J., Mukherjee, A., & Chandrasekaran, N. (2018). Antimicrobial potency of high-energy emulsified black pepper oil nanoemulsion against aquaculture pathogen. *Aquaculture, 491*, 210-220.

Jones, B. C., & Carton, A. G. (2015). Effects of dietary enrichment with alpha-tocopherol acetate and post-harvest filleting on lipid oxidation and flesh quality of tropical farmed barramundi (*Lates calcarifer*). *Aquaculture, 448*, 280-287.

Kabalnov, A., & Wennerström, H. (1996). Macroemulsion stability: The Oriented Wedge Theory Revisited. *Langmuir, 12*(2), 276-292.

Karthik, P., Ezhilarasi, P. N., & Anandharamakrishnan, C. (2017). Challenges associated in stability of food grade nanoemulsions. *Crit Rev Food Sci Nutr, 57*(7), 1435-1450.

Kashani, H. N., Moghaddam, F., & Mehramizi, A. (2012). HPLC - UV chromatography determination of benzaldehyde arising from benzyl alcohol used as preservative in injectable formulations. *Asian J Pharm Clin Res*, *5*(2), 98-100.

Komaiko, J. S., & McClements, D. J. (2016). Formation of food-grade nanoemulsions using low-energy preparation methods: a review of available methods. *Compr Rev Food Sci Food Saf, 15*(2), 331-352.

Landry, K. S., Micheli, S., McClements, D. J., & McLandsborough, L. (2015). Effectiveness of a spontaneous carvacrol nanoemulsion against *Salmonella enterica Enteritidis* and *Escherichia coli* O157:H7 on contaminated broccoli and radish seeds. *Food Microbiol, 51*, 10-17.

Lee, L., & Norton, I. T. (2013). Comparing droplet breakup for a high-pressure valve homogeniser and a microfluidizer for the potential production of food-grade nanoemulsions. *J Food Eng, 114*(2), 158-163.

Leong, T. S., Wooster, T. J., Kentish, S. E., & Ashokkumar, M. (2009). Minimising oil droplet size using ultrasonic emulsification. *Ultrason Sonochem, 16*(6), 721-727.

Li, J., Chang, J. W., Saenger, M., & Deering, A. (2017). Thymol nanoemulsions formed via spontaneous emulsification: physical and antimicrobial properties. *Food Chem, 232*, 191-197.

Li, W., Chen, H., He, Z., Han, C., Liu, S., & Li, Y. (2015). Influence of surfactant and oil composition on the stability and antibacterial activity of eugenol nanoemulsions. *LWT - Food Sci Technol, 62*(1), 39-47.

López-Montilla, J. C., Herrera-Morales, P. E., Pandey, S., & Shah, D. O. (2002). Spontaneous Emulsification: mechanisms, physicochemical aspects, modeling, and applications. *J Disper Science Technol, 23*(1-3), 219-268.

Lu, W. C., Huang, D. W., Wang, C. R., Yeh, C. H., Tsai, J. C., Huang, Y. T., & Li, P. H. (2018). Preparation, characterization, and antimicrobial activity of nanoemulsions incorporating citral essential oil. *J Food Drug Anal, 26*(1), 82-89.

Ma, Q., Davidson, P. M., & Zhong, Q. (2016). Antimicrobial properties of microemulsions formulated with essential oils, soybean oil, and Tween 80. *Int J Food Microbiol, 226*, 20-25.

Manthey-Karl, M., Lehmann, I., Ostermeyer, U., & Schroder, U. (2016). Natural chemical composition of commercial fish species: characterisation of pangasius, wild and farmed turbot and barramundi. *Foods, 5*(3), 1-14.

McClements, D. J. (1994). Ultrasonic determination of depletion flocculation in oil-in-water emulsions containing a non-ionic surfactant. *Colloids Surf A Physicochem Eng Asp, 90*(1), 25-35.

McClements, D. J. (2011). Edible nanoemulsions: fabrication, properties, and functional performance. *Soft Matter, 7*(6), 2297-2316.

McClements, D. J., & Rao, J. (2011). Food-grade nanoemulsions: formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Crit Rev Food Sci Nutr, 51*(4), 285-330.

Moghimi, R., Aliahmadi, A., McClements, D. J., & Rafati, H. (2016). Investigations of the effectiveness of nanoemulsions from sage oil as antibacterial agents on some food borne

pathogens. *LWT - Food Sci Technol, 71*, 69-76.

Nabavi, S. F., Di Lorenzo, A., Izadi, M., Sobarzo-Sanchez, E., Daglia, M., & Nabavi, S. M. (2015). Antibacterial effects of cinnamon: from farm to food, cosmetic and pharmaceutical industries. *Nutrients, 7*(9), 7729-7748.

National Bureau of Agricultural Commodity, Food Standards Ministry of Agriculture, & Cooperatives. (2015). Quick Frozen Fish Fillets. the Royal Gazette.

Oliver, J. D. (2005). The viable but nonculturable state in bacteria. *J Microbiol, 43*, 93-100.

Ozogul, Y., Yuvka, İ., Ucar, Y., Durmus, M., Kösker, A. R., Öz, M., & Ozogul, F. (2017). Evaluation of effects of nanoemulsion based on herb essential oils (rosemary, laurel, thyme and sage) on sensory, chemical and microbiological quality of rainbow trout(*Oncorhynchus mykiss*) fillets during ice storage. *LWT - Food Sci Technol, 75*, 677-684.

Pena, A. A., & Miller, C. A. (2006). Solubilization rates of oils in surfactant solutions and their relationship to mass transport in emulsions. *Adv Colloid Interface Sci, 123-126*, 241-257.

Qian, C., & McClements, D. J. (2011). Formation of nanoemulsions stabilized by model food-grade emulsifiers using high-pressure homogenization: factors affecting particle size. *Food Hydrocolloids, 25*(5), 1000-1008.

Rao, J., & McClements, D. J. (2010). Stabilization of phase inversion temperature nanoemulsions by surfactant displacement. *J Agric Food Chem, 58*(11), 7059-7066.

Ribeiro-Santos, R., Andrade, M., Madella, D., Martinazzo, A. P., de Aquino Garcia Moura, L., de Melo, N. R., & Sanches-Silva, A. (2017). Revisiting an ancient spice with medicinal purposes: cinnamon. *Trends Food Sci Technol, 62*, 154-169.

Roger, K., Cabane, B., & Olsson, U. (2010). Formation of 10−100 nm size-controlled emulsions through a sub-PIT cycle. *Langmuir, 26*(6), 3860-3867.

Roger, K., Cabane, B., & Olsson, U. (2011). Emulsification through surfactant hydration: the PIC process revisited. *Langmuir, 27*(2), 604-611.

Ryu, V., McClements, D. J., Corradini, M. G., & McLandsborough, L. (2018). Effect of ripening inhibitor type on formation, stability, and antimicrobial activity of thyme oil nanoemulsion. *Food Chem, 245*, 104-111.

Saberi, A. H., Fang, Y., & McClements, D. J. (2013). Fabrication of vitamin E-enriched nanoemulsions: factors affecting particle size using spontaneous emulsification. *J Colloid*

Interface Sci, 391, 95-102.

Salton, M. R. (1953). Studies of the bacterial cell wall. IV. The composition of the cell walls of some Gram-positive and Gram-negative bacteria. *Biochim Biophys Acta, 10*(4), 512-523.

Salvia-Trujillo, L., Rojas-Graü, A., Soliva-Fortuny, R., & Martín-Belloso, O. (2015). Physicochemical characterization and antimicrobial activity of food-grade emulsions and nanoemulsions incorporating essential oils. *Food Hydrocolloids, 43*, 547-556.

Shaaban, H. A., & Edris, A. E. (2015). Factors affecting the phase behavior and antimicrobial activity of carvacrol microemulsions. *J Oleo Sci, 64*(4), 393-404.

Sienkiewicz, M., Lysakowska, M., Denys, P., & Kowalczyk, E. (2012). The antimicrobial activity of thyme essential oil against multidrug resistant clinical bacterial strains. *Microb Drug Resist, 18*(2), 137-148.

Siripatrawan, U. (2016). Active food packaging from chitosan incorporated with plant polyphenols. *Novel Approaches of Nanotechnology in Food* (pp. 465-507). Elsevier.

Solans, C., & Solé, I. (2012). Nano-emulsions: formation by low-energy methods. *Curr Opin Colloid Interface Sci, 17*(5), 246-254.

Sudareva, N. N., & Chubarova, E. V. (2006). Time-dependent conversion of benzyl alcohol to benzaldehyde and benzoic acid in aqueous solutions. *J Pharm Biomed Anal, 41*(4), 1380-1385.

Sugumar, S., Ghosh, V., Mukherjee, A., & Chandrasekaran, N. (2016). Chapter 9 - Essential Oil-Based Nanoemulsion Formation by Low- and High-Energy Methods and Their Application in Food Preservation against Food Spoilage Microorganisms. In V. R. Preedy (Ed.), *Essential Oils in Food Preservation, Flavor and Safety* (pp. 93-100). San Diego: Academic Press.

Taylor, P. (1998). Ostwald ripening in emulsions. *Adv Colloid Interface Sci, 75*(2), 107-163.

Terjung, N., Loffler, M., Gibis, M., Hinrichs, J., & Weiss, J. (2012). Influence of droplet size on the efficacy of oil-in-water emulsions loaded with phenolic antimicrobials. *Food Funct, 3*(3), 290-301.

Tookwinas, S., Srichantulk, N., & Choongan, C. (1994). *Coastal aquaculture in Thailand.* Paper presented at the Proceedings of the SeminarWorkshop on Aquaculture Development in Southeast Asia and Prospects for Seafarming and Searanching, Tigbauan, Iloilo,

Philippines: SEAFDEC Aquaculture Department.

Turek, C., & Stintzing, F. C. (2013). Stability of essential oils: a review. *Compr Rev Food Sci Food Saf, 12*(1), 40-53.

Wang, X., Jiang, Y., Wang, Y.-W., Huang, M.-T., Ho, C.-T., & Huang, Q. (2008). Enhancing anti-inflammation activity of curcumin through O/W nanoemulsions. *Food Chem, 108*(2), 419-424.

Weerakkody, N. S., Caffin, N., Turner, M. S., & Dykes, G. A. (2010). *In vitro* antimicrobial activity of less-utilized spice and herb extracts against selected food-borne bacteria. *Food Control, 21*(10), 1408-1414.

Weiss, J., Canceliere, C., & McClements, D. J. (2000). Mass transport phenomena in oil-in-water emulsions containing surfactant micelles: Ostwald Ripening. *Langmuir, 16*(17), 6833-6838.

Xu, C., Ren, H., Wang, S., & Peng, X. (2004). Proteomic analysis of salt-sensitive outer membrane proteins of *Vibrio parahaemolyticus*. *Res Microbiol, 155*(10), 835-842.

Xue, J., Michael Davidson, P., & Zhong, Q. (2015). Antimicrobial activity of thyme oil co-nanoemulsified with sodium caseinate and lecithin. *Int J Food Microbiol, 210*, 1-8.

Yildirim, S. T., Oztop, M. H., & Soyer, Y. (2017). Cinnamon oil nanoemulsions by spontaneous emulsification: formulation, characterization and antimicrobial activity. *LWT - Food Sci Technol, 84*, 122-128.

Yuan, Y., Gao, Y., Zhao, J., & Mao, L. (2008). Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions. *Food Res Int, 41*(1), 61-68.

Zhang, Y., Liu, X., Wang, Y., Jiang, P., & Quek, S. (2016). Antibacterial activity and mechanism of cinnamon essential oil against *Escherichia coli* and *Staphylococcus aureus*. *Food Control, 59*, 282-289.

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