FOAM-MAT-FREEZE DRIED CLOVE ESSENTIAL OIL NANOEMULSION FOR INHIBITION OF SPOILAGE MICROORGANISMS IN WHITELEG SHRIMP *Litopenaeus vannamei*

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A Dissertation Submitted in Partial Fulfillment of the Requirements

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นาโนอิมัลชันน้ำมันหอมระเหยกานพลูที่ผ่านการทำแห้งแบบแช่เยือกแข็งในรูปโฟมแมทเพื่อการยับยั้งจุลินทรีย์ที่ทำให้เกิดการเน่าเสียในกุ้งขาว *Litopenaeus vannamei*

นายปุณชรัชต์ ปิลอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	FOAM-MAT-FREEZE DRIED CLOVE ESSENTIAL OIL NANOEMULSION FOR INHIBITION OF SPOILAGE MICROORGANISMS IN WHITELEG SHRIMP *Litopenaeus vannamei*
By	Mr. Puncharat Pilong
Field of Study	Biotechnology
Thesis Advisor	Professor UBONRATANA SIRIPATRAWAN, Ph.D.

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ปุณชรัชต์ ปิลอง : นาโนอิมัลชันน้ำมันหอมระเหยกานพลูที่ผ่านการทำแห้งแบบแช่เยือกแข็งในรูปโฟมแมทเพื่อการยับยั้งจุลินทรีย์ที่ทำให้เกิดการเน่าเสียในกุ้งขาว *Litopenaeus vannamei*. (FOAM-MAT-FREEZE DRIED CLOVE ESSENTIAL OIL NANOEMULSION FOR INHIBITION OF SPOILAGE MICROORGANISMS IN WHITELEG SHRIMP *Litopenaeus vannamei*) อ.ที่ปรึกษาหลัก : ศ. ดร. อุบลรัตน์ สิริภัทราวรรณ

งานวิจัยนี้พัฒนา FFD-CN เพื่อใช้ยับยั้งการเจริญเติบโตของจุลินทรีย์ที่ทำให้อาหารเน่าเสีย โดยแบ่งงานวิจัยออกเป็นสามขั้นตอน ขั้นตอนแรกเป็นการหาสภาวะที่เหมาะสมในการผลิต CN โดยวัดขนาดอนุภาค, ดัชนีการกระจายตัว, ค่าความต่างศักย์, และความหนืด พบว่า การใช้น้ำมันกานพลู 1 เปอร์เซ็นต์, ทวีนความเข้มข้น 3 เปอร์เซ็นต์ และความดันที่ใช้งาน 10,000 ปอนด์ต่อตารางนิ้ว เป็นสูตรที่เหมาะสมที่สุด โดยให้นาโนอิมัลชันที่มีขนาดอนุภาค 30.76 นาโนเมตร, ดัชนีการกระจายตัว 0.179, ค่าความต่างศักย์ -50.01 มิลลิโวลต์, และความหนืด 7.81 เซนติพอยส์ และ CN มีความเสถียรต่อการรวมตัวของอนุภาคและการแยกเฟสตลอดการเก็บรักษา CN มีฤทธิ์ต้านจุลชีพต่อเชื้อ *S. aureus, E. coli และ P. aeruginosa* ดีกว่าน้ำมันกานพลู ขั้นตอนต่อมาเป็นการพัฒนาเทคนิคการทำแห้งแบบเยือกแข็งในรูปโฟมแมทเพื่อรักษาความเสถียรและฤทธิ์ต้านจุลินทรีย์ของ CN พบว่า สภาวะที่เหมาะสมที่สุด คือ เมทโทเซล 3 เปอร์เซ็นต์, อุณหภูมิในการทำแห้ง 60 องศาเซลเซียส และเวลาในการทำแห้ง 72 ชั่วโมง FFD-CN มีขนาดอนุภาค 26.14 นาโนเมตร, ดัชนีการกระจายตัว 0.193, ค่าแอคติวิตีของน้ำ 0.273, ความสามารถในการละลาย 88.71 เปอร์เซ็นต์, และความหนืด 12.85 เซนติพอยส FFD-CN มีความคงตัวที่ดีและความสามารถในการต้านจุลินทรีย์ซึ่งวิเคราะห์โดยใช้ความเข้มข้นต่ำสุดในการยับยั้งของเชื้อ *E. coli*, *P. aeruginosa* และ *S. aureus* ได้มากกว่า CN ขั้นตอนสุดท้ายเป็นการศึกษาประสิทธิภาพของ FFD-CN ในการรักษาคุณภาพของกุ้งขาวในระหว่างการเก็บรักษาแบบแช่เย็นที่อุณหภูมิ 4 ± 2 องศาเซลเซียส เมื่อเปรียบเทียบกับ CN และน้ำกลั่น (กลุ่มควบคุม) พบว่า FFD-CN มีประสิทธิภาพมากกว่า CN ในการยับยั้งการเจริญเติบโตของจุลินทรีย์ในกุ้งขาว การชะลอการเปลี่ยนแปลงของสารระเหยไตรเมทิลลามีน, สารประกอบไนโตรเจนที่ระเหยได้ทั้งหมด, เนื้อสัมผัส, สี, และ ค่าความเป็นกรดด่าง การวิเคราะห์สารประกอบที่ระเหยได้ของตัวอย่างทั้งหมดแสดงให้เห็นว่า FFD-CN สามารถรักษาความเข้มข้นของสารยูจีนอลได้มากกว่า CN และน้ำมันกานพลู และพบว่า กุ้งกลุ่มควบคุม และกุ้งที่ผ่านการแช่ด้วย CN และ FFD-CN มีอายุการเก็บรักษา 6, 8, และ 10 วัน ตามลำดับ สรุปได้ว่าการทำ CN จากของเหลวให้อยู่ในรูปของแข็งที่ผ่านการทำแห้งแบบแช่เยือกแข็งในรูปโฟมแมทสามารถรักษาคุณภาพและยืดอายุการเก็บของกุ้งขาวได้อย่างมีประสิทธิภาพ

สาขาวิชา	เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต .....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORD: Clove essential oil nanoemulsion/ Foam/ Freeze-drying/ Antimicrobial activity/ Bacterial morphology

Puncharat Pilong : FOAM-MAT-FREEZE DRIED CLOVE ESSENTIAL OIL NANOEMULSION FOR INHIBITION OF SPOILAGE MICROORGANISMS IN WHITELEG SHRIMP *Litopenaeus vannamei* . Advisor: Prof. UBONRATANA SIRIPATRAWAN, Ph.D.

This research developed foam-mat freeze-dried clove essential oil nanoemulsion (FFD-CN) for inhibiting food spoilage microorganisms. This research comprised three parts. Firstly, the fabrication of clove essential oil nanoemulsion (CN) was optimized. The characteristics including particle size, polydispersity index (PDI), Zeta-potential, and viscosity of the fabricated nanoemulsions were observed. The optimal CN having a droplet size of 30.76 nm, PDI of 0.179, Zeta-potential of -50.01 mV, and viscosity of 7.81 cP was achieved at 1% v/v clove oil and 3% v/v Tween® 80 concentration and 10,000 psi operating pressure. The CN exhibited stability against particle aggregation and phase separation throughout storage. Moreover, the CN exhibited better antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* when compared to clove oil. Secondary, the foam-mat freeze-drying (FFD) technique was used to maintain stability and antimicrobial activity of CN. The optimal condition was found at 3% (v/v) of foaming agent (Methocel^{TM}), 60 °C drying temperature, and 72 h drying time. The obtained FFD-CN had the droplet size of 26.14 nm, PDI of 0.193, water activity (a_w) of 0.273, solubility of 88.71%, and viscosity of 12.85 cP. The FFD-CN exhibited good stability and maintained antimicrobial activity, as analyzed using minimum inhibitory concentration (MIC), against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* better than CN. Lastly, the FFD-CN was investigated for its efficiency to maintain the quality of whiteleg shrimp during chilled storage (4 ± 2 °C) in comparison to CN and distilled water (control). The CN and FFD-CN were found to be effective in inhibiting the growth of bacteria in whiteleg shrimp. FFD-CN was more effective than CN in delaying the changes in trimethylamine (TMA), total volatile basic nitrogen (TVB-N), total viable count (TVC), texture, color, and pH. The analysis of volatile compounds of all samples showed that FFD-CN could maintain a higher concentration of eugenol than CN and clove oil. The results also suggested that Control, CN, and FFD-CN treated shrimp had the shelf life of 6, 8, and 10 days, respectively. The FFD-CN can effectively be used as a natural preservative for maintaining the quality and shelf life of whiteleg shrimp.

Field of Study:	Biotechnology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....

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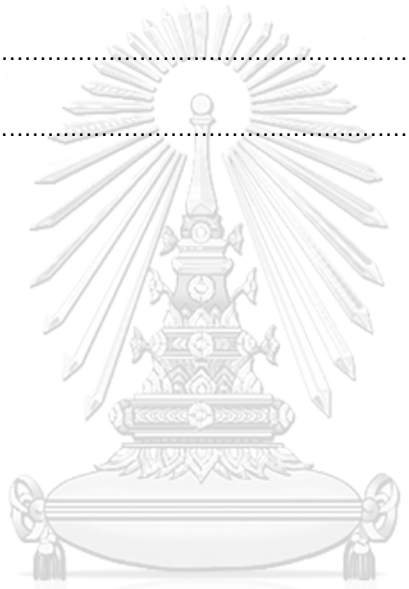

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LIST OF ABBREVIATIONS

RSM	Response surface methodology
CCD	Central composite design
BBD	Box Behnken design
CRD	Completely randomized design
CFU	Colony forming unit
a_w	Water activity
SEM-EDS	Scanning electron microscope and energy dispersive x-ray spectrometer
GC-MS	Gas chromatography- mass spectrometry
SPME	Solid phase microextraction
NIST	National Institute of Standards and Technology library data
TISTR	Thailand Institute of Scientific and Technological Research
MIC	The minimum inhibitory concentration
FFD	Foam-mat freeze-drying
CN	Clove essential oil nanaemulsion
FFD-CN	Foam-mat freeze-dried clove essential oil nanaemulsion
USFDA	United States Food and Drug Administration
GRAS	Generally Recognized as Safe
pH	Potential of hydrogen ion
TVC	Total viable count
TMA	Trimethylamine
TVB-N	Total volatile basic nitrogen
L*	Lightness
a*	Redness
b*	Yellowness

CHAPTER I

INTRODUCTION

1.1 Background, motivation and linking of the study

Clove (*Syzygium aromaticum*) is one of the most valuable spices that have been used for centuries as flavoring, antioxidant, and antimicrobial agents on various food products (Zahi, Liang, &Yuan, 2015). The major components of clove oil are eugenol and cinnamaldehyde. Eugenol is a phenolic compound that can exhibit excellent antimicrobial activity by changing protein properties and inhibiting the protease activity of spoilage bacteria (Cortes-Rojas et al., 2014; Xu-GJ et al., 2016). Whereas, an aldehyde group in cinnamaldehyde is capable of interfering with bacterial DNA and protein functions (Cortes-Rojas et al., 2014). Clove oil is recognized as an effective antimicrobial agent against a wide range of microorganisms by causing leakage of small cellular electrolytes, affecting membrane permeability, and resulting in a decrease of bacterial metabolism (Xu-GJ et al., 2016). However, it is hydrophobic and has a potent odor, which can affect food's organoleptic characteristics. Therefore, clove essential oil cannot be used directly in foods (Chuesiang et al., 2018).

Nanoemulsion consists of two phases, oil (dispersed phase) and water (continuous phase), which one is small droplets in the other phase. Due to the size of droplets (20-200 nm), nanoemulsion exhibited a number of advantages over conventional emulsion in terms of transparency and high stability against particle aggregation (Chuesiang et al., 2018). Antimicrobial activity of the nanoemulsion is greatly increased due to an increase in the surface area/volume ratio of encapsulated bioactive compounds, leading to the better fusion between nanoemulsion and bacterial cell wall, and thus, causing the disruption of bacterial cells (Zahi et al., 2015). Nanoemulsions can be produced by using mechanical devices such as high-pressure valve homogenizers, microfluidizers, and ultrasonicators to generate the disruptive force which can break up the oil and water and lead to the formation of tiny oil droplets with a narrow size distribution (Khandare et al., 1994). Among those methods, the microfluidizer which uses a very high shearing action to form fine emulsions, has been reported as an effective way to form a nano-size of a droplet

(Ronkart et al., 2010; Tang et al., 2013). Many studies have shown that emulsification by microfluidizers provides stable nanoemulsions with smaller particle sizes and narrower sizes distributions at lower surfactant levels when compared to those emulsified by traditional homogenization (Liu et al., 2009). However, the nanoemulsions are kinetically unstable, and the size of oil droplets can be changed at a slow rate during the storage period.

Therefore, the nanoemulsion system to encapsulate clove essential oils in order to minimize those effects before using inhibiting microbials in food was studied. The characteristics and antimicrobial activity of microfluidized clove essential oil nanoemulsion are presented in Chapter 2, which is published in *Journal of Food Processing and Preservation.*

To maintain stability and antimicrobial activity in food, in addition to the clove essential oil nanoemulsion (CN) by microfluidizer method, another main objective of maintaining stability and antimicrobial activity in food is foam-mat freeze-drying (FFD) method for potential development in terms of active packaging to focus on maintaining the quality of products. Currently, the FFD method is mostly demanded to maintain active compounds and antimicrobial activity, and it continues to be an area of interest for researchers in the fields of food packaging and food microbiology. Therefore, the FFD made from methocel™ incorporated with clove essential oil nanoemulsion was developed.

The FFD consists of two phases, air (dispersed phase) and liquid (continuous phase), of which the dispersed phase is larger than the continuous phase (Ruengdech & Siripatrawan, 2022). The principle of FFD is to transform liquid products into stable foam by whipping the product mixture, followed by freeze-drying (Muthukumaran et al., 2008). The freeze-drying process gives many advantages by reducing oxidation, preserving flavor and volatiles, increasing solubility, extending shelf life, and less weight of the products. The FFD can be returned to the nanoemulsion system again after dissolving in water (Thuwapanichayanan et al., 2008). The qualities of the foam have been observed to be affected by factors such as foaming agent type and concentration, drying time, and drying temperature. FFD has

been reported to improve the storage stability and solubility of essential oil emulsions (Mahdi et al., 2021; Ruengdech & Siripatrawan, 2022).

In this work, CN as a natural antimicrobial activity was incorporated into FFD, and the effect of the FFD approach on preserving the stability and antimicrobial activity of CN was studied. The study of this part of the research is described in Chapter 3, which is published in *Journal Food Bioscience.*

Although the antimicrobial activity of CN has been studied, no research has been reported on the properties of foam-mat freeze-dried clove essential oil nanoemulsion (FFD-CN) on the quality of whiteleg shrimp. In the present study, whiteleg shrimp was used as a food model to investigate the effectiveness of CN and FFD-CN on the inhibition of food spoilage bacteria. A nanoemulsion of clove essential oil is expected to enhance the antimicrobial properties of clove essential oil without any alterations to its properties or the organoleptic properties of whiteleg shrimp.

The whiteleg shrimp (*Litopenaeus vannamei*) is an important economic animal, which is highly demanded as food for people in Thailand and many countries (Imaizumi et al., 2022; Zhang et al., 2015). However, whiteleg shrimp have a short shelf life and spoiled during the process of post-harvesting, storing, and transportation. The quality deterioration and safety loss of whiteleg shrimp can be issued mainly by the growth of food spoiled bacteria (Masri et al., 2021; Zhang et al., 2015), which are found in the muscle, intestinal, and digestive systems of shrimp. For instance, H_2S from bacteria cause spoilage in shrimp (Liu et al., 2020). Chemical treatment, pasteurization, and high-hydrostatic pressure (Li et al., 2015; Shaikhmahamud et al., 2022; Wang et al., 2018) have also been reported to inhibit food spoilage in shrimp. In addition, using high pressure to inhibit food spoilage bacteria may increase melanosis (Montero et al., 2001). However, due to the negative impacts on the nutritive values and sensory attributes of shrimp, those methods are unacceptable to most consumers (Bindu et al., 2013; Shaikhmahamud et al., 2022).

Finally, improving the properties of foam-mat freeze-dried clove essential oil nanoemulsion on the quality of whiteleg shrimp was also investigated. This research is presented in Chapter 4.

1.2 Objectives of research

1. To study the optimal condition for the fabrication of clove essential oil nanoemulsion.
2. To study the optimal condition for foam-mat freeze-dried clove essential oil nanoemulsion.
3. To study the efficiency of clove essential oil nanoemulsion for inhibition of spoilage bacteria in whiteleg shrimp.

1.3 Scope of the study

This research contains three main parts.

1. The condition of clove essential oil was prepared at 1% (v/v). The nanoemulsion was studied at varying Tween® 80 concentrations (1%, 2%, and 3% v/v) and operating pressures (5,000 psi, 10,000 psi, and 15,000 psi). The characteristics of clove essential oil nanoemulsions including mean droplet diameter, particle size distribution curve, PDI, ζ-potential, viscosity, composition, stability, and antimicrobial activity were determined.

2. The condition of foam-mat freeze-dried clove essential oil nanoemulsion was prepared from 1% (v/v) clove essential oil nanoemulsion, and optimized by varying foaming agent concentrations (1%, 2%, and 3% v/v), freeze-drying temperatures (25 °C, 40 °C, and 60 °C), and drying times (24 h, 48 h, and 72 h). The characteristics of foam-mat freeze-dried clove essential oil nanoemulsion including particle size distribution curve, PDI, a_w, solubility, viscosity, composition, stability, and antimicrobial activity were determined.

3. The effects of foam-mat freeze-dried clove essential oil nanoemulsion maintain the quality on whiteleg shrimp was prepared at 1% (v/v) of clove essential oil nanoemulsion and Methocel^{TM} at 3% (v/v) at drying temperatures of 60 °C for 72 h. The samples were examined for physical, chemical, and microbiological changes on days 0, 2, 4, 6, 8, and 10 of the storage periods.

1.4 Benefits of this study

The success of this research can lead to the potential development of foam-mat freeze-dried clove essential oil nanoemulsion which can effectively preserve stability and antimicrobial activity. The developed foam can be applied as food packaging to maintain the quality of whiteleg shrimp.

CHAPTER II
MANUSCRIPT I

Characteristics and antimicrobial activity of microfludized clove essential oil nanoemulsion optimized using response surface methodology

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Abstract

Clove essential oil nanoemulsion with optimal antimicrobial activity and stability was formulated and analyzed using response surface methodology with central composite design (CCD). The optimal clove oil nanoemulsion was obtained from microfluidizer (10,000 psi of operating pressure) of 1% (v/v) clove oil, and 3% (v/v) of Tween® 80 (surfactant), with a droplet size of 30.76 nm, 0.179 polydispersity index (PDI), -50.01 mV Zeta-potential, and 7.81 cP viscosity. It also exhibited good stability against particle aggregation and phase separation during 48 h of storage at 5 ^{o}C. Clove essential oil nanoemulsion had higher amount of eugenol, but lower amount of benzyl alcohol and caryophyllene than those contained in bulk oil. The clove oil nanoemulsion exhibited better antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, when compared to clove oil. Bacterial cell morphological destruction caused by clove oil nanoemulsion or clove oil was also observed using a scanning electron microscope and energy dispersive x-ray spectrometer (SEM-EDS). The results suggested that fabrication of clove essential oil into nanoemulsion improved its antimicrobial activity and has the potential to be used as a natural preservative for food applications.

Keywords: Clove essential oil nanoemulsion, Microfluidics, Antimicrobial, Cell morphology, SEM-EDS.

Novelty impact statement

The experiments in this research were designed to determine the feasibility of using the clove oil nanoemulsion as antimicrobial agent directly in food and its stability if used under different food processing conditions, storages or usages. Our study is, however, not only the first to optimize the important factors required to formulate antimicrobial clove essential oil nanoemulsion using microfluidization but also the first to report the chemical stability of clove essential oil after nanoemulsion formation and storage. In addition, the bacterial cell morphology was visualized using a scanning electron microscope and energy dispersive x-ray spectrometer (SEM-EDS) to verify the antimicrobial efficiency of clove essential oil nanoemulsion, clove

essential oil, and kanamycin sulfate (as control), which has never been reported in the literature.

2.1 Introduction

Plant essential oils are secondary metabolites of plants that contain various non-volatile and volatile compounds. Clove (*Syzygium aromaticum*) essential oil is a highly prized spice that has long been used as a food preservative in a variety of foods due to its antibacterial and antioxidant properties (Cortes-Rojas *et al.,* 2014). The antimicrobial mechanisms of clove essential oil are attributed to its ability to increase membrane permeability which leads to the leakage of small cellular electrolytes and results in a decrease in bacterial metabolism (Xu-GJ *et al.*, 2016). The antioxidant activity of clove essential oil has also been reported by several studies (Chaieb *et al.,* 2007; Chen *et al.*, 2016; Gulcin *et al.*, 2012), due to the ability of eugenol to reduce oxygen concentration and catalytic metal ions, thereby, stop the lipid oxidation at the initial or propagation step of oxidations (Gulcin *et al.,* 2012). Even though clove essential oil is recognized as an effective antimicrobial and antioxidant agent, some limitations of using clove essential oil directly into food have been reported such as its hydrophobicity and strong odor which may change the original appearance and odor of food (Donsi & Ferrari, 2016). Therefore, a delivery system such as a nanoemulsion can be considered as an alternative way to encapsulate clove essential oil in order to minimize those effects before using in food products.

Nanoemulsion consists of two-phases, oil (dispersed phase) and water (continuous phase), where one phase exists as small droplets in the other phase. The nanoscale size of oil droplets (< 200 nm) in nanoemulsion exhibits several advantages, including transparency, high bioactivity, and high stability against particle aggregation (Chuesiang *et al.*, 2018) . Antimicrobial activity of the nanoemulsion is greatly increased due to an increase in the surface area /volume ratio of encapsulated bioactive compounds, leading to a better fusion between nanoemulsion and bacterial cell wall, and consequently, disrupting the bacterial cells (Zahi, Liang, &Yuan, 2015). Surfactants are generally added to the oil-water mixture to improve the kinetic stability of such a system. A surfactant is an amphiphilic

molecule with a hydrophilic head group (polar area) and a lipophilic tail group (nonpolar region) with a high affinity for oil (Anton & Vandamme, 2011). Typically, Tween® 80 is often employed as surfactant for nanoemulsion systems due to its high degree of compatibility with other components and low toxicity (Shahavi *et al.*, 2019). Nanoemulsions can be produced by using mechanical devices such as ultrasonicators, homogenizers, microfluidizers, or hydrodynamic cavitation and microchannel. These instruments generate a disruptive force that breaks up water and oil into small droplets (Cao *et al.*, 2020; Ronkart *et al.*, 2010; Tang & Sivakumar, 2013). Among these devices, microfluidizer has been reported as an effective way to form nanoemulsion because the increasing operating pressure of the microfluidizer generates high shear force causing the decrease of emulsion droplet size (Ronkart *et al.*, 2010; Tang *et al.*, 2013). Many studies have shown that emulsification by microfluidizer provided stable nanoemulsions with smaller oil droplet size and narrower size distribution at lower surfactant levels when compared to those emulsified by traditional homogenization (Tang *et al.*, 2013). Although the microfluidizer is capable of reducing the droplet size within a short time, it may not provide stable nanoemulsions without the condition optimization (Raviadaran *et al*., 2018). Therefore, the purpose of this current study was first to optimize the nanoemulsion fabrication parameters including surfactant (Tween® 80) concentration and operating pressure of the microfluidizer for fabrication of stable clove essential oil nanoemulsion using response surface methodology (RSM) with central composite design. The characteristics including mean droplet diameter, particle size distribution, polydispersity index (PDI), ζ-potential, and viscosity of the clove essential oil nanoemulsion were determined. Moreover, the antimicrobial activity of the optimized nanoemulsion was then studied against *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*).

2.2 Materials and methods

2.2.1 Materials

Pure clove essential oil batch No. 0009115315 and Tween® 80 were purchased from TTK Science Co., Ltd. (Laksi, Bangkok, Thailand). *Staphylococcus aureus* (TISTR 746), *Escherichia coli* (TISTR 527), and *Pseudomonas aeruginosa*

(TISTR 2370) were obtained from the Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani, Thailand.

2.2.2. Preparation of clove essential oil nanoemulsions

Clove essential oil nanoemulsions were prepared using 1% (v/v) clove essential oil (disperse phase) , Tween® 80 (surfactant), and deionized water (continuous phase) . The fabrication parameters affecting the characteristics of the nanoemulsion were studied by varying Tween® 80 concentration (1%, 2%, and 3% v/v) and operating pressure (5,000 psi, 10,000 psi, and 15,000 psi), following the method of Khan *et al*. (2014). Clove essential oil nanoemulsion was firstly prepared by mixing clove essential oil, Tween® 80, and deionized water using a magnetic stirrer (Ika RCT basic, Guangzhou machine tool works Ltd, Guangdong, Guangdong, China) set at 400 rpm for 3 min (Ghosh *et al*., 2013). The mixture was then subject to the microfluidizer (Microfluidics LM 20 high pressure homogenizer, Microfluidics Instrument Ltd, Westwood, MA, USA) set at 20 kHz. After fabrication, the characteristics of clove essential oil nanoemulsions including mean droplet diameter, particle size distribution curve, PDI, ζ-potential, viscosity, composition, stability, and antimicrobial activity were determined.

2.2.3 Optimal condition for nanoemulsion fabrication using CCD-RSM

In this research, RSM was used as a statistical method which uses quantitative data from the experiments to determine regression model and to optimize response (output) variables (i.e. particle size, PDI, ζ-Potential, and viscosity) which were affected by independent (input) variables (i.e. surfactant concentration and operating pressure of microfluidizer). The parameters used for fabrication of clove essential oil nanoemulsion were analyzed by a response surface methodology design called central composite design (CCD). The CCD-RSM which can be used for fitting a quadratic surface was used to optimize the nanoemulsion process parameters (e.g. surfactant concentration and operating pressure of microfluidizer) with a minimum number of experiments and to observe the interaction between these process parameters. Using the CCD-RSM, the optimization involved three steps, including

statistical design of experiments, estimating the coefficients in a mathematical model, and predicting the response and evaluating the efficacy of the model.

In this study, a 3^2 factorial design was implemented in order to evaluate the optimal formulation and process parameters for clove essential oil nanoemulsion. The independent variables used for this study were surfactant (Tween® 80) concentration (X_1) and operating pressure of microfluidizer (X_2) of which each variable was investigated at 3 levels. The dependent variables were droplet size (Y_1), PDI (Y_2), ζ-Potential (Y_3), and viscosity (Y_4) of the clove essential oil nanoemulsions. The units and the coded levels of the independent variables are shown in **Table 2.1**. **Table 2.2** displays the central composite design of different microfluidizer conditions. The experimental data were mean of triplicate determination.

The CCD-RSM **(Equation 1)** was performed to investigate the effect of independent variables and their interactions on the responses. A second-order polynomial equation was applied to indicate the responses as a function of the independent variables.

$$Y = c_0 + c_1X_1 + c_2X_2 + c_{12}X_1X_2 + c_{11}X_1^2 + c_{22}X_2^2 \quad (1)$$

where Y is the measured response associated with each factor level combination, c_0 is the constant, c_1 and c_2 are the linear coefficients, c_{11} and c_{22} are the quadratic coefficients of observed experimental values, c_{12} is the interaction coefficients among two factors, and X_1 and X_2 are the mentioned levels of independent variables. The coefficients of the second-order polynomial model were determined by multiple regression analysis for the values of responses obtained from the experiments. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and p-value. A set of 13 verification runs at the optimal conditions were then conducted to confirm that mean droplet diameter, PDI, ζ-potential, and viscosity of the nanoemulsion were met by the set of fabrication settings (Pongsumpun *et al.*, 2020).

TABLE 2.1 Coded variables with central composite design for optimization of clove essential oil nanoemulsions.

Independent variables	Symbol	Coded variables		
		-1	0	1
Tween® 80 concentration (%v/v)	X_1	1	2	3
Operating pressure (psi)	X_2	5,000	10,000	15,000

TABLE 2.2 The central composite design of different microfluidizer conditions.

Run	Tween® 80 concentration (X_1)	Operating pressure (X_2)
1	2	2,928.93
2	3.41	10,000
3	3	15,000
4	2	10,000
5	1	5,000
6	1	15,000
7	0.59	10,000
8	2	10,000
9	2	10,000
10	2	10,000
11	3	5,000
12	2	10,000
13	2	17,071.07

2.2.4 Measurement of particle size, PDI, ζ-potential, and viscosity

The mean droplet diameter, particle size distribution curve, and PDI of clove essential oil nanoemulsions were measured by dynamic light scattering (DLS) (Zetasizer Nano ZS90, Malvern Instruments Ltd, Malvern, Worcestershire, UK) comprising a helium-neon gas laser (λ=633 nm) for a detector angle of 173° at 25 °C. Before use, each sample was diluted with 1:100 ratios of deionized water to prevent multiple scattering. All measurements were conducted in triplicate.

The assessment of surface electrical charge between the oil droplets and the continuous phase of clove essential oil nanoemulsion was reported in terms of the ζ-potential. Briefly, each sample was firstly diluted with 1:100 ratios of deionized water to prevent multiple scattering. Phase analysis light scattering (PALS) was then carried out-using the Zetasizer Nano ZS90 (Bhargava *et al*., 2015).

The viscosity of clove essential oil nanoemulsion was investigated using Brookfield digital viscosity meter (Brookfield Laboratories Inc, Middleboro, MA, USA). Firstly, the nanoemulsion (250 mL) was poured into a 500 mL beaker mug prior to insert the spindle (s85) at 100 rpm down into the solution. Clove essential oil nanoemulsion was sheared alternating with an up and down in the ramp at 25 °C. The result of viscosity was reported as centipoise (cP) (Chen *et al*., 2016).

2.2.5 Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The composition of clove essential oil nanoemulsion, clove essential oil nanoemulsion after storage (5 °C for 48 h), and clove essential oil were analyzed using gas chromatography-mass spectrometer (GC-MS; Agilent 7890B, Agilent Technologies Inc, Santa Clara, CA, USA) interfaced the mass spectrometry detector (Agilent 7000C, Agilent Technologies Inc, Santa Clara, CA, USA), and headspace auto-sampler (Agilent Technologies Inc., Santa Clara, CA, USA). According to the method described by Zhang *et al.* (2016) with minor modifications, the samples (2 mL) of clove essential oil nanoemulsion and clove essential oil were added into a 20 mL headspace vial (Chromselection, Vicenza, Veneto, Italy), which was then tightly closed with an aluminum crimp cap attached with a polytetrafluoroethylene (PTFE) silicone septum (Chromselection, Agilent Technologies Inc., Santa Clara, CA, USA) using a hand crimper (Model 20001, Kebby Industries Inc., Rockford, IL, USA). After heating the samples with low shaker mode at an equilibrium temperature of 130 °C for 40 min, the gas phase was injected into the GC-MS for analysis. The operating conditions were used as follows: separating column: a HP-5ms capillary column (30 m × 0.25 mm, and film thickness 0.25 μm); carrier gas: helium (flow rate: 0.8 mL/min); split ratio 100:1; injector and detector temperatures: 250 °C; and sample size: 0.5 μL. The MS analysis parameters were energy ionization Ion source with 70 eV of electron energy and temperatures 230 °C, solvent delay of 2 min, and m/z 33-

500 amu. The chemical constituents of clove essential oil nanoemulsion and clove essential oil were identified by the NIST2011 library data.

2.2.6 Stability testing

The stability of clove essential oil nanoemulsion was performed following the method of Ghosh *et al*. (2013) by centrifuging the nanoemulsions set to 10,000 rpm for 30 min. After 6 cycles of centrifugation, the samples were then stored in an incubator at 5 ℃ or 45 ℃ for 48 h. Characteristic properties including size, PDI, Zeta potential, and viscosity were then observed to determine the stability of clove essential oil nanoemulsions. Stability measurements were carried out in triplicate.

2.2.7 Antimicrobial activity

The antimicrobial activity of clove essential oil nanoemulsion was tested against Gram-positive bacteria, *S. aureus*, and Gram-negative bacteria *E. coli* and *P. aeruginosa*, according to the method of Zahi, Liang, & Yuan, (2015) with some modificaions. The bacterial culture was stored at 4 ℃ on the nutrient agar slant. Each bacterial culture was prepared by transferring a ring loop of cells from the agar slant to a test tube containing 20 mL of nutrient broth and incubated at 37 ℃ for 24 h. A single colony of cells was then prepared by streaking a culture onto the nutrient agar. After incubation at 37 ℃ for 24 h, a colony was then transferred with a ring loop from nutrient agar to the nutrient broth and incubated again at 37 ℃ for 24 h. The initial turbidity of each culture was then adjusted to 0.1 using a UV spectrophotometer at 600 nm to obtain the bacteria at the concentration of 8 log CFU/mL. The minimum inhibitory concentration (MIC) of clove essential oil nanoemulsion and clove essential oil was then determined using broth dilution method. Briefly, 6 mL of clove essential oil nanoemulsion (1% clove essential oil in the nanoemulsion) or clove essential oil were added to the first tube containing 6 mL of nutrient broth. A serial of two-fold dilution was then prepared in the tubes containing the same volume of nutrient broth. The 6 mL of the first tube of each sample was added as a serial dilution to each prepared tube. After that, 1 mL of each aliquot culture was added to the prepared clove essential oil nanoemulsion or clove essential oil in each tube and mixed by vortexing. The concentration of clove essential oil in nanoemulsions was ranging

from 248 mg/mL to 1.93 mg/mL. Whereas the concentration of clove essential oil was ranging from 2,709.68 mg/mL to 21.16 mg/mL. MIC was determined as the lowest concentration of clove essential oil nanoemulsions or clove essential oil that inhibits the growth of bacteria. The standard plate count method, which involves the spreading of sample-bacteria mixture (0.1 mL) from each prepared tubes onto the nutrient agar plate prior to incubate at 37 ^{o}C for 24 h was then carried out to enumerate the bacteria. The concentration of clove essential oil nanoemulsions or clove essential oil that showed no growth of bacteria on the plate was selected as the MIC value. In addition, the bacterial cell morphology was visualized using a scanning electron microscope and energy dispersive x-ray spectrometer (SEM-EDS; JSM-IT500HR, JED-2300, Akishima, Tokyo, Japan) to verify the antimicrobial efficiency of clove essential oil nanoemulsion, clove essential oil, and kanamycin sulfate (positive control). In the present study, Kanamycin sulfate (50µg/mL) was used as a positive control and was prepared in the same manner as those of clove essential oil nanoemulsions and clove essential oil. The experiments were carried out in triplicate.

2.2.8. Statistical analysis

The experimental results from triplicate measurements were expressed as mean values and standard deviation (mean $\pm$ SD). The SPSS Software (version 26.0, IBM incorporation, Armonk, NY, USA) was used to perform a one-way analysis of variance (ANOVA). The significance of the difference between means was ascertained using Duncan's new multiple range test when $p \leq 0.05$. A stepwise procedure was employed to generate the three-dimensional surface plots for examining the relationship between a response and two independent variables using Design-expert Statistics Software (Version 7.0, Stat Soft Inc., Minneapolis, MN, USA).

2.3 Results and discussion

2.3.1 Characteristic of nanoemulsions

The effect of surfactant concentration (1%, 2%, and 3% v/v) and operating pressures (5000 psi, 10000 psi, and 15000 psi) on the properties including mean

droplet diameter, PDI, ζ-Potential, and viscosity of the microfluidized clove essential oil nanoemulsions was studied.

2.3.1.1. Particle size and PDI

The particle size and PDI of the microfluidized clove essential oil nanoemulsions were determined. The results showed that the mean droplet diameter of the nanoemulsions decreased with an increase of surfactant concentration. The transparent nanoemulsion was observed with the smallest mean droplet diameter of 30.76 ± 0.46 nm and PDI of 0.179 ± 0.03 when 3% (v/v) surfactant was used **(Figures 2.1 A-B).** This effect may have occurred because Tween®80 can decrease an interfacial tension between oil and water, hence, decreases the free energy for nanoemulsion formation and promotes the formation of small droplets with low PDI and narrow size distribution (Koocheki & Kadkhodaee, 2011). As previously reported by several studies, an increase in the concentration of surfactant affects the formation of smaller oil droplets (Pongsumpun, Iwamoto, & Siripatrawan, 2020; Saberi, Fang, & McClements, 2013; Yuan *et al*., 2008). The operating pressure was also found to have an impact on the mean droplet diameter and PDI of the nanoemulsions. While too low (5,000 psi) and too high (15,000 psi) operating pressure caused an increase in the size and PDI, the smallest mean droplet diameter and PDI were found when the optimal operating pressure (10,000 psi) was used. In general, using higher operating pressure has been reported to decrease the size of emulsion droplet due to the strong generated disruptive force (Raviadaran *et al*., 2018). However, the increase of droplet size with increasing operating pressure could occur due to the collision and coalescence of droplets during nanoemulsion formation as a result of high Brownian movement and slow surfactant adsorption (Jafari, He, & Bhandari, 2007). As described by Jafari, He, & Bhandari, (2007), droplet coalescence occurs when the incomplete surfactant-covered droplets collide. This phenomenon is called “over processing” and can be prevented by optimizing the surfactant type and concentration (Jafari, He, & Bhandari, 2007).

2.3.1.2. ζ-Potential

The ζ-Potential is a parameter used to measure the electrical charge on the nanoemulsion droplets. It is an indicator of the stability of nanoemulsion (Pongsumpun, Iwamoto, & Siripatrawan, 2020). In the present study, clove essential oil nanoemulsion containing different concentrations of surfactant was prepared at a constant level of clove essential oil (1% (v/v)) using a microfluidizer at 5,000 - 15,000 psi. The results found that the average ζ-potential of clove essential oil nanoemulsion decreased dramatically with an increase in the concentration of surfactant. Whereas the average ζ-potential was observed decreasing with no significant difference ($p > 0.05$) when the operating pressure increased from 5,000 to 15,000 psi **(Table 2.3)**. The ζ-potential of clove essential oil nanoemulsion prepared with 3% (v/v) Tween® 80 at 10,000 psi was -50.01 ±1.01 mV. The negative charge on a droplet of clove essential oil nanoemulsion could be attributed to the presence of free fatty acids and other polar constituents (Bhargava *et al*., 2015). Besides, as suggested by Garcıa-Marquez *et al*. (2016), the high shear is more effective to breakdown the droplet, thus, the decreasing of the ζ-potential with the increasing of operating pressure could occur due to the increase in the repulsion between charged droplets. Since the nanoemulsion with more than ± 30 mV of ζ-potential is considered stable (Raviadaran *et al*., 2018), the results in this experiment indicated that the fabricated clove essential oil nanoemulsions (ζ-Potential of -50.01 mV) had high stability against particle aggregation.

2.3.1.3. Viscosity

The viscosity of clove essential oil nanoemulsions fabricated with various surfactant concentrations and operating pressures was studied. The results in **Table 2.3** showed an increase in viscosity of the nanoemulsions with elevated surfactant concentration and operating pressure. The results can be first attributed to the properties of Tween®80, a viscous liquid, which has been reported with the ability to trap water molecules in its cross-link chains (Ghosh *et al*., 2013). Whereas the observed thickening behavior of the nanoemulsion along with the increase in the operating pressure could be attributed to the droplet coalescence, that occurred during nanoemulsion formation as aforementioned (Purwanti *et al*., 2018). Moreover, the viscosity of nanoemulsions has been reported to be dependent on several factors such

as the rheology of component phases, droplet size, droplet charge, and shear stress (McClements, 2005).

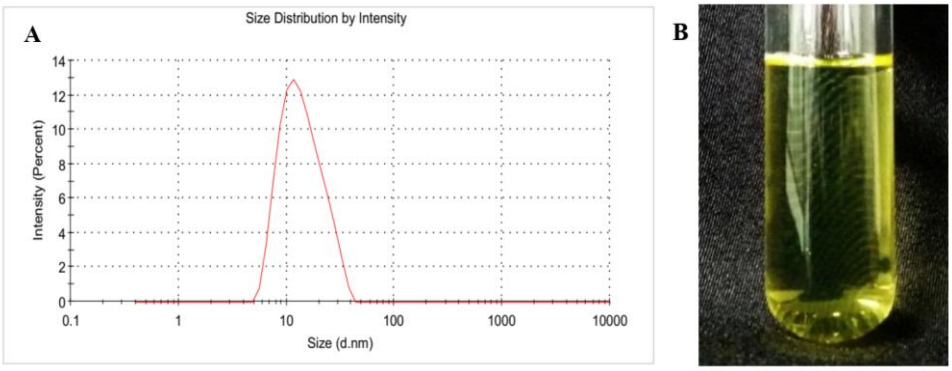

Figure 2.1 (A) Particle size distribution of clove essential oil nanoemulsion prepared with clove essential oil: Tween® 80 at a concentration of 1:3% (v/v) and (B) image of clove essential oil nanoemulsion fabricated from clove essential oil: surfactant at 1: 3% (v/v) and operating pressure at 10,000 psi.

2.3.2 Optimal condition for nanoemulsion fabrication using RSM

In order to fabricate a stable clove essential oil nanoemulsion, RSM with the central composite design was used to optimize the condition for nanoemulsion fabrication. In this study, 13 experimental sets constructed with different levels of surfactant concentration and operating pressure are presented in **Table 2.3**. The polynomial response surface models of droplet size, PDI, ζ-potential, and viscosity as a function of Tween® 80 concentration and operating pressure are shown in the three-dimensional plots **(Figures 2.2 A-D)**. The actual relationship between responses: droplet size (Y_1), polydispersity index (Y_2), ζ- potential (Y_3), and viscosity (Y_4) and significant variables indicated the variability in the responses that could be explained by the second-order polynomial predictive model (**Equations. A- D) (Table 2.4)** with a satisfactory coefficient of determination (R^2 = 0.81, 0.81, 0.95, and 0.95, respectively). The p-value of 0.017, 0.016, 0.0001, and < 0.0001 for Y1-Y4, respectively, also confirmed the fit of the polynomial model. The results showed that mean droplet diameter of 30.76 nm, PDI of 0.179, ζ-potential of -50.01 mV, and viscosity of 7.81 cP were predicted to be the minimal value. The predicted optimal

surfactant concentration and operating pressure for the fabrication of stable clove essential oil nanoemulsion, were 3% (v/v) and 10,000 psi, respectively. This was, therefore, the most desirable condition that could provide clove essential oil nanoemulsion with minimum mean droplets and PDI, minimum ζ-Potential, and optimum viscosity.

TABLE 2.3 Central composite design used for optimization of clove essential oil nanoemulsion for the particle size and size distribution curve**,** PDI, ζ-potential, and viscosity.

Run	Tween® 80 (%v/v)	Operating pressure (psi)	Size (nm)*	PDI*	ζ-potential (mV)*	Viscosity (cP)*
1	2	2,928.93	37.89 ± 0.03^{c}	0.310 ± 0.05^{bc}	-30.01 ± 1.02^{b}	5.50 ± 0.47^{b}
2	3.41	10,000	30.76 ± 0.46^{d}	0.179 ± 0.04^{e}	-50.01 ± 1.01^{d}	7.81 ± 0.24^{a}
3	3	15,000	36.60 ± 0.41^{c}	0.200 ± 0.02^{de}	-47.92 ± 1.71^{d}	7.82 ± 0.37^{a}
4	2	10,000	36.50 ± 0.81^{c}	0.270 ± 0.06^{cde}	-38.15 ± 0.98^{c}	5.73 ± 0.28^{b}
5	1	5,000	43.90 ± 0.03^{b}	0.410 ± 0.02^{a}	-22.08 ± 1.01^{a}	2.41 ± 0.35^{e}
6	1	15,000	45.57 ± 0.18^{a}	0.380 ± 0.03^{ab}	-30.11 ± 1.73^{b}	2.84 ± 0.14^{e}
7	0.59	10,000	36.92 ± 1.35^{c}	0.290 ± 0.06^{bc}	-31.00 ± 1.00^{b}	2.60 ± 0.32^{e}
8	2	10,000	36.50 ± 0.81^{c}	0.270 ± 0.06^{cde}	-38.15 ± 0.98^{c}	5.73 ± 0.28^{b}
9	2	10,000	36.50 ± 0.81^{c}	0.270 ± 0.06^{cde}	-38.15 ± 0.98^{c}	5.73 ± 0.28^{b}
10	2	10,000	36.50 ± 0.81^{c}	0.270 ± 0.06^{cde}	-38.15 ± 0.98^{c}	5.73 ± 0.28^{b}
11	3	5,000	36.85 ± 0.35^{c}	0.280 ± 0.06^{cd}	-38.06 ± 1.71^{c}	7.70 ± 0.61^{a}
12	2	10,000	36.50 ± 0.81^{c}	0.270 ± 0.06^{cde}	-38.15 ± 0.98^{c}	5.73 ± 0.28^{b}
13	2	17,071.07	43.65 ± 1.18^{b}	0.370 ± 0.05^{ab}	-36.12 ± 2.63^{c}	5.77 ± 0.05^{b}

*Mean values ± one standard deviation derived from three replications. Different data letters within a same column indicate statistically significant differences ($p \leq 0.05$).

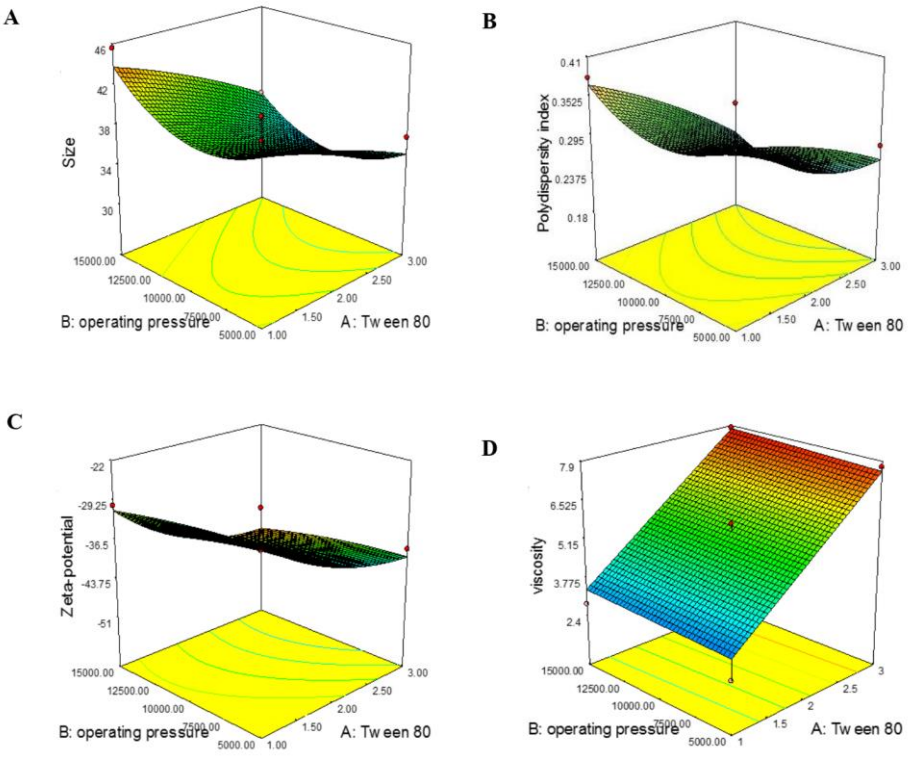

Figure 2.2 Response surface and contour plots of the interactions between Tween® 80 and operating pressure on the droplet size (A), PDI (B), ζ-potential (C), and viscosity (D) of the clove essential oil nanoemulsion.

TABLE 2.4 Second-order polynomial models for the droplet size (Y_1), polydispersity index (Y_2), ζ -potential (Y_3), and viscosity (Y_4) of clove essential oil nanoemulsions.

Equation	Response	Second-order polynomial model	R^2	*P*-value
A	Droplet size	Coded: $Y_1 = 36.50-3.09x_1+1.20x_2 -0.48x_1x_2-0.47x_1^2+2.99x_2^2$	0.81	0.017
		Actual: Droplet size = $48.44-0.23X_1-1.96E-003X_2-9.60E-005X_1X_2-0.47X_1^2+1.19E-007X_2^2$		
B	Polydispersity index	Coded: $Y_2 = 0.27-0.05x_1-3.14E-003x_2-0.01x_1x_2-0.01x_1^2 +0.04x_2^2$	0.81	0.016
		Actual: polydispersity index = $0.47+6.80E-003X_1-2.96E-005X_2-2.50E-006X_1X_2-1.00E-002X_1^2+1.70E-009X_2^2$		
C	ζ -potential	Coded: $Y_3 = -38.15-7.58x_1-3.32x_2-0.46x_1x_2-0.62x_1^2+3.10x_2^2$	0.95	0.0001
		Actual: ζ -potential = $8.23-4.20X_1-2.96E-003X_2-9.15E-005X_1X_2-0.61X_1^2+1.24E-007X_2^2$		
D	viscosity	Coded: $Y_4 = 5.47+2.20\ x_1+0.12x_2$	0.95	< 0.0001
		Actual: viscosity = $0.82+2.20X_1+2.32E-005X_2$		

Y = coded value of response, x_1 = coded value of Tween® 80 concentration (% v/v) and x_2 = coded value of operating pressure (psi), X_1 = actual value of Tween® 80 concentration (% v/v) and X_2 = actual value of operating pressure (psi).

2.3.3 Chemical compound identification

The major constituents of clove essential oil, clove essential oil nanoemulsion, and clove essential oil nanoemulsion after storage (5 °C for 48 h) were similar. They contained eugenol, benzyl alcohol, and caryophyllene but with different percentages of these compositions (**Figures 2.3 (A-C)**). Clove essential oil nanoemulsion contained a higher amount of eugenol, but a lower amount of benzyl alcohol and caryophyllene when compared to clove essential oil. This result was different from that previously reported by Cui *et al*. (2016), of which the GC characteristic peak of eugenol was not observed after proteoliposome encapsulation because of complete encapsulation with no leakage of oil. In this study, the observed higher content of eugenol in the nanoemulsion (70.69 %) when compared to that of clove essential oil (60.11 %) might be attributed to the high surface area/volume ratio of eugenol which occurred through the plenty amount of fine clove essential oil droplets. However, to our knowledge, the effect of nanoemulsion on the observed higher content of a bioactive compound containing in clove essential has never been reported. Therefore, a specific mechanistic study may be needed in this area.

In the present study, the observed not many differences in the amount of eugenol, benzyl alcohol, and caryophyllene, containing in the clove essential oil nanoemulsions before and after storage at 5 °C for 48 h indicated the encapsulation efficiency and chemical stability of the clove essential oil nanoemulsion. This result is in line with the study of Chuesiang *et al*. (2019), indicating the chemical stability of encapsulated cinnamon oil in nanoemulsion form after storage for 31 days.

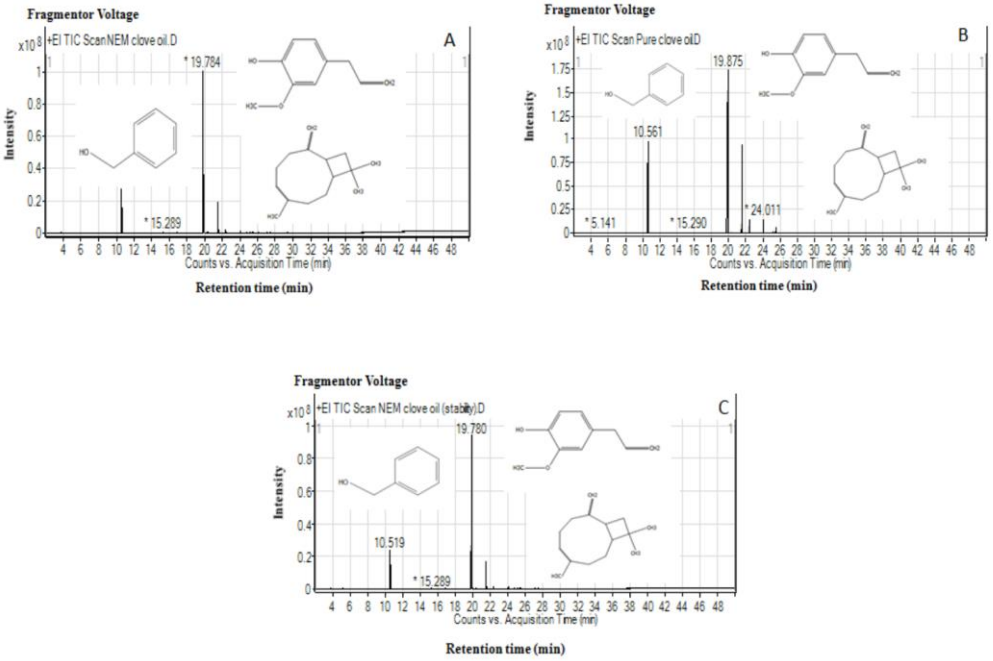

Figure 2.3 GC chromatograms of (A) clove essential oil: eugenol (retention time =19.79 min; 60.11 % total peak area); benzyl alcohol (retention time =10.56 min;18.77% total peak area); and, caryophyllene (retention time =24.01 min; 14.22 % total peak area , (B) clove essential oil nanoemulsion: eugenol (retention time =19.86 min; 70.69 % total peak area); benzyl alcohol (retention time=10.56 min;13.39% total peak area); and, caryophyllene (retention time=24.00 min; 11.15 % total peak area, and (C) clove essential oil nanoemulsion after stored: eugenol (retention time =19.78 min; 70.69 % total peak area); benzyl alcohol (retention time =10.51 min;14.99% total peak area); and, caryophyllene (retention time=24.00 min; 11.15 % total peak area.

2.3.4 Stability of clove essential oil nanoemulsion

The stability of clove essential oil nanoemulsions was tested under accelerated conditions by centrifugation before storage for 48 h at 5 °C or 45 °C. Then, the particle size, PDI, size distribution, ζ-potential, and viscosity of clove essential oil nanoemulsions were determined. At 5 ^{o}C, except for the ζ-potential and viscosity, there was no significant ($p > 0.05$) change in the particle size, PDI, and size distribution of nanoemulsion throughout 12 days of storage when compared to those of initial clove essential oil nanoemulsions. The smallest mean droplet diameter, PDI,

ζ-potential, and viscosity of nanoemulsion were observed at 30.74±0.33 nm, 0.172±0.04, -46.48±0.88 mV, 8.34±0.16 cP, respectively, when 3% v/v surfactant concentration and 10,000 psi of operating pressure were used (**Figure 2.4).** The increase in the ζ-potential and viscosity of the nanoemulsion during storage at 5 °C was probably resulted from the droplet aggregation due to the temperature change (Shafiq *et al*., 2007).

An increase in droplet size, PDI, and ζ-potential, but a decrease in viscosity of the nanoemulsion were observed when stored the nanoemulsion at 45 °C. These results could be due to the coalescence of the emulsion droplets as the effect of high temperatures (Pongsumpun, Iwamoto, & Siripatrawan, 2020; Saberi, Fang, & McClement, 2013). Ostwald ripening is one of the phenomena that cause the coalescence of the droplets in nanoemulsions. The slight increase in droplet size could be related to the molecular diffusion of essential oil from small to large droplets, which occurs as the differences in Laplace pressure and composition between droplets (Chuesiang *et al*., 2018).

In the present study, nanoemulsions tended to be more turbid when stored at 5 °C. However, there was no phase separation or the presence of creaming or sedimentation observed in clove essential oil nanoemulsions at either 5 ^{o}C or 45 °C **(Figures 2.5)** Therefore, the clove essential oil nanoemulsion was considered to be stable in terms of droplet size with the tested storage time and temperatures.

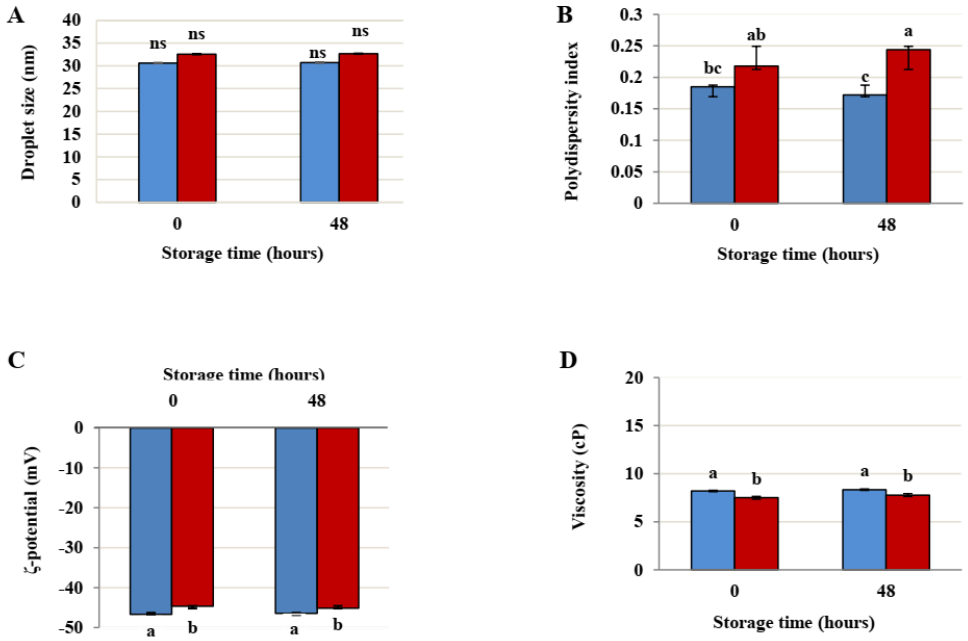

Figure 2.4 Droplet size (A), PDI (B), ζ-potential (C), and viscosity (D) of clove essential oil nanoemulsion, exported to a stability testing of 6 cycles of centrifugation and stored at 5 °C (■) or 45 °C (■) for 0 and 48 h. $^{a\text{-}c}$ means within the same measurement with different annotations are significantly different ($p \leq 0.05$). ^{ns} mean not significantly different ($p > 0.05$).

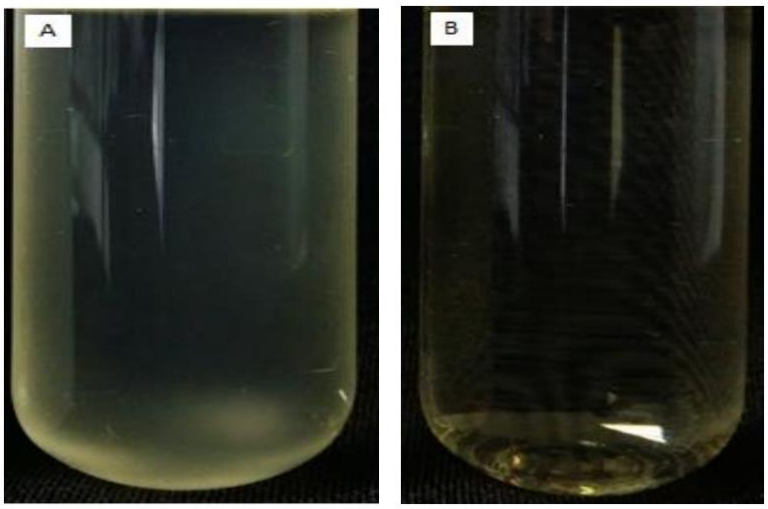

Figure 2.5 Appearance of clove essential oil nanoemulsion prepared with clove essential oil: Tween® 80 at the concentration of 1: 3% (v/v) the pressure at 10,000 psi, exported to stability tested 6 cycles of centrifugation and incubation at 5 °C (A) or 45 °C (B) and storage for 48 h.

2.3.5 Antimicrobial activity

The MICs of clove essential oil nanoemulsion, clove essential oil, and Kanamycin sulfates against *S. aureus, E. coli,* and *P. aeruginosa* were evaluated and are shown in **Table 2.5.** Clove essential oil nanoemulsions exhibited a higher inhibitory effect (lower MICs) toward *S. aureus*, *E. coli,* and *P. aeruginosa* when compared to those of clove essential oil. These results occurred probably because of the ability of the nanoemulsion system to deliver the bioactive compounds to the microbial cell membrane via fine droplets of clove essential oil (Moghimi *et al*., 2015). According to Donsì & Ferrari (2016), the small clove oil droplets caused the rapid diffusion of the bioactive compounds to the bacteria cell, hence, affecting the disruption of bacteria (Anwer *et al*., 2014). Previous reports also showed a rapid inactivation of eugenol essential oil nanoemulsion against *S. aureus* (Cortes-Rojas *et al*., 2014). These results confirmed that the antimicrobial activity of clove essential oil could be improved by the nanoemulsion system. In this study, Kanamycin sulfate, an antibiotic against the broad variety of gram-negative and gram-positive bacteria, was used as a positive control. The clove essential oil nanoemulsion exhibited a higher

inhibitory effect (lower MICs) toward gram-negative *E. coli*, and *P. aeruginosa* when compared to those of Kanamycin sulfates.

TABLE 2.5 The minimal inhibitory concentration of clove essential oil, clove essential oil nanoemulsion and kanamycin sulfates against *E. coli*, *S. aureus*, and *P. aeruginosa*.

Minimal inhibitory concentration (mg/mL)			
Microorganism	Clove essential oil	Clove essential oil nanoemulsion	Kanamycin sulfates (50 mg/mL)
Escherichia coli	169.35[a]	7.75[a]	3.12[a]
Staphylococcus aureus	84.67[b]	3.87[b]	0.78[c]
Pseudomonas aeruginosa	84.67[b]	3.87[b]	1.56[b]

* Different data letters within a same column indicate statistically significant differences ($p < 0.05$).

Moreover, cell morphological deformation of *E. coli*, *S. aureus*, and *P. aeruginosa* when exposed to clove essential oil, clove essential oil nanoemulsion, or kanamycin sulfate at 37 ^{o}C for 30 min was also evident as shown on the SEM-EDS images (**Figure 2.6**). The bacterial cell morphological destruction is probably because clove oil phenolic compounds can inhibit a great number of bacteria by causing protein to denature and microbial cell membrane leakage (Lin, Dai, & Cui, 2017). Therefore, the clove essential oil nanoemulsion has a high potential to use as an antimicrobial agent against a broad range of bacteria.

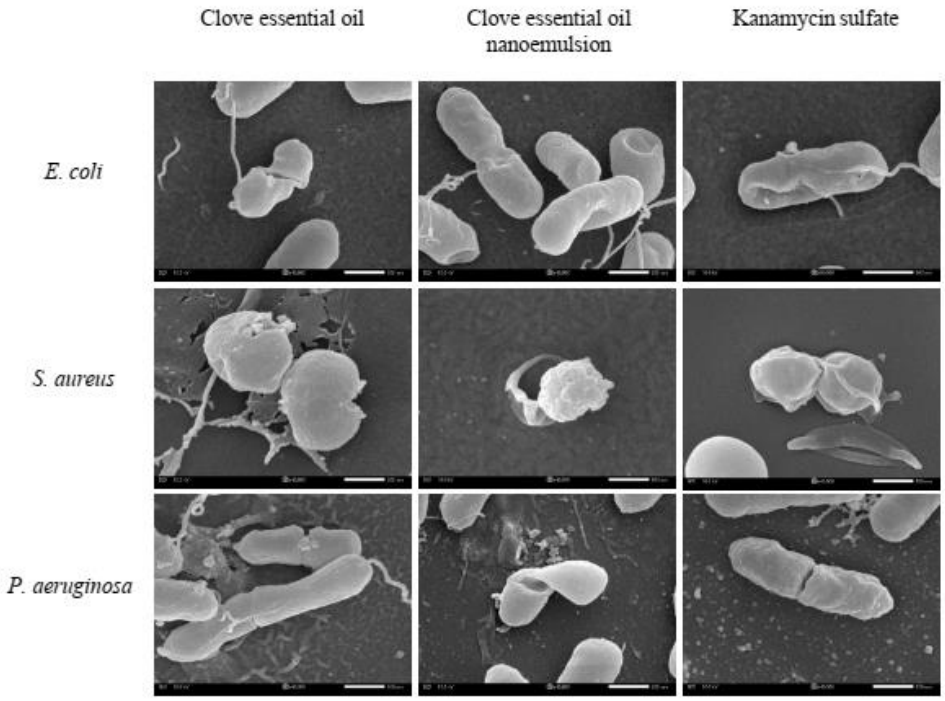

Figure 2.6 Scanning electron microphotographs of *E. coli*, *S. aureus*, and *P. aeruginosa* after treated with clove essential oil, clove essential oil nanoemusion, and kanamycin sulfate (positive control), and incubated at 37 ^{o}C for 30 min.

2.4 Conclusion

The optimal condition for the formation of clove essential oil nanoemulsion using the microfluidizer was 1% (v/v) clove essential oil, 3% (v/v) Tween®80 and 10,000 psi of operating pressure. The smallest mean droplet diameter (30.76 nm), and PDI (0.179) were achieved with -50.01 mV of ζ-potential and 7.81 cP of viscosity. The nanoemulsion was stable when stored for 48 h at 5 °C or 45 °C. GC-MS revealed that clove essential oil nanoemulsion was chemically stable during storage at 5 °C for 48 h. The antimicrobial activity of clove essential oil nanoemulsions against *S. aureus*, *E. coli*, and *P. aeruginosa*, was higher (lower MICs) than that of clove essential oil. Moreover, bacterial cell morphological deformation of all tested bacteria when exposed to clove essential oil nanoemulsion or clove essential oil was also evident. This research has shown that clove essential oil nanoemulsion has a high potential to be used as a natural antimicrobial agent.

2.5 Acknowledgement

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2.6 Conflict of interest

The authors have declared no conflict of interest for this article.

2.7 Data availability statement

The research data are not shared.

CHAPTER III
MANUSCRIPT II

Foam-mat freeze-drying approach for preserving stability and antimicrobial activity of clove essential oil nanoemulsion

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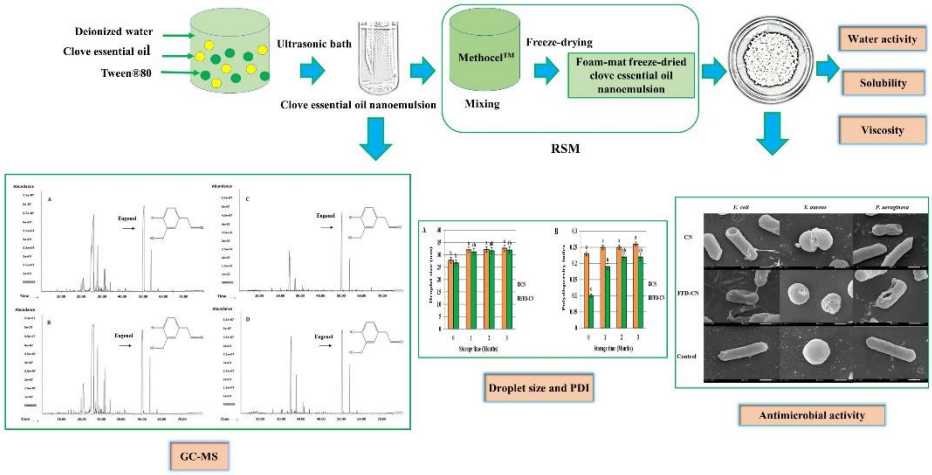

Figure 3.1 Graphical abstract

Abstract

Foam-mat freeze-drying technique was used to maintain stability and antimicrobial activity of clove essential oil nanoemulsion (CN). The CN was prepared from 1% (v/v) clove essential oil, 3% (v/v) Tween® 80, and deionized water as a continuous phase, using an ultrasonication. The optimal condition for fabrication of foam-mat freeze-dried clove essential oil nanoemulsion (FFD-CN) was achieved using response surface methodology (RSM) with Box-Behnken design (BBD). The optimal condition was found at 3% (v/v) of foaming agent (Methocel^{TM}), 60 °C drying temperature, and 72 h drying time. The obtained FFD-CN had the droplet size of 26.14 nm, polydispersity index (PDI) of 0.193, water activity (a_w) of 0.273, solubility of 88.71%, and viscosity of 12.85 cP. The FFD-CN exhibited good stability and maintained antimicrobial activity, as analyzed using minimum inhibitory concentration (MIC), against gram-negative *Escherichia coli*, *Pseudomonas aeruginosa* and gram-positive *Staphylococcus aureus* during 3 months storage at 25 °C. When observed using a scanning electron microscope and energy dispersive x-ray spectrometer (SEM-EDS), bacterial cell morphological destruction caused by both FFD-CN and CN was evident. Gas chromatography-mass spectrometry (GC-MS) results suggested that FFD-CN can preserve eugenol, a major volatile bioactive compound in clove oil, in its structure better than liquid-formed nanoemulsion.

Transformation of liquid CN into FFD-CN can effectively preserve stability and antimicrobial activity throughout the storage.

Keywords:
Clove essential oil nanoemulsion,
Foam,
Freeze-drying,
Antimicrobial activity,
Bacterial morphology

3.1 Introduction

Clove (*Syzygium aromaticum*) essential oil has gained increasing interest as a natural food additive to replace chemical additives in various food products (Milind & Deepa, 2011; Ulanowska & Olas, 2021). Clove essential oil contains key active phenolic compounds such as eugenol, a major aromatic phenolic compound in clove oil, and caryophyllene, a member of bicyclic sesquiterpene, which have antibacterial properties against gram-positive and gram-negative bacteria and fungi (Anwer et al., 2014; Milind & Deepa, 2011; Zhang et al., 2016). The antimicrobial activity of eugenol has been reported to be attributed to the hydroxyl (OH) group in its structure (Ulanowska & Olas, 2021), causing an alteration of protein characteristics of bacterial cell membrane and inhibition of protease activity of bacterial cell (Amelia et al., 2017; Liu et al., 2017; Somrani et al., 2021). Clove essential oil is commercially used as a food additive under the classification of Generally Recognized as Safe (GRAS) by the United States Food and Drug Administration (USFDA). However, due to its low water solubility and strong odor, there have been some limitations of using clove essential oil directly in food. As a result, a delivery technology such as a nanoemulsion could be used to encapsulate clove essential oil in order to reduce those effects before being used in food products (Donsi & Ferrari, 2016).

Nanoemulsion of clove essential oil is a system obtained by two-phase: oil (dispersed phase) and water (continuous phase) (Purwanti et al., 2016). Many studies have shown that the suitable droplet sizes for nanoemulsions are in the range of 20-200 nm. The nanoemulsion is translucent and has high stability against particle

aggregation (Chuesiang et al., 2018). Despite the fact that the nanoemulsion system contains small oil droplets, the liquid state of the nanoemulsion cannot retain the stability of the size of the small droplets during storage. Therefore, a drying method such as foam-mat freeze-drying (FFD) can be considered as an alternative way to encapsulate clove essential oil nanoemulsion (CN) in order to minimize those effects (Raharitsifa & Ratti, 2010).

Freeze-drying is a low-temperature drying method that can produce high-quality dried products (Thuwapanichayanan et al., 2008). In the freeze-drying process, the original flavors and volatiles as well as vitamins and bioactive compounds are retained. Furthermore, freeze-drying is regarded as one of the best methods for drying. Freeze-dried products are characterized by low bulk density, high porosity, and good rehydration properties, which are important for dried food (Ratti, 2001; Sablani & Rahman, 2002). Dry food products have lower weight than their liquid form, allowing for lower shipping cost, and, due to their low water activity, have longer shelf life (Ratti, 2001; Thuwapanichayanan et al., 2008). The FFD has been developed to encapsulate essential oils and bioactive compounds from plants, such as nutraceuticals, vitamins, colors, and flavors (Norcino et al., 2020). Among several drying methods, the FFD method is considered a good alternative for making power-formed nanoemulsion because it uses low temperature that allows the product's quality to be maintained (Seerangurayara et al., 2018). The principle of FFD is to transform a liquid product into a stable foam by whipping the mixture of product and wall materials and then dehydrating it by freeze-drying (Muthukumaran et al., 2008; Ruengdech & Siripatrawan, 2022). The qualities of the foam have been observed to be affected by factors such as foaming agent type and concentration, drying time, and drying temperature. FFD has been reported to improve the storage stability and the solubility of essential oil emulsion (Mahdi et al., 2021; Ruengdech & Siripatrawan, 2022).

Therefore, the objective of this study was to optimize the foam-mat freeze-dried clove essential oil nanoemulsion (FFD-CN) fabrication by investigating various parameters, including foaming agent (Methocel™) concentration, drying temperature, and drying time of the FFD. The characteristics of the FFD-CN, including mean droplet diameter, particle size distribution, polydispersity index (PDI), water activity

(a_w), solubility, and viscosity, were determined. The antimicrobial activity of the optimized FFD-CN was also investigated against *Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus*.

3.2 Materials and methods

3.2.1 Materials

Pure clove essential oil batch No. 0009115315 was purchased from TTK Science Co., Ltd. (Laksi, Bangkok, Thailand). Tween® 80 and Methocel™ were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). *E. coli* (ATCC O157), *S. aureus* (ATCC 25923), and *P. aeruginosa* (ATCC 19429) were obtained from the American Type Culture Collection (Rockville, MD, USA).

3.2.2 Preparation of CN

The optimum formulation of the CN was prepared from 1% (v/v) clove essential oil, 3% (v/v) Tween® 80, and deionized water as a continuous phase following the method described by Pilong et al. (2022), using an ultrasonic bath (Branson 3800 series, Branson Ultrasonics, Danbury, CT, USA). The ultrasonication was operated at a fixed operation frequency of 43 kHz at 40 °C for 20 min.

3.2.3 Optimization of FFD-CN fabrication

FFD-CN was prepared using Methocel™ as a foaming agent. The fabrication parameters affecting the characteristics of the FFD-CN were optimized by varying foaming agent concentrations (1%, 2%, and 3% v/v), freeze drying temperatures (25 °C, 40 °C, and 60 °C), and drying times (24 h, 48 h, and 72 h). The CN was mixed with Methocel™ in a 250-watt kitchen blender (Oster®, Sunbeam Products, Inc., Boca Raton, FL, USA) at maximum speed at 25 °C for 5 min, following the methods suggested by Pinto et al. (2018) and Rattanapitigorn et al. (2016). After that, 500 mL of the sample was placed in an aluminum tray (20 × 30 cm) with 3 cm high borders. A thermocouple was used to measure the temperature at the center of the samples during drying in a freeze dryer (Virtual Wizard 2.0, SP Scientific, Warminster, PA, USA) for the processing time of 30 min.

The optimal condition for FFD-CN fabrication was determined by investigating the effects of foaming agent (Methocel^{TM}) concentration (X_1), drying temperature (X_2), and drying time (X_3) on the responses, including droplet size (Y_1), PDI (Y_2), a_w (Y_3), solubility (Y_4), and viscosity (Y_5) of the FFD-CN. The RSM with Box-Behnken design was performed to investigate the effect of independent variables and the interaction between independent variables on the responses **(Equation 1).**

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (1)$$

where Y is the response of the factor level combination, b_0 is a constant, b_1, b_2, b_3 are the linear coefficients, b_{11}, b_{22}, b_{33} are the quadratic coefficients, b_{12}, b_{13}, b_{23} are the interaction coefficients, and X_1, X_2, and X_3 are the independent variables. The goodness of fit of the model was indicated by the coefficient of determination (R^2) and p-value. The model fit well when R^2 is close to one and a p-value < 0.05 (Pongsumpun et al., 2020).

3.2.4 Determination of FFD-CN properties and stability

The FFD-CN was transferred to an aluminum-coated plastic bag, heat sealed, and stored in a desiccator (DE-20, Ltd., Waltham, MA, USA**)** with silica gel at 25 °C (Pinto et al., 2018) for up to 3 months. The properties of the obtained FFD-CN, including particle size, PDI, a_w, solubility, viscosity, stability, and antimicrobial activity, were investigated during the storage at 25 °C for 0-3 months. The volatile compounds of the FFD-CN were also analyzed in comparison to the CN using solid phase microextraction-gas chromatography/mass spectrometry (SPME-GC-MS). The a_w of the FFD-CN was measured at 25 °C using a water activity analyzer (Aqualab, Decagon Modulo 3TE, Pullman, WA, USA).

Before use, one gram of FFD-CN was dissolved in 100 mL of deionized water with continuous stirring using a centrifuge (VWR^{TM}centrifuge, Mega Star 1.6, Avantor, Radnor, PA, USA) at 12,300 x g (2,500 rpm in a M2 rotor) for 5 min at room temperature (22-25 °C). The FFD-CN was rehydrated with deionized water at the ratio of 1:100 to avoid interparticulate interaction. The particle size and PDI were measured using dynamic light scattering DLS (Zetasizer NanoZS,

Malvern Instruments Ltd, Malvern, Worcestershire, UK) comprising a helium-neon gas laser (λ=633 nm) with a detector angle of 173° at 25 °C, following the method of Pilong et al. (2022).

The solubility was determined according to the method of Pinto et al. (2018) with minor modifications. The sample (100 mL) was transferred to an aluminum plate and incubated in a hot air oven (ED Series, VWR™, Ltd., Radnor, PA, USA) at 80 °C for 24 h. The percentage of solubility was calculated using **Equation 2**.

$$\%\text{Solubility}=100-\frac{M_{DRY}}{M_{INITIAL}}\times 100 \qquad (2)$$

where M_{DRY} is the final mass and $M_{INITIAL}$ is the initial mass.

The viscosity was measured using a Brookfield digital viscosity meter (DV-E model, Brookfield Laboratories Inc., Middleboro, MA, USA). The FFD-CN sample (300 mL) was placed in a 500 mL beaker prior to inserting the spindle (s85) at 100 rpm into the solution at 25 °C. The measurement was performed, and the average viscosity was reported as centipoise (cP) (Rattanapitigorn et al., 2016).

3.2.5 Analysis of volatile compounds of FFD-CN

Volatile compounds were collected using the solid phase microextraction (SPME) technique as previously described by Chen et al. (2019) and Chuesiang et al. (2022) with minor modifications. One gram of FFD-CN was dissolved in 100 mL of deionized water with continuous stirring using a centrifuge at 12,300 x g for 5 min at room temperature. Briefly, 2 mL of CN, CN after 3 months of storage, FFD-CN, and FFD-CN after 3 months of storage were placed into a 20 mL glass vial (Chromselection, Vicenza, Veneto, Italy). The vial was then tightly closed with an aluminum crimp cap attached to a polytetrafluoroethylene (PTFE) silicone septum (Chromselection, Agilent Technologies Inc., Santa Clara, CA, USA) using a hand crimper (Model 20001, Kebby Industries Inc., Rockford, IL, USA). After 10 min of sample equilibration at 40 °C, the 50/30 µm divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber, 23 Ga Autosampler (Gray)

(Supelco, Inc., Bellefonte, PA, USA) was put into the headspace of the sample vial for 30 min and then immediately inserted into the GG-MS (Autosystem XL, PerkinElmer Inc., Waltham, MA, USA) injection port with the spitless mode at 250 °C for 2 min.

The GC column was a DB-wax capillary column (30 m × 250 µm, and 0.25 µm film thickness) (Agilent 7980A Restek, Santa Clara, CA, USA). The GC oven temperature gradient was initially set at 50 °C (hold for 5 min), increased to 125 °C (hold for 3 min) at 3 °C min^{-1}, then increased to 180 °C (hold for 3 min) at 2 °C min^{-1}, and finally increased to 250 °C (hold for 5 min) at 6 °C min^{-1}. Helium was used as the carrier gas with a flow rate at 1 mL/ min; the splitless ratio of 100:1; injector and detector temperatures: 250 °C; and sample size: 1 µL. The MS (5977B Turbomass MS, PerkinElmer Inc., Waltham, MA, USA) was coupled to the GC. The ion source temperature was 230 °C and the MS was scanned at 70 eV over the 40 to 500 mass ranges. The chemical constituents of CN and FFD-CN were tested and identified by the National Institute of Standards and Technology library data (NIST2017).

3.2.6. Analysis of antimicrobial activity of FFD-CN

The antimicrobial activity of FFD-CN was determined against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus*) during storage for 3 months. All these bacterial cultures were prepared following the method of Chuesiang et al. (2019) and stored at 4 °C on the nutrient agar slant before being used. Active cultures were prepared by transferring a ring loop of cells from the agar slant to a test tube containing 50 mL of nutrient broth and incubating overnight at 37 °C for 24 h. A single colony of bacterial cells was prepared by streaking a culture onto the nutrient agar. After incubation at 37 °C for 24 h, a colony was then transferred with a ring loop from the nutrient agar to the nutrient broth and incubated again at 37 °C for 24 h. After 24 h of incubation, the initial turbidity was adjusted to 0.1 at 600 nm using a UV spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) to obtain the initial number of the bacterium at approximately 8 log CFU/mL.

One gram of FFD-CN was dissolved in 100 mL of deionized water with continuous stirring using a centrifuge at 12,300 x g for 5 min at room temperature.

The minimum inhibitory concentrations (MICs) of FFD-CN (1% CN in the FFD-CN) and CN were prepared by broth dilution following the method described by Pilong et al. (2022). FFD-CN or CN (6 mL) and nutrient broth (6 mL) were added to the first test tube. A serial two-fold dilution was determined in the second tube containing the same nutrient broth. After that, the microbial suspension (8 log CFU/mL) was added to the tube. Each prepared tube received a serial dilution of 6 mL from the first tube of each sample. After that, 1 mL of each aliquot culture was added to the prepared FFD-CN or CN in each tube and mixed by vortexing. The concentration of CN or FFD-CN ranged from 248 mg/mL to 1.93 mg/mL. MIC was determined as the lowest concentration of FFD-CN or CN that inhibits the growth of bacteria. The standard plate count method was used to count the bacteria. This procedure involves transferring the sample-bacteria mixture (0.1 mL) from each prepared tube to a nutrient agar dish and then incubating it at 37 °C for 24 h. The concentration of FFD-CN or CN showing no bacterial growth on the plate was selected as the MIC value.

In addition, the bacterial cell morphology was visualized using a scanning electron microscope and energy dispersive x-ray spectrometer (SEM-EDS; JSM-IT500HR, JED-2300, Akishima, Tokyo, Japan) following the method of Ozogul et al. (2020) and Zhang et al. (2016) with slight modifications to verify the antimicrobial efficiency of FFD-CN, CN, and distilled water (control).

3.2.7 Statistical analysis

All experiments were performed in triplicate. The data was recorded as mean values and standard deviation (mean ± SD). The RSM was conducted to optimize the FFD-CN fabrication using the Design-expert program (version 7.0, Stat Soft Inc., Minneapolis, MN, USA). A Box-Behnken design was used to determine the effect of foam-mat freeze-drying on the responses, including droplet size, PDI, a_w, solubility, and viscosity properties of FFD-CN. The IBM SPSS Statistical Software (version 26.0, IBM SPSS Inc., Armonk, NY, USA) was used to perform a one-way analysis of variance (ANOVA) being carried out and differences between means being compared using Duncan's multiple range test when $p \leq 0.05$.

3.3 Results and discussion

3.3.1 Optimal condition for FFD-CN fabrication

The CN with a mean droplet diameter of 30.76 nm and PDI of 0.179 were obtained from the condition using 1% (v/v) of clove essential oil and 3% of surfactant**.** FFD-CN was then fabricated by varying foaming agent concentrations (1-3% v/v), drying temperatures (25-60 °C), and drying time (24-72 h). Thereafter, the characteristics including mean droplet diameter, PDI, a_w, solubility, and viscosity of the FFD-CN were determined.

3.3.1.1 Particle size and PDI of FFD-CN

The mean droplet diameter of the FFD-CN decreased with increasing foaming agent concentration. This effect may be caused by Methocel™, a foaming agent which reduces the surface tension between two liquids, facilitates foam formation, and promotes the formation of small droplets with low PDI and narrow size distribution (Saberi et al., 2013; Wang et al., 2009). Pinto et al. (2018) also reported that an increase in foaming agent concentration promoted the greater stability of foam. The drying temperature was found to have an impact on the mean droplet diameter and PDI of the FFD-CN. An increase in drying temperature decreased the size and PDI of the droplet. The smallest mean droplet diameter and PDI were found when the process operated at 60 °C. These results could be explained by the fact that increasing drying temperature increases interior air volume (Sankat & Castaigne, 2004), leading to a fast-drying and homogeneous encapsulated CN. Drying time was also found to have an impact on the mean droplet diameter and PDI of FFD-CN foam. A bigger mean droplet diameter was found at lower drying times (24 and 48 h), and the optimal operating drying time was 72 h. This may be because increasing the drying time causes larger foam thickness due to more liquid loss, leading to greater CN encapsulation in FFD. Hertzendorf & Moshy (1970) stated that drying time was found to be proportional to the bubble size and thickness of foam mat dried food. Furthermore, foaming the liquid FFD-CN provides more surface area for evaporation, reducing the drying time of FFD-CN (Rattanapitigorn et al., 2016).

3.3.1.2 a_w of FFD-CN

The a_w of the FFD-CN increased with increasing foaming agent concentration but decreased with an increase in drying temperature and drying time. Salahi et al. (2014) who studied the encapsulation of cantaloupe reported a significant decrease in the a_w with an increasing drying temperature. A decrease in a_w with higher drying temperatures may contribute to the closer of constituent molecules leading to an inhibition of hydrophilic site to interact with water. However, our results indicated that drying time did not significantly affect the a_w. The lowest (0.260±0.04) a_w value of FFD-CN was obtained when prepared with 3% (v/v) Methocel^{TM} at 60 °C, and a drying time of 72 h.

3.3.1.3 Solubility of FFD-CN

The solubility of the FFD-CN increased with decreasing foaming agent. This could be because an increase in foaming agent concentration resulted in increasing the surface area of the foam due to integrating air, causing increased foam thicknesses, and reduced solubility. The results were consistent with those in the previous work reported by Thirupathi et al. (2008), indicating the solubility of the foam mat dried egg powders increased when decreasing foam thickness. In addition, decreased solubility was found at lower drying temperatures (25 and 40 °C) and the optimal operating drying temperature was 60 °C. It has been reported that the solubility increased as the drying temperature increased, probably because a higher temperature resulted in foam with a higher porosity, providing a larger surface contact area between foam and water (Pinto et al., 2018). However, our results indicated that the drying time did not significantly affect the solubility.

3.3.1.4 Viscosity of FFD-CN

The viscosity of FFD-CN increased with elevated foaming agent concentration, drying temperature, and drying time. The results can be attributed to the properties of Methocel^{TM}, a viscous polymer synthetic, which has been reported to have an ability as a binder and water retention in foam (Rattanapitigorn et al., 2016). An increase in foaming agent concentration resulted in a higher viscosity of foam solution

due to the difficulty of retaining air during the process, causing lower expansion and higher thickness (Bag et al., 2011; Rattanapitigorn et al., 2016). In addition, the drying temperature affected the foam viscosity. An increase in drying temperature could lead to foaming of the liquid sample with a higher surface area for evaporation, resulting in a higher density and viscosity of foam (Muthukumaran et al., 2008).

3.3.1.5 RSM for optimization of FFD-CN fabrication

In this study, the RSM with the Box-Behnken design was used to optimize the conditions for the fabrication of FFD-CN. The droplet size, PDI, a_w, solubility, and viscosity of FFD-CN fabricated using different Methocel^{TM} concentrations, drying temperatures, and drying times are shown in **Table 3.1.** The details of the RSM computation are provided in the supplementary file. The satisfactory coefficients of determination (R^2 = 0.74, 0.51, 0.97, 0.78, and 0.99, respectively). The lack of fit test ($p < 0.05$) indicated that the models fitted well. The results suggested that the optimal conditions for the FFD-CN fabrication were 3% (v/v) foaming agent, 60 °C drying temperature, and 72 h drying time. The optimal conditions gave the FFD-CN with the smallest mean droplet size diameter of 26.14 nm, PDI of 0.193, a_w of 0.273, solubility of 88.71%, and viscosity of 12.85 cP.

The accuracy of the model was tested by conducting the experiments under the obtained optimal conditions, the experimental values for droplet size, PDI, a_w, solubility, and viscosity value were 28.67 nm, 0.118, 0.247, 88.10%, and 13.02 cP, respectively, which were in line with the predicted response values from the RSM-BBD. Thus, the equation derived from the RSM-BBD could optimize the conditions for the fabrication of the FFD-CN.

Table 3.1 Box-Behnken design used for optimization of foam-mat freeze-dried clove essential oil nanoemulsion (FFD-CN) with droplet size, polydispersity index (PDI), water activity (a_w), solubility, and viscosity values.

Run	Natural variables			Response				
	Methocel^{TM} (% v/v)	Drying temperature (°C)	Drying time (h)	Size (nm)*	PDI*	a_w*	Solubility (%) *	Viscosity (cP)*
1	1	60	48	36.6 ± 0.2^{e}	0.43 ± 0.05^{b}	0.25 ± 0.04^{d}	91.9 ± 0.1^{d}	4.08 ± 0.03^{g}
2	1	25	24	57.5 ± 0.2^{a}	0.47 ± 0.03^{a}	0.41 ± 0.01^{ab}	92.5 ± 0.1^{b}	3.09 ± 0.01^{h}
3	2	42.5	48	36.5 ± 0.3^{e}	0.16 ± 0.01^{e}	0.31 ± 0.01^{cd}	90.2 ± 0.1^{g}	8.29 ± 0.02^{f}
4	2	42.5	48	36.5 ± 0.3^{e}	0.16 ± 0.01^{e}	0.31 ± 0.01^{cd}	90.2 ± 0.1^{g}	8.29 ± 0.02^{f}
5	3	60	72	27.5 ± 0.2^{m}	0.11 ± 0.01^{g}	0.26 ± 0.04^{d}	89.4 ± 0.1^{i}	13.1 ± 0.1^{a}
6	3	25	48	34.6 ± 0.3^{g}	0.2 ± 0.1^{efg}	0.45 ± 0.05^{a}	90.5 ± 0.1^{f}	12.03 ± 0.02^{d}
7	1	25	48	37.6 ± 0.3^{j}	0.15 ± 0.01^{ef}	0.40 ± 0.01^{ab}	91.6 ± 0.1^{e}	4.05 ± 0.05^{g}
8	2	42.5	48	36.5 ± 0.3^{e}	0.16 ± 0.01^{e}	0.31 ± 0.01^{cd}	90.2 ± 0.1^{g}	8.29 ± 0.02^{f}
9	2	42.5	48	36.5 ± 0.3^{e}	0.16 ± 0.01^{e}	0.31 ± 0.01^{cd}	90.2 ± 0.1^{g}	8.29 ± 0.02^{f}
10	3	60	48	32.4 ± 0.5^{h}	0.12 ± 0.02^{fg}	0.31 ± 0.02^{cd}	89.3 ± 0.03^{i}	12.17 ± 0.01^{d}
11	3	42.5	72	31.4 ± 0.2^{i}	0.12 ± 0.02^{efg}	0.36 ± 0.04^{bc}	89.37 ± 0.01^{h}	12.5 ± 0.5^{c}
12	3	25	72	32.5 ± 0.2^{h}	0.13 ± 0.02^{efg}	0.41 ± 0.01^{ab}	87.27 ± 0.02^{k}	12.72 ± 0.03^{b}
13	1	60	24	38.5 ± 0.5^{c}	0.14 ± 0.03^{efg}	0.26 ± 0.03^{d}	92.7 ± 0.1^{a}	3.04 ± 0.02^{h}
14	2	42.5	48	36.5 ± 0.3^{e}	0.16 ± 0.01^{e}	0.31 ± 0.01^{cd}	90.2 ± 0.1^{g}	8.29 ± 0.02^{f}
15	1	60	72	35.5 ± 0.1^{f}	0.22 ± 0.01^{d}	0.25 ± 0.04^{d}	90.2 ± 0.1^{g}	4.11 ± 0.01^{g}
16	3	25	24	37.4 ± 0.3^{d}	0.14 ± 0.01^{efg}	0.45 ± 0.04^{a}	89.2 ± 0.1^{j}	11.62 ± 0.01^{e}
17	1	42.5	24	48.4 ± 0.2^{b}	0.36 ± 0.02^{c}	0.3 ± 0.1^{d}	92.4 ± 0.1^{c}	3.01 ± 0.02^{h}

*Mean values ± one standard deviation derived from three replications. Different data letters within a same column indicate statistically significant differences ($p \leq 0.05$).

3.3.2 Stability of FFD-CN during storage

The stability of CN and FFD-CN were tested for particle size and PDI during storage for 3 months at 25 °C (**Fig. 3.2A-B**). Water activity, solubility, and viscosity (after rehydration) of the FFD-CN were also measured during the storage for 3 months (**Fig. 3.3A-C**). At time zero, the initial droplet size, PDI, a_w, solubility, and viscosity of the FFD-CN were 31.91±0.79 nm, 0.220±0.06, 0.260±0.10, 88.44±0.70%, and 13.54±0.42 cP, respectively. After 3 months, there were no significant ($p > 0.05$) changes in the a_w, solubility, and viscosity. When re-dissolved, the particle size and PDI of the FFD-CN showed significant differences ($p \leq 0.05$) during the storage when compared to those of the initial samples. However, the particle size and PDI of the FFD-CN remained under the range of optimal nanoemulsion. The minor changes in particle size and PDI may be attributed to the

coalescence of the air from small to large bubbles as a result of the differences in the internal pressures between bubbles (Damodaran, 2005; Lomakina & Mikova, 2006) or the coalescence of the oil droplets in the clove oil nanoemulsion due to the Ostwald ripening (Chuesiang et al., 2018).

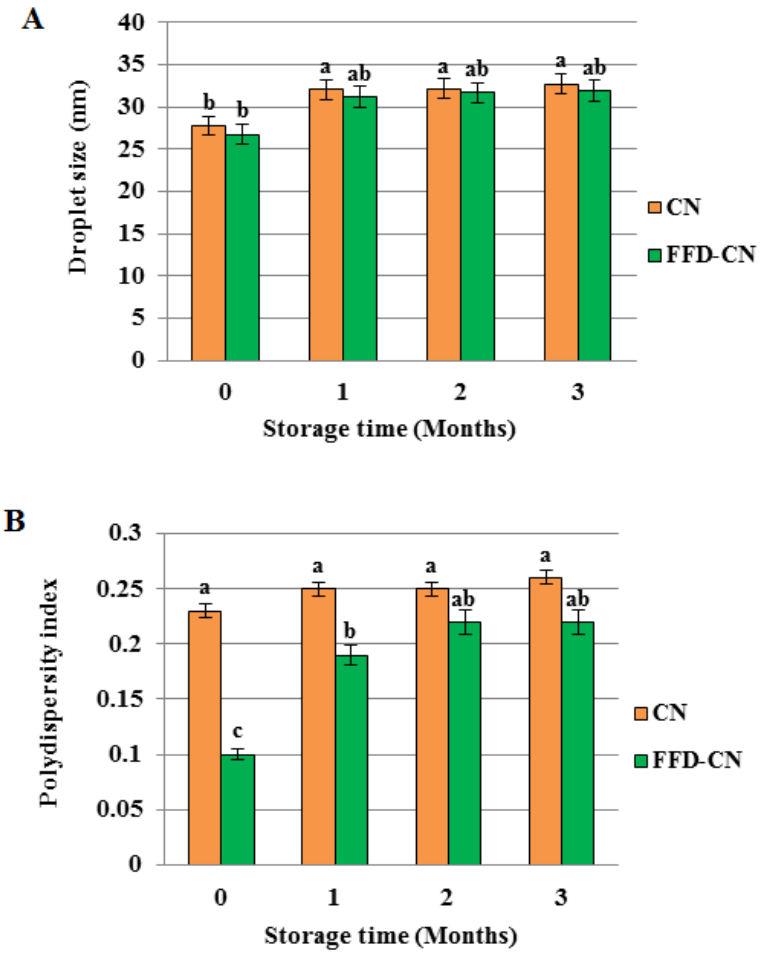

Fig. 3.2 (A) Droplet size and (B) PDI of CN and FFD-CN after being dissolved in deionized water during storage at 25 °C for 3 months. Different letters (a, b, c) indicate significant differences ($p \leq 0.05$).

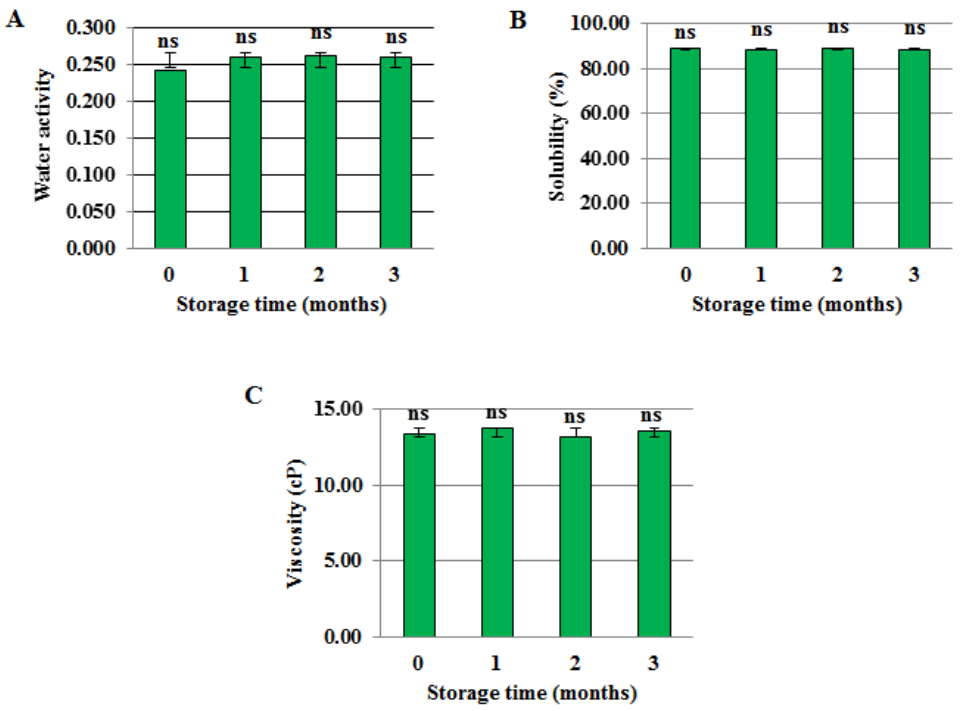

Fig. 3.3 (A) a_w, (B) solubility, and (C) viscosity of FFD-CN after being dissolved in deionized water during storage at 25 °C for 3 months. ^{ns} means not significantly different ($p > 0.05$).

3.3.3 Volatile compounds of FFD-CN

Fig. 3.4 shows GC chromatograms of the volatile profiles of CN and FFD-CN before and after 3 months of storage at 25 ^{o} C. They contained four similar major volatile compounds (e.g., eugenol, caryophyllene, humulene, and phenol, 2-methoxy-4-(2-propenyl)-acetate), but with different intensities. Eugenol was the major volatile phenolic compound found in CN and FFD-CN. Before and after 3 months storage, CN contained 29.98% and 24.49% of eugenol, whereas FFD-CN contained 51.26% and 43.18% at 0 and 3 months storage, respectively. After 3 months storage, eugenol encapsulated in CN decreased by 18.93%, whereas that encapsulated in FFD-CN decreased by 15.76%, when compared to those at the initial time. The lower loss of eugenol in FFD-CN than that in CN suggested that clove essential oil was better preserved in the matrix of FFD-CN than in the liquid-formed CN. These results agree

with those of Mahdi et al. (2021) who studied the nanocapsules of essential oil emulsion using free drying process and found that the wall materials used for freeze-dried essential oil encapsulation may increase the stability of some essential oil nanoemulsion. The observed difference in the eugenol content in CN and FFD-CN after storage at 25 ^{o}C for 3 months suggested the better encapsulation efficacy and chemical stability of the FFD-CN when compared to that of liquid-formed nanoemulsion.

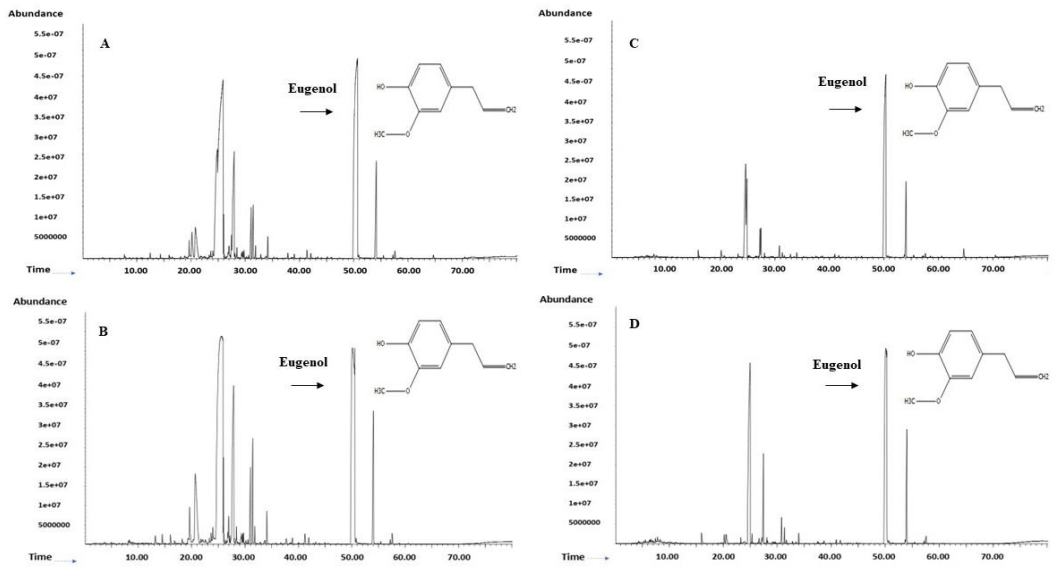

Fig. 3.4 GC chromatograms of volatile compounds of (A) CN: eugenol (retention time=50.35 min; 29.98% total peak area); caryophyllene (retention time=25.30 min; 44.05% total peak area); and humulene (retention time =27.95 min; 6.66% total peak area), (B) CN after stored: eugenol (retention time=50.35 min; 24.49% total peak area); caryophyllene (retention time=25.30 min; 42.80% total peak area); and humulene (retention time =27.88 min; 8.39% total peak area), (C) FFD-CN: eugenol (retention time=50.35 min; 51.26% total peak area); methylvinyl (2-methylpent-3-yloxy) (retention time=25.30 min; 25.49% total peak area); phenol, 2-methoxy-4- (2-propenyl)-acetate (retention time=54.06 min; 7.26% total peak area); caryophyllene (retention time=24.01 min; 0.02% total peak area); and humulene (retention time =27.44 min; 2.16% total peak area), and (D) FFD-CN after stored: eugenol (retention time=50.35 min; 43.18% total peak area); 10,10-Dimethyl-2,6- dimethylenebicyclo (7.2.0) undecane (retention time=25.30 min; 30.17% total peak area); phenol, 2-

methoxy-4- (2-propenyl)-acetate (retention time=54.02 min; 7.53% total peak area); caryophyllene (retention time=23.92 min; 0.02% total peak area); and humulene (retention time =27.45 min; 5.05% total peak area).

3.3.4 Antimicrobial activity of FFD-CN

The MIC of CN and FFD-CN against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus*) was evaluated during storage for 3 months using the minimal inhibitory concentration assay as presented in **Table 3.2.** Both CN and re-dissolved FFD-CN exhibited antimicrobial activities against both Gram negative (*E. coli* and *P. aeruginosa*) and Gram positive (*S. aureus*) which are known to be due to the action of the hydroxyl group of eugenol and other phenolic compounds in clove essential oil (Ulanowska & Olas, 2021). The inhibition of the clove oil nanoemulsion against Gram-positive bacteria may result from the lack of the outer membrane of the cell wall of Gram-positive bacteria, enhancing the diffusion of the nano-sized clove essential oil into the bacterial cell. The antimicrobial effect of clove oil on Gram-negative bacteria has been reported to be caused by the binding of eugenol's hydroxyl group to enzymes such as amylase, histidine decarboxylase, and protease, resulting in an inhibition the enzymes' action (Swamy et al., 2016; Ulanowska & Olas, 2021). The phenolic compounds in clove essential oil can also alter the bacterial cell structure and increase the cytoplasmic membrane permeability, leading the leakage of intracellular components, changing cell morphology and consequently causing cell death (Anwer et al., 2014; Burt, 2004).

According to **Table 3.2**, the MIC of CN and FFD-CN were similar at the initial time. After 2 months of storage at 25 °C, the antimicrobial activities of CN against *E. coli*, *S. aureus*, and *P. aeruginosa* decreased (MIC increased), whereas those of FFD-CN did not change during 3 months of storage. The results occurred probably because eugenol in the nanoemulsion system was effectively encapsulated in the form of FFD-CN, as evident by the GC-MS results of the volatile profiles of CN and FFD-CN. It was possible that the Methocel^{TM} which was used as wall material for foam-mat freeze-dried encapsulation may increase the stability of the essential oil (Mahdi et al., 2021). Moreover, according to Cruz-Tiradoa et al. (2020), the foam structure can accelerate the diffusion of essential oils from within the foams to the

surface. When rehydrated, the expansion of the FFD-CN structure facilitates the migration of phenolic compounds from the internal layers to the surface of the foam. Consequently, the FFD-CN system can deliver the bioactive compounds to the microbial cell membrane via fine droplets of clove essential oil (Moghimi et al., 2015).

Table 3.2 The minimal inhibitory concentrations of CN and FFD-CN against *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), and *Pseudomonas aeruginosa* (*P. aeruginosa*).

sample	months	Minimal inhibitory concentration (mg/mL)		
		E. coli	*S. aureus*	*P. aeruginosa*
CN	0	7.75^{a}	3.87^{b}	3.87^{b}
	1	7.75^{a}	3.87^{b}	3.87^{b}
	2	7.75^{a}	15.50^{a}	3.87^{b}
	3	7.75^{a}	15.50^{a}	7.75^{a}
FFD-CN	0	7.75^{a}	3.87^{b}	3.87^{b}
	1	7.75^{a}	3.87^{b}	3.87^{b}
	2	7.75^{a}	3.87^{b}	3.87^{b}
	3	7.75^{a}	3.87^{b}	3.87^{b}

Clove essential oil nanoemulsion (CN); Foam-mat freeze-dried clove essential oil nanoemulsion (FFD-CN). Different data letters within a same column indicate statistically significant differences ($p < 0.05$).

3.3.5 Morphology of bacteria cell responded to FFD-CN

The effect of CN and FFD-CN on the inhibition of *E. coli*, *S. aureus*, and *P. aeruginosa* were investigated. All tested bacterial cells treated and untreated with CN and FFD-CN were observed using SEM-EDS and the images are shown in **Fig. 3.5.** FFD-CN and CN exhibited antimicrobial activity against *E. coli*, *S. aureus*, and *P. aeruginosa* by causing morphological destructions of the bacterial cell membrane, leading to the leakage of internal cellular materials. The alterations of bacterial cell morphology were not observed in all untreated bacteria. The antimicrobial effects of clove oil nanoemulsion on the bacterial cell agree well with the previous reports of the

effects of various essential oil emulsions in the literature (Lin et al., 2017; Ulanowska & Olas (2021).

The SEM-EDS micrographs suggested both FFD-CN and CN had the antimicrobial effects against all the tested bacteria. These results can be explained by the fact that the small oil droplets of the nanoemulsion enhanced the ability of the antimicrobial agents in clove essential oil to interact with the bacterial cells (Lu et al., 2018). Moreover, the results may be attributed to the properties of Methocel^{TM} used in FFD-CN, which has been reported to have an ability as a binder due to its viscous polymeric structure and thus can effectively retain bioactive compounds of the clove oil (Rattanapitigorn et al., 2016). The results suggested that the FFD-CN can maintain the antimicrobial activity of the clove essential oil nanoemulsion.

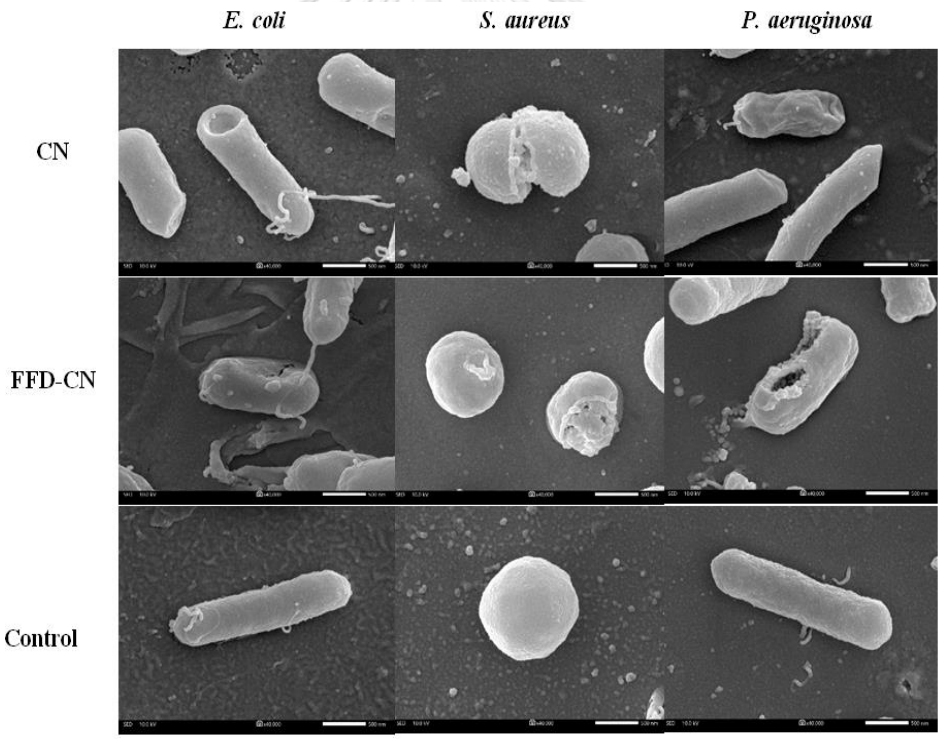

Fig. 3.5 Scanning electron microphotographs of *E. coli*, *S. aureus,* and *P. aeruginosa* untreated (control) and treated with FFD-CN and CN.

3.4 Conclusion

The optimum conditions for the fabrication of FFD-CN were 3% (v/v) Methocel^{TM}, 60 °C drying temperature, and 72 h drying time. These optimal conditions were achieved using RSM Box-Behnken design. The particle size and PDI of the FFD-CN showed some changes during 3 months storage at 25 °C, but they remained under the range of optimal nanoemulsion. The lower loss of eugenol content in FFD-CN than that in CN after storage at 25 °C for 3 months, as analyzed using SPME-GC-MS, suggested the better encapsulation efficacy and chemical stability of the FFD-CN than those of the liquid-formed nanoemulsion. The bacterial cell morphological deformation was evident after being exposed to FFD-CN or CN. After 3 months storage, the antimicrobial abilities of FFD-CN against *E. coli*, *P. aeruginosa*, and *S. aureus* were higher (lower MIC) than those of CN. The FFD-CN exhibited good stability and maintained antimicrobial activity during the storage. These results suggested that transformation of liquid CN into FFD-CN can effectively preserve stability and antimicrobial activity throughout the storage.

3.5 Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

3.6 Data availability

The authors do not have permission to share data.

3.7 Acknowledgement

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3.8 Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102369.

CHAPTER IV
MANUSCRIPT III

Foam-mat freeze-dried clove essential oil nanoemulsion for preserving quality of whiteleg shrimp

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Abstract

Foam-mat freeze-dried clove essential oil nanoemulsion (FFD-CN) was investigated for its efficiency to maintain quality of whiteleg shrimp during chilled storage (4 ± 2 °C), in comparison to clove essential oil nanoemulsion (CN) and distilled water (control). The whiteleg shrimps were treated with CN, FFD-CN or Control and measured for trimethylamine (TMA), total volatile basic nitrogen (TVB-N), total viable count (TVC), texture, color, and pH. In addition, solid phase microextraction-gas chromatography/mass spectrometry (SPME-GC-MS) was used to investigate the volatile compounds of the shrimp. CN and FFD-CN were found to be effective in inhibiting bacteria in whiteleg shrimp. FFD-CN was more effective than CN in

delaying the changes in TMA, TVB-N, TVC, texture, color, and pH. Based on the TVB-N value, Control, CN, and FFD-CN treated shrimp had the shelf life of 6, 8, and 10 days, respectively. The SPME-GC-MS results suggested that FFD-CN could maintain a higher concentration of eugenol than CN, suggesting that eugenol in the nanoemulsion system was effectively encapsulated in FFD. These results indicated that both FFD-CN and CN can effectively be used as a natural preservative for maintaining the quality of whiteleg shrimp. However, FFD-CN outperformed CN for preserving the quality of whiteleg shrimp.

Keywords: Natural preservative; Foam; Freeze-drying; Nanoemulsion; Antimicrobial activity

4.1 Introduction

Whiteleg shrimp (*Litopenaeus vannamei*) is a valuable economic animal that is in high demand as a food source in Thailand and many countries (Imaizumi et al., 2022; Zhang et al., 2015). However, whiteleg shrimp has a short shelf life and its quality gradually decreases during the process of post-harvest, storage, and transportation. The quality deterioration and safety loss of whiteleg shrimp are caused mainly by bacteria (Masri et al., 2021; Zhang et al., 2015), which are found in the muscles, intestinal, and digestive systems of shrimp. For instance, H_2S and indole from bacteria cause spoilage in shrimp (Liu et al., 2020). Chemical treatment, pasteurization, and high-hydrostatic pressure (Li et al., 2015; Shaikhmahamud et al., 2022; Wang et al., 2018) have been reported to inhibit food spoilage in shrimp. However, using high pressure to inhibit food spoilage bacteria may increase melanosis, an unappealing discoloration, on shrimp (Montero et al., 2001). The chemicals that can be used in food systems to inhibit melanosis in food are limited by their off-flavors, off-odors, and toxicity (Chen et al., 1991b). The pasteurization processes used in the industry do not kill all microorganisms in foods, but they kill the pertinent target pathogens and reduce spoilage organisms that may grow during storage (Silva & Gibbs, 2010). Moreover, thermal processes such as pasteurization may result in a loss of moisture, textural properties, and sensory attributes (Wang et al., 2018). Due to the negative

impacts on the nutritive values and sensory attributes of the shrimp, those methods cause unacceptable shrimp quality to most consumers (Bindu et al., 2013; Shaikhmahamud et al., 2022). Currently, a natural antimicrobial agent from plant essential oil, has been proposed as an alternative method to control the safety and quality of whiteleg shrimp.

Clove essential oil, which contains eugenol and eugenyl acetate, is recognized as an effective antimicrobial agent and has antimicrobial activity against a wide range of microorganisms (Zahi, Liang, &Yuan, 2015). However, it is hydrophobic and has a potent odor, which can affect food's organoleptic characteristics. Therefore, clove essential oil cannot be used directly in foods (Chuesiang et al., 2018). Emulsions or nanoemulsions of essential oils can be more compatible with food. Due to its small particle size, nanoemulsion provides more benefits than a traditional emulsion system, such as increasing bioactivity, improving diffusion, and reducing effects on food's organoleptic quality. Small droplets, which range in size from 20 to 200 nm, are transparent and stable for gravity separation or particle aggregation (Chuesiang et al., 2018; Pilong et al., 2022). Nanoemulsions present stable kinetics in terms of small size and narrow distribution and are capable of adapting to inhibit bacteria in food. However, the liquid state of the nanoemulsion cannot maintain the stability of the small droplet size during storage. It has been suggested that transforming liquid nanoemulsion into dried state by using foam-mat freeze-drying (FFD) solves such problems (Pilong et al., 2023).

The two phases of FFD are air (dispersed phase) and liquid (continuous phase) (Ruengdech & Siripatrawan, 2022). FFD is to turn a liquid product into a stable foam by whipping the mixture of product and surfactant and then dehydrating it by freeze-drying. The obtained FFD has low bulk density, high porosity which can encapsulate bioactive ingredients, and long-term stability when rehydrated (Muthukumaran et al., 2008). To date, the formation of FFD-CN and its effectiveness in inhibiting spoilage bacteria and preserving the quality of whiteleg shrimp have never been studied. This research is the first to apply FFD-CN as a natural preservative to preserve the quality of whiteleg shrimp. The physical, chemical, and microbiological properties of the FFD-CN treated whiteleg shrimp was investigated in comparison to those treated with CN, and distilled water (control).

4.2 Materials and methods

4.2.1 Materials

Pure clove essential oil batch No. 0009115315 was purchased from CT Chemicals Ltd. (Laksi, Bangkok, Thailand). A food-grade non-ionic polysorbate 80 surfactants (Tween® 80) and Methocel™ were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Whiteleg shrimp was purchased at Talaadthai market, Pathum Thani, Thailand. The shrimp was packed in a foam box with immediate ice and transported to the laboratory within approximately 1 hour. The ratio of shrimp to ice was 1 : 2 (w/w). The peptone water and plate count agar were purchased from Laboratories Pvt. Ltd. (Dindori, Nashik, India). The methanol and formic acid were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). The hydrochloric acid (HCl) and ethanol were purchased from Qchemical Co., Ltd. (Nong Khaem, Bangkok, Thailand). The boric acid, trichloroacetic acid (TCA), potassium carbonate, formaldehyde, bromocresol green, and methyl red were purchased from Elago Enterprises Pty. Ltd. (Cherrybrook, New South Wales, Australia).

4.2.2 Fabrication of FFD-CN

The CN was prepared from 3% (v/v) non-ionic surfactant (Tween®80), 1% (v/v) clove essential oil, and deionized water as a continuous phase, using an ultrasonic bath (Power sonic 410, Hwashin Tech Ltd, Gangnam-gu, Seoul, Korea) following the method described by Pilong et al. (2023). The ultrasonication was operated at a fixed operation frequency of 43 kHz at 40 °C for 20 min. For the FFD-CN preparation, the CN was mixed with Methocel™ at 3% (v/v) in a 250-watt kitchen blender (Oster®, Sunbeam Products, Inc, Boca Raton, FL, USA), with the maximum speed at 25 °C for 5 minutes. After that, 500 mL of the sample was placed in an aluminum tray (20 cm × 30 cm) and dried in a freeze dryer (Stoppering tray dryer, Labcondo Ltd, Fort Scott, KS, USA) at 60 °C drying temperatures for 72 h. The mean droplet diameter, polydispersity index (PDI), stability, appearance, and minimum inhibitory concentration (MIC) against foodborne pathogens of the FFD-CN, CN, and clove oil were characterized in our previous report.

4.2.3 Preparation of whiteleg shrimp

The whiteleg shrimp (*Litopenaeus vannamei*) was chosen in the present study because it is an important food source in Thailand (Imaizumi et al., 2022; Zhang et al., 2015). The head of the shrimp was removed. After that, the shrimps were washed clean with distilled water and graded according to size; they were not peeled or cut at the tail. The shrimp had approximately 20 ± 2 g in weight and 2.12 ± 2 cm x 10 ± 2 cm in width x length, was randomly divided into 3 groups. Before use, the FFD-CN was dissolved in deionized water to have the final concentration similar to that of CN. Each group was separately dipped in FFD-CN solution, CN, or distilled water for 10 minutes at 25 °C. After dipping, the shrimp was drained for 3 minutes (Khaledian et al., 2021; Yuan et al., 2016). The samples (60 g) were packaged in a polyethylene bag (15 cm x 23 cm), heat-sealed, and kept at 4 ± 2 °C. The samples from all treatments were randomly samples for physical, chemical, and biological analyses on days 0, 2, 4, 6, 8, and 10 of the storage periods.

4.2.4 Microbiological analysis

The total viable count (TVC) of whiteleg shrimp was determined according to the method of Baek et al. (2021). Twenty grams of the whiteleg shrimp from each treatment was aseptically transferred to a stomacher bag containing 180 mL of sterile peptone water. The mixture was homogenized for 2 min at 230 rpm in a stomacher (Stomacher®400 circulator, Seward, MO, USA). Then, 10-fold dilutions were made. After that, 1 mL of each dilution sample was added to sterile plate count agar using the spread plate method and incubated at 37 °C for 48 h. The experiment was carried out in triplicate. The microbial count was expressed as log CFU/g of shrimps, after counting the colonies.

4.2.5 Physical properties

4.2.5.1 Color measurement

The color of the whiteleg shrimp was measured using a chromameter (CR-400, Konika Minolta Sensing Inc., Chiyoda-ku, Tokyo, Japan) with a light source that satisfies the de l'Eclairage (CIE) standard for illumination D65 (Yuan et al., 2016). Redness/greenness (a*, +a* = red, -a* = green), lightness (L*, 100 = white, 0 = black), and yellowness/blueness (b*, +b* = yellow, -b* = blue) values of the

samples were obtained from five locations of each shrimp on days 0, 2, 4, 6, 8, and 10 of the storage. The mean color values were calculated from five replicates. The total color differences (ΔE values) indicate the amount of color difference between whiteleg shrimp on the first and 10 days of chilled storage. The ΔE value of whiteleg shrimp was calculated using **Equation 1**.

$$\Delta E = \sqrt{(L_t^* - L_{t0}^*)^2 + (a_t^* - a_{t0}^*)^2 + (b_t^* - b_{t0}^*)^2} \quad (1)$$

where t0 and t specify the amount of parameters on the first and 10th days of storage, respectively (Khaledian et al., 2021).

4.2.5.2 Texture analysis

The texture of the whiteleg shrimp was measured using a calibrated TA-XT2 texture analyzer (TA-XT plus, Godalming, Surrey, UK) at 25 °C (Xu et al., 2018). It was compressed using a uniaxial compression test and a 100 mm compression plate (P/100). The constant speed test was 1 mm/s; sample deformation was 50%; and trigger force was 0.05 N. The hardness of the sample was obtained from each shrimp on days 0, 2, 4, 6, 8, and 10 of storage. The parameters of hardness were tested and calculated from five replicates.

4.2.6 Chemical properties

4.2.6.1 Measurement of TVB-N and TMA

The TVB-N and TMA values of the samples were determined following the Conway method (Conway, 1936) and Sanguandeekul et al. (2008). Briefly, 4% trichloroacetic acid (TCA) solution was mixed with 5 g of minced whiteleg shrimp for 30 min at 25 °C. The mixture was filtered through Whatman No. 1 and 1 mL was pipetted to the outside ring of a Conway glass dish. A saturated potassium carbonate solution was added to the outside ring of the dish on the opposite side of the sample. One milliliter of boric acid was added to the inside ring of the dish. In addition, the TMA value was added to 1 mL of the formaldehyde in the sample solution before incubating at 37 °C for 1 hour. After that, the inner solution was then titrated with 0.01 N hydrochloric acid (HCl) until the solution color changed

from green to pink. A blank was 4% TCA. The concentrations of TVB-N and TMA (in mg N/100 g sample) were calculated using **Equation 2** and **Equation 3**, respectively.

$$\text{TVB-N (mgN/100g)} = \frac{(N_{HCL})(A_N)(V_S - V_B)(V_{TCA})(100)}{W_S} \quad (2)$$

$$\text{TMA (mgN/100g)} = (V_S\text{-}V_B) \times (N_{HCL} \times A_N) \times \frac{\left[\left(W_S \times \frac{M}{100}\right) + V_{TCA}\right] \times 100}{W_s} \quad (3)$$

where V_S is the titration volume between HCl and sample, V_{TCA} is the total volume of sample and TCA solution, V_B is the titration volume between HCl and blank, N_{HCL} is the normality of HCl, A_N is the atomic weight of nitrogen, W_S is the weight of the sample, and M is the percentage of sample moisture.

4.2.6.2 pH measurement

Ten grams of the whiteleg shrimps were homogenized with 100 mL of distilled water and pH was measured in triplicate using a digital pH meter (Mettler-Toledo, Ltd., Nanikon, Zurich, Switzerland).

4.2.6.3 Volatile profile of whiteleg shrimp

The volatile compounds of the whiteleg shrimps were collected using solid phase microextraction (SPME) technique as previously described by Soncin et al. (2009) with minor modifications. Briefly, 2 g of whiteleg shrimps were cut and part into a 20 mL glass vial (Chromselection, Vicenza, Veneto, Italy). The vial was then firmly sealed with an aluminum crimp cap containing a PTFE silicone septum (Chromselection, Agilent Technologies Inc., Santa Clara, CA, USA) using a hand crimper (Model 20001, Kebby Industries Inc., Rockford, IL, USA). After 10 min of sample equilibration at 40 °C, the 23 Ga Autosampler (Gray) (Supelco, Inc., Bellefonte, PA, USA), equipped with a 50/30 m divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber, was immediately introduced into the GC (Auto system XL, PerkinElmer Inc., Waltham, MA, USA) injection port while operating in the spitless mode at 250 °C for 2 min.

The GC column was a DB-wax capillary column (30 m x 250 µm, and 0.25 µm film thickness) (Agilent 7980A Restek, Santa Clara, CA, USA). The temperature of the GC oven was gradually raised from 50 °C (hold for 5 min) to 125 °C (hold for 3 min) at 3 °C min^{-1}, to 180 °C (hold for 3 min) at 2 °C min-1, and ultimately to 250 °C (hold for 5 min) at 6 °C min^{-1}. At a flow rate of 1 mL/min, a split ratio of 100:1, injector and detector temperatures of 250 °C, and a sample volume of 1 L, helium was employed as the carrier gas. The GC (5977B Turbomass MS, PerkinElmer Inc., Waltham, MA, USA) and MS was connected. The MS was scanned at 70 eV for the mass range of 40 to 500 while the ion source temperature was 230 °C. The chemical constituents of whiteleg shrimp treated with CN, FFD-CN, and distilled water were tested after storage for 0 and 6 days at 4 ± 2 ^{o} C. Data from the National Institute of Standards and Technology Library (NIST2017) were used to identify the volatile compounds.

4.2.7 Statistical analysis

The experimental results from three replications were expressed as mean values and standard deviation. A completely randomized design (CRD) was used to determine the effect of FFD-CN on whiteleg shrimp, including the physical, chemical, and microbiological properties of shrimp. The analysis of variance (ANOVA) was analyzed to examine the statistically significant differences between the means being compared using Duncan's new multiple range test the $p \leq 0.05$. The statistical analysis was performed using IBM SPSS Statistics Software (version 26.0, IBM SPSS Inc., Armonk, NY, USA).

4.3 Results and discussion

4.3.1 CN and FFD-CN characteristics

The CN and FFD-CN used in this study were prepared following the method developed by Pilong et al. (2022) and Pilong et al. (2023). The CN of non-aggregated tiny spherical droplets of 30.76 nm with a narrow PDI of 0.179 was observed throughout the continuous phase of the nanoemulsion, of which its mean droplet

diameter did not significantly ($p > 0.05$). The FFD-CN was non-aggregated tiny spherical droplets of 27.46 nm with a PDI of 0.108 after dissolution, of which its mean droplet diameter did not significantly ($p > 0.05$) change during storage for 3 months at 25 °C. The CN and FFD-CN were used for minimum inhibitory concentration (MIC) after preparation and after 3 months of storage at 25 °C. The MIC of FFD-CN against *S. aureus, E. coli*, and *P. aeruginosa* was 3.87, 7.75, and 3.87 mg/mL, respectively. The MIC of CN against *S. aureus, E. coli*, and *P. aeruginosa* was 15.50, 7.75, and 7.75 mg/mL, respectively. As previously reported when the FFD-CN was examined by gas chromatography-mass spectrometry (GC-MS), the FFD-CN can effectively maintain stability and antimicrobial activity throughout 3 months storage at 25 °C and can preserve eugenol, a major volatile bioactive compound in clove essential oil, in its structure better than a liquid-formed nanoemulsion.

4.3.2 Microbiological properties of shrimp treated with CN or FFD-CN

The TVC of the whiteleg shrimp was determined during storage for 0, 2, 4, 6, 8, and 10 days. The results of TVC expressed as log CFU/g are displayed in **Fig. 4.1**. The initial number of TVC in shrimp treated with distilled water was 4.40 log CFU/g, whereas those of shrimp treated with CN and FFD-CN were 2.94 and 2.64 log CFU/g, respectively. The TVC of the shrimp from the control group was 7 log CFU/g on day 6 which exceeded the safety limit for shrimp as suggested by Khodanazary (2019). Whereas, the TVC of the shrimp treated with CN and FFD-CN was below 5 log CFU/g throughout the storage period. Based on the TVC values, control samples had a shelf life of less than 6 days, whereas FFD-CN and CN treated samples had TVC below the safety limit throughout 10 days of storage. Since FFD-CN can effectively encapsulate eugenol nanoemulsion in its matrix, the FFD-CN contained a higher amount of bioactive compounds including eugenol, caryophyllene, and humulene when compared to CN. When the FFD-CN was dissolved, the nanoemulsion can deliver the bioactive compounds to the microbial cell membrane via fine droplets of clove oil, affecting the bioactivity of bacteria (Anwer et al., 2014). Moreover, the tiny droplets of the nanoemulsion can accomplish slow-releasing and antimicrobial

activity throughout the storage process (Pilong et al., 2023). Our results agree well with the study of Beak et al. (2021) found that shrimp coated with nanoparticles of sodium alginate containing grapefruit seed extract had better quality than shrimp without nanoparticle coating, and its TVC was not exceeded 7 log CFU/g during 8 days of storage, which is the safety limit for shrimp.

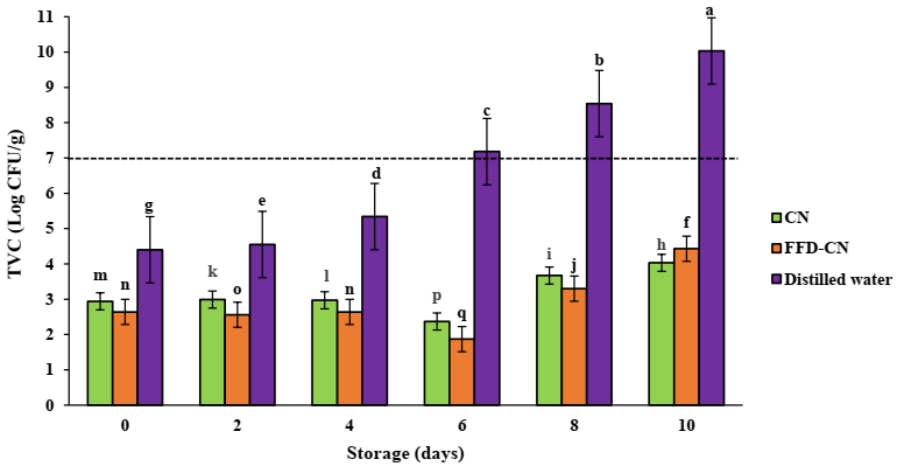


Figure 4.1 TVC of whiteleg shrimp treated with CN, FFD-CN, and distilled water during chilled storage. Different data letters in the figure indicate statistically significant differences ($p \leq 0.05$). *The maximal TVC concentration (7 log CFU/g) in whiteleg shrimp is shown by the dashed line.

4.3.3 Physical properties of shrimp treated with CN and FFD-CN

4.3.3.1 Color

The color of whiteleg shrimp, which influence the consumer's purchasing decision, can be measured to determine their quality. After treatment, the ΔE values of the whiteleg shrimp treated with FFD-CN and CN were not significantly different ($p \leq 0.05$) from those before treatment. During chilled storage, the ΔE values of the CN, FFD-CN, and control samples increased with time storage (**Fig. 4.2A**). The ΔE value of the control sample was highest at the end of storage ($p \leq 0.05$). However, an increase in ΔE value of the whiteleg shrimp was slower for the FFD-CN treated shrimp than those for the CN treated. The reasons could be attributed to the delivery efficacy of clove essential oil in the nanoemulsion system, which was encapsulated in

the FFD, can gradually release essential oil from the nanoemulsion system with time (Pilong et al., 2023). Similarly, Yuan et al. (2016) reported that the chitosan coating combined with pomegranate peel extract on the quality of shrimp, which had a lower ΔE value.

4.3.3.2 Texture

The hardness of the whiteleg shrimp is shown in **Fig. 4.2B**. The hardness of shrimp treated with CN and FFD-CN had no significant difference ($p > 0.05$) during storage at 4 ± 2 ^{o}C, whereas the hardness of control gradually decreased during storage. The deterioration in shrimp muscle may be brought on by the hydrolysis of cellular compounds by bacterial and intracellular enzymes during storage (Abdou et al., 2018), which decreases the muscle's ability to bind water (Masniyom et al., 2005). The obtained results demonstrate the efficiency of the FFD to encapsulate and protect clove essential oil in the CN from adverse chemical reactions (Chuesiang et al., 2020; Pilong et al., 2023).

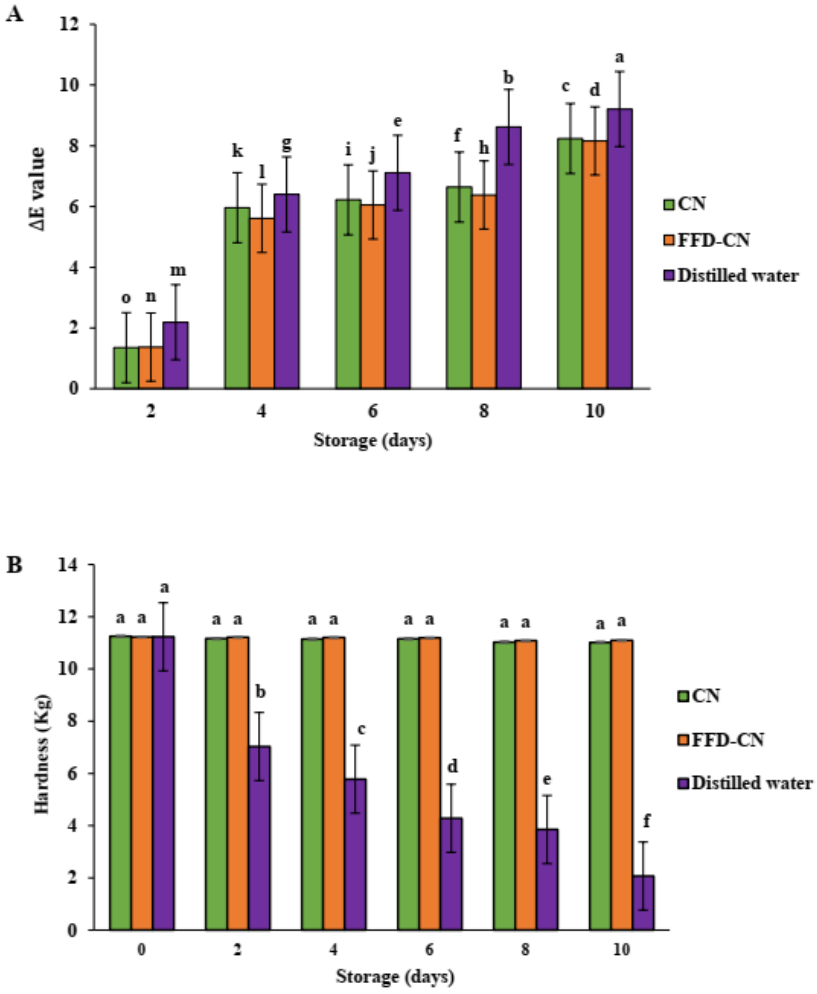


Figure 4.2 The ΔE values (A) and hardness (B) of whiteleg shrimp treated with CN, FFD-CN, and distilled water during chilled storage. Different data letters in the figure indicate statistically significant differences ($p \leq 0.05$).

4.3.4 Chemical properties of shrimp treated with CN and FFD-CN

4.3.4.1 TVB-N and TMA

The TVB-N values of whiteleg shrimp during storage are shown in **Fig. 4.3A**. TVB-N is created by microorganisms and enzymatic activities involved in the degradation of proteins and nonproteins nitrogenous compounds (Ozogul et al., 2017). At the end of storage, the TVB-N of the control samples increased significantly ($p \leq 0.05$) from 17.97 to 42.63 mg/100 g sample. Whereas samples from

the CN and FFD-CN groups had TVB-N values changed from 16.14 to 34.71 and 17.05 to 28.93 mg/100 g sample, respectively, after 10 days of storage. As suggested by Khodanazary (2019), the value of TVB-N for shrimp freshness was limited at a 30 mg/100 g sample to be in an acceptable quality. Therefore, based on the TVB-N value, the Control sample exceeded the acceptable limit after 8 days whereas the CN and FFD-CN treated samples were acceptable throughout the storage. It should be noted that an increase in TVB-N values of the shrimp treated with FFD-CN was slower than those treated with CN throughout the storage. The results occurred probably because the active components in the clove essential oil nanoemulsion was effectively encapsulated in the form of FFD, which, when dissolved in deionized water, was able to gradually release the active components from the nanoemulsion system (Muthukumaran et al., 2008).

The initial TMA value of whiteleg shrimp sample in this study was 3.08 mg/100 g sample and reached 10.63, 9.79, and 13.98 mg/100 g sample on day 10 of storage when treated with CN, FFD-CN, and Control, respectively **(Fig. 4.3B)**. For shrimp, the value of 10 mg/100 g is typically regarded as the limit of acceptable quality, meaning sample with a TMA value higher than 10 mg/100 g should be rejected due to quality degradation caused by off-flavor (Zhang et al., 2015). The TMA values of CN and FFD-CN treated samples were observed to be significantly ($p \leq 0.05$) lower when compared to those of control, indicating that the use of CN and FFD-CN could delay the changes in TMA of the samples. Clove essential oil has eugenol as its major component, which can give an antimicrobial action by altering protein characteristics and preventing the protease activity of bacteria (Pilong et al., 2022). In addition, a slower increase in TMA value was found in the FFD-CN-treated sample when compared to that of the CN-treated samples. The GC-MS results (section 4.3.4.3) suggested that FFD-CN had higher eugenol concentrations than CN. Thus, FFD was able to not only guardedly encapsulate but also gradually and efficiently release the active components of clove essential oil in the nanoemulsion (Muthukumaran et al., 2008). These results suggested that the transformation of liquid CN into FFD-CN can effectively preserve the eugenol and its antimicrobial activity throughout the storage (Pilong et al., 2023).

4.3.4.2 pH

The changes in the pH values of CN, FFD-CN, and distilled water treated whiteleg shrimp during storage are shown in **Fig. 4.3C**. The increase in pH of the FFD-CN and CN treated samples were slower than that of the control groups. The pH values of shrimp increased with storage time as a result of the buildup of alkaline substances including triethylamine, dimethylamine, and ammonia produced during the deterioration process of shrimp (Khodanazary, 2019; Liu et al., 2020; Ozogul et al., 2017). Various studies have reported that different coatings and preservatives such as sweet potato starch, corn starch, and chitosan can slow down an increase in pH of shrimp during storage by inhibiting microbial growth and preventing the activity of endogenous proteolytic enzymes (Alotaibi & Tahergorabi, 2018; Aseel et al., 2017). The results occurred probably because bioactive compounds including eugenol in the clove essential oil was effectively encapsulated in the form of FFD-CN and gradually released from the nanoemulsion system with time (Pilong et al., 2023; Zahi et al., 2015).

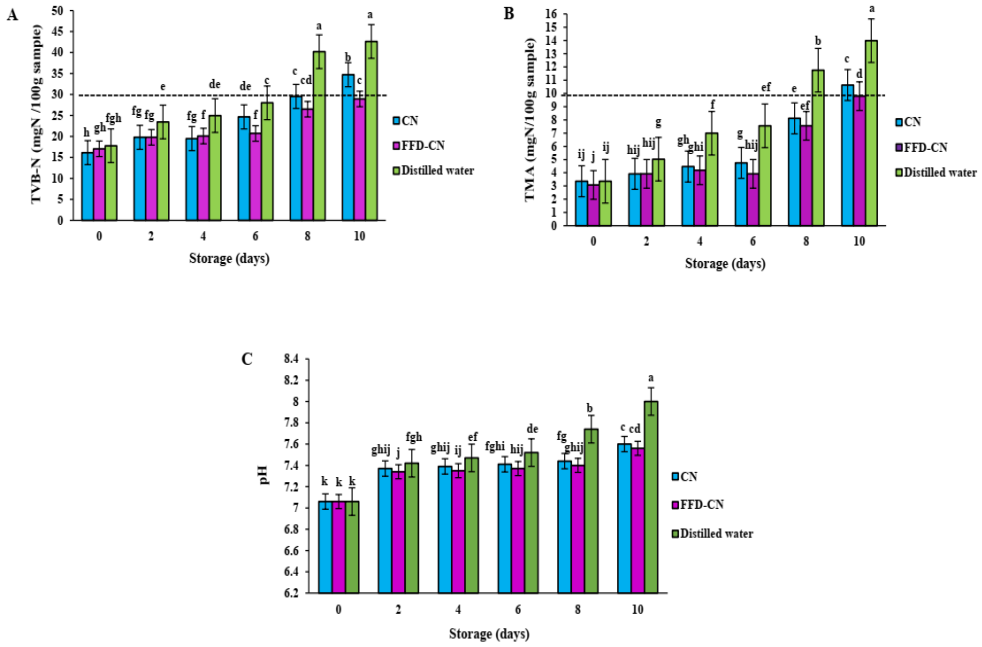
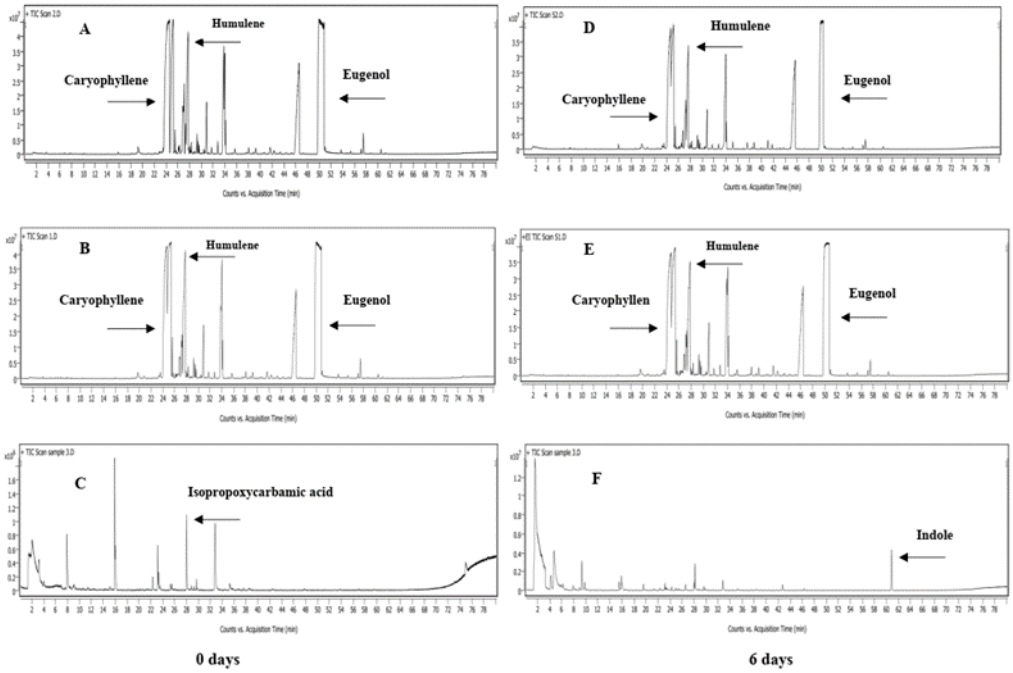


Figure 4.3 TVB-N values (A), TMA values (B), and pH (C) of whiteleg shrimp treated with CN, FFD-CN, and distilled water during chilled storage. Different data letters in the figure indicate statistically significant differences ($p \leq 0.05$). The limit value of TVB-N for whiteleg shrimp freshness is 30 mg/100 g sample. The dashed line represents the limit value of TMA for whiteleg shrimp freshness (10 mg/100 g sample).

4.3.4.3 Volatile compounds of treated whiteleg shrimp

GC chromatograms of the volatile profiles of the whiteleg shrimp treated with CN, FFD-CN, and distilled water were determined on day 0 and 6 of the storage, as shown in **Figs. 4.4A-F**. Based on the TVB-N value, the sample began to spoil on day 6 of storage. FFD-CN contained a higher amount of eugenol, caryophyllene, and humulene when compared to CN. The higher eugenol content (30.93% and 28.76%) in the FFD-CN was found when compared to that of CN (29.94% and 25.70%) on days 0 and 6, respectively. The results occurred probably because eugenol in the nanoemulsion system was effectively encapsulated in FFD. These suggested that clove essential oil was better preserved in the matrix of FFD-CN than in the liquid-

formed CN, as evident by GC-MS results. These results agree with those of Pilong et al. (2023), who studied the encapsulation of CN using a freeze-drying process and found that the wall materials used for FFD-CN encapsulation may increase the stability of CN. According to Cruz-Tiradoa et al. (2020), foam structure expedites the diffusion of essential oils from within the foam to the surface. When dissolved in water, phenolic compounds migrate from the internal layer of foam to the foam's surface as a result of the expanding of the FFD-CN structure. The presence of indole was found in control samples after 6 days of storage, which may be caused by bacterial growth during the storage at 4 ± 2 ^{o}C (Snellings et al., 2003). However, indole was not detected in FFD-CN and CN treated samples probably because eugenol, caryophyllene, and humulene found in FFD-CN and CN have the potential to delay bacterial growth (Satyal et al., 2012). The result suggests that FFD-CN outperformed CN. The formation of CN into FFD was able to improve the stability and antimicrobial activity of the nanoemulsion, which can effectively be used as a natural preservative for maintaining the quality of whiteleg shrimp.



4.4 GC chromatograms of volatile compounds on whiteleg shrimp treated with CN (A, D), FFD-CN (B, E), and distilled water (C, F) after storage at 4 ± 2 °C for 0 and 6 days, respectively. (A) CN: eugenol (retention time = 50.35 min; 29.94% total peak area); caryophyllene (retention time = 24.20 min; 11.26% total peak area); and humulene (retention time = 27.77 min; 7.10% total peak area, (B) FFD-CN: eugenol (retention time =50.35 min; 30.93% total peak area); caryophyllene (retention time = 25.30 min; 35.41% total peak area); and, humulene (retention time = 27.81 min; 8.94% total peak area), (C) distilled water: Isopropoxycarbamic acid (retention time =28.01 min; 10.27% total peak area), (D) CN: eugenol (retention time = 50.35 min; 25.70 % total peak area); caryophyllene (retention time = 25.30 min; 35.35 % total peak area); and humulene (retention time = 27.27 min; 1.59 % total peak area, (E) FFD-CN: eugenol (retention time =50.35 min; 28.76 % total peak area); caryophyllene (retention time = 25.30 min; 34.07 % total peak area); and, humulene (retention time = 27.82 min; 8.40 % total peak area), and (F) distilled water: Indole (retention time = 60.86 min; 3.08% total peak area).

4.4 Conclusion

The FFD-CN can retard bacterial growth and delayed the production of TVB-N values, TMA values, and pH, including preventing the alteration of the color and

texture qualities of the sample more effectively when compared to those of CN. The volatile compounds of all samples showed that FFD-CN could maintain a higher concentration of eugenol than CN, suggesting better encapsulation efficacy and chemical stability of the FFD-CN than those of the liquid-formed nanoemulsion. Whereas the quality of distilled water-treated shrimps was significantly changed. FFD-CN and CN could maintain the qualities of the whiteleg shrimp for 10 and 8 days, respectively, when compared to the Control sample. Therefore, FFD-CN has a high potential to be used as a natural antimicrobial agent to maintain the quality of shrimp.

4.5 Acknowledgements

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CHAPTER V
CONCLUSION AND FUTURE WORKS

5.1 Conclusions

This research presented the optimal conditions for the formation of clove essential oil nanoemulsion using the microfluidizer was 1% (v/v) clove essential oil, 3% (v/v) Tween®80, and 10,000 psi of operating pressure. The smallest mean droplet diameter (30.76 nm), and PDI (0.179) were achieved with -50.01 mV of ζ-potential and 7.81 cP of viscosity. The nanoemulsion was stable when stored for 48 h at 5 °C or 45 °C. GC-MS revealed that clove essential oil nanoemulsion was chemically stable during storage at 5 °C for 48 h. The antimicrobial activity of clove essential oil nanoemulsions against *S. aureus, E. coli*, and *P. aeruginosa*, was higher (lower MICs) than that of clove essential oil. Moreover, bacterial cell morphological deformation of all tested bacteria when exposed to clove essential oil nanoemulsion or clove essential oil was also evident. This research has shown that clove essential oil nanoemulsion has a high potential to be used as a natural antimicrobial agent.

Improving the FFD technique was used to maintain the stability and antimicrobial activity of CN. The optimum conditions for the fabrication of FFD-CN were 3% (v/v) Methocel™, 60 °C drying temperature, and 72 h drying time. These optimal conditions were achieved using RSM Box-Behnken design. The particle size and PDI of the FFD-CN showed some changes during 3 months storage at 25 °C, but they remained under the range of optimal nanoemulsion. The lower loss of eugenol content in FFD-CN than that in CN after storage at 25 °C for 3 months, as analyzed using SPME-GC-MS, suggested the better encapsulation efficacy and chemical stability of the FFD-CN than those of the liquid-formed nanoemulsion. The bacterial cell morphological deformation was evident after being exposed to FFD-CN or CN. After 3 months storage, the antimicrobial abilities of FFD-CN against *E. coli*, *P. aeruginosa*, and *S. aureus* were higher (lower MIC) than those of CN. The FFD-CN exhibited good stability and maintained antimicrobial activity during the storage. These results suggested that transformation of liquid CN into FFD-CN can effectively preserve stability and antimicrobial activity throughout the storage.

The effect of FFD-CN on the preserving qualities of the whiteleg shrimp during chilled storage was investigated. The FFD-CN can retard bacterial growth and delayed the production of TVB-N values, TMA values, and pH, including preventing the alteration of the color and texture qualities of the sample more effectively when compared to those of CN. The volatile compounds of all samples showed that FFD-CN could maintain a higher concentration of eugenol than CN, suggesting better encapsulation efficacy and chemical stability of the FFD-CN than those of the liquid-formed nanoemulsion. Whereas the quality of distilled water-treated shrimps was significantly changed. FFD-CN and CN could maintain the qualities of the whiteleg shrimp for 10 and 8 days, respectively, when compared to the Control sample. Therefore, FFD-CN has a high potential to be used as a natural antimicrobial agent to maintain the quality of shrimp.

5.2 Future works

To focus on the application of the developed FFD-CN for stability and antimicrobial agents in the food packaging industry, these FFD-CN should be continuously studied. The use of freeze-drying techniques with Methocel^{TM} was successful in improving the FFD-CN properties such as physical, chemical, stability, and antimicrobial activity in whiteleg shrimp. However, these properties probably change the storage period. For the long-term application of the FFD-CN in the food or food packaging industry, these properties should be investigated.

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