



UNIVERSITI PUTRA MALAYSIA

***PREPARATION OF ESSENTIAL OIL NANOEMULSIONS AND
APPLICATION AS EDIBLE COATING ON GUAVA***

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AS EDIBLE COATING ON GUAVA**

By
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ABSTRACT

Nanoemulsions possess powerful nano-scale properties that make them attractive for diverse applications that can be produce using low energy method or high energy method. This study aims to determine the effect of two oil phase which is essential oils (EO) and virgin coconut oil (VCO) as the carrier oil to produce nanoemulsion using high energy method which is high pressure homogenizer. The characterization of nanoemulsions produced was done by observing the appearance of nanoemulsions, analysing the droplets size of nanoemulsions and the stability of nanoemulsions during a month of storage at 25°C. The most stable nanoemulsions for each essential oil which are Formulation 2 for clove EO nanoemulsion and Formulation 4 for lemon EO were then used to incorporate with chitosan to produce edible coating on guava. These coatings were applied onto guavas by dipping the fruits into the solutions for 3 minutes. Guavas were dried in and stored in an air-conditioned room maintained at 25°C. Five samples of guava which are coated with chitosan only (CH), coated with chitosan containing clove nanoemulsion (CH-CEO (HPH)), coated with chitosan containing clove essential oil (CH-CEO), coated with chitosan containing lemon nanoemulsion (CH-LEO (HPH)) and coated with chitosan containing lemon essential oil (CH-LEO). The microbiological analysis of all sample were determined by total plate count and yeast and mould counts. Guava properties were analysed for coating effectiveness on weight loss, firmness and pH during storage at temperature (25°C) for 10 days. The results obtained indicated that CH-CEO (HPH) and CH-LEO (HPH) are significantly ($p<0.05$) effective to reduce the total plate counts and yeast and mould counts to prolong the shelf life of guava. They also had slowed the increases of weight loss and yellowness and slowed the decline

of lightness and greenness of guava at day 10 of 25°C of storage temperature, hence preserving the guava better compared to those coated with chitosan.

ABSTRAK

Nanoemulsi mempunyai ciri-ciri skala nano yang menjadikannya menarik untuk pelbagai aplikasi dan ia boleh dihasilkan menggunakan kaedah tenaga rendah atau kaedah tenaga yang tinggi. Kajian ini bertujuan untuk menentukan kesan dua fasa minyak yang merupakan minyak pati (EO) dan minyak kelapa dara (VCO) sebagai minyak pembawa untuk menghasilkan nanoemulsi menggunakan kaedah tenaga tinggi iaitu homogenizer tekanan tinggi. Pencirian nanoemulsi yang dihasilkan dilakukan dengan memerhatikan penampilan nanoemulsi, menganalisis ukuran saiz nanoemulsi dan kestabilan nanoemulsi selama sebulan penyimpanan pada 25°C. Nanoemulsi yang paling stabil untuk setiap minyak pati iaitu Formulasi 2 untuk nanoemulsi cengkih dan Formulasi 4 untuk nanoemulsi lemon, kemudiannya digunakan untuk dicampur dengan kitosan untuk menghasilkan lapisan yang boleh dimakan pada jambu batu. Salutan ini digunakan pada jambu dengan mencelupkan buah-buahan ke dalam larutan selama 3 minit. Guavas dikeringkan dan disimpan dalam bilik berhawa dingin yang dikekalkan pada suhu 25 ° C. Lima sampel jambu yang bersalut dengan kitosan sahaja (CH), disalut dengan kitosan yang mengandungi nanoemulsi cengkih (CH-CEO (HPH)), disalut dengan kitosan yang mengandungi minyak pati cengkih (CH-CEO), disalut dengan kitosan yang mengandungi lemon nanoemulsi (CH -LEO (HPH)) dan disalut dengan kitosan yang mengandungi minyak pati lemon (CH-LEO). Analisis mikrobiologi bagi semua sampel ditentukan oleh jumlah kiraan plat dan rajah yis dan acuan. Sifat jambu batu dianalisis untuk

keberkesanan lapisan terhadap penurunan berat badan, ketegasan dan pH semasa penyimpanan pada suhu (25°C) selama 10 hari. Keputusan yang diperoleh menunjukkan bahawa CH-CEO (HPH) dan CH-LEO (HPH) secara signifikan ($p < 0.05$) berkuat kuasa untuk mengurangkan bilangan plat jumlah dan ragi dan acuan untuk memanjangkan jangka hayat jambu. Mereka juga telah memperlahankan peningkatan berat badan dan ketegasan dan memperlambat penurunan ringan dan kehijauan jambu pada hari 10 dari 25°C suhu penyimpanan, dengan demikian mereka memelihara jambu batu lebih baik dibandingkan dengan yang dilapisi kitosan.



Table of Contents

APPROVAL SHEET	2
ACKNOWLEDEGEMENT	i
ABSTRACT	ii
ABSTRAK	iii
LIST OF FIGURE	viii
LIST OF TABLE	x
CHAPTER 1	1
INTRODUCTION	1
1.1. Background	1
1.2. Problem statement	4
1.3. Objectives	5
1.4. Scope of study	5
CHAPTER 2	6
LITERATURE REVIEW	6
2.1. Essential oils as antimicrobial agent	6
2.1.1. Lemon essential oil	7
2.1.2. Clove essential oil	8
2.2. Essential oil nanoemulsion as edible coating	10
2.2.1. Nanoemulsion	10
2.2.2. Methods of preparation of nanoemulsion	11
2.2.3. Application of essential oil nanoemulsion as edible coating	16
2.3. Chitosan as biopolymer matrix	17
CHAPTER 3	20
METHODOLOGY	20
3.1. Materials	20

3.2. Nanoemulsion Fabrication	20
3.2.1. Particle size analysis	21
3.2.2. Stability	22
3.3. Preparation of edible coating solution	22
3.4. Application of edible chitosan–nanoemulsified EO coatings to guavas.....	22
3.5. Microbiological evaluation	23
3.6. Physico-chemical analysis	24
3.6.1. Fresh weight loss analysis.....	24
3.6.2. Firmness determination.....	24
3.6.3. pH determination.....	25
3.6.4. Colour parameters	25
3.6.5. Physical appearance	26
3.7. Statistical analysis	26
CHAPTER 4	27
RESULTS AND DISCUSSIONS	27
4.1. Preparation and characterization of essential oils nanoemulsions	27
4.1.1. The appearance of essential oils nanoemulsions.....	27
4.1.2. The effect of oil phase composition on droplet size of nanoemulsions	29
4.1.3. The effect of oil phase composition on stability of CEO-VCO emulsions and LEO-VCO emulsions during a month of storage.....	31
4.1.4. Conclusion for optimal formulations to be choose for application as edible coating	33
4.2. The application of essential oil nanoemulsion as edible coating on guava.	34
4.1.1. The physical appearance of guava during storage time at 25°C	34
4.1.2. Effect of treatments on microbiological properties of guava during storage time at 25°C	35
4.1.3. Weight loss of guava during storage time at 25°C	38
4.1.4. Firmness of guava during storage time at 25°C	39

4.1.5.	Colour properties of guava during storage at 25°C.....	41
4.1.6.	The effect of treatment on the pH of guava during storage time at 25°C 44	
CHAPTER 5		45
CONCLUSION AND RECOMMENDATIONS.....		45
REFERENCES.....		47
APPENDICES		50



LIST OF FIGURE

Figure 2.1: Antimicrobial mode of action of EOs against different cell targets of microorganisms	7
Figure 2.2: Structures of classical phytochemicals derived from EOs with known antimicrobial activity: the terpene a) limonene and the terpenoids b) carvacrol, c) thymol, d) menthol, e) citral, f) geraniol, g) eugenol, h) cinnamaldehyde.	10
Figure 2.3: Schematic of high pressure homogenizer (HPH) used for making nanoemulsions.....	15
Figure 3.1: High pressure homogenizer (HPH- Avestin Canasa C50)	21
Figure 3.2 Preparation of edible coating solution	22
Figure 3.3: Coating application on guava	23
Figure 3.4: Texture analyzer for firmness analysis.....	25
Figure 4.1: Nanoemulsion of five formulation starting with formulation 1 to 5 from left to right. (a) Clove essential oil nanoemulsions, and (b) lemon essential oil nanoemulsions.....	29
Figure 4.2: Graph of size of clove and lemon essential oil nanoemulsions.....	30
Figure 4.3: Graph of the stability of clove essential oil (CEO) nanoemulsion from Formulation 1 to Formulation 5 during a month.....	32
Figure 4.4: Graph of the stability of lemon oil nanoemulsions during a month	32

Figure 4.5: Log CFU/g graph (a) for total plate count and (b) for mould and yeast count.....	37
Figure 4.6: Weight loss (%) of guava. CH: guava coated with chitosan; CH-CEO (HPH): guava coated with chitosan containing clove EO nanoemulsion; CH-CEO: guava coated with chitosan containing clove EO; CH-LEO (HPH): guava coated with chitosan containing lemon EO nanoemulsion; CH-LEO: guava coated with chitosan containing lemon EO.....	39
Figure 4.7: Graph of the guava firmness. CH: guava coated with chitosan; CH-CEO (HPH): guava coated with chitosan containing clove EO nanoemulsion; CH-CEO: guava coated with chitosan containing clove EO; CH-LEO (HPH): guava coated with chitosan containing lemon EO nanoemulsion; CH-LEO: guava coated with chitosan containing lemon EO.....	40
Figure 4.8: (a) L* parameter, (b) a* parameter and (c) b* parameter of colour properties of guava. CH: guava coated with chitosan; CH-CEO (HPH): guava coated with chitosan containing clove EO nanoemulsion; CH-CEO: guava coated with chitosan containing clove EO; CH-LEO (HPH): guava coated with chitosan containing lemon EO nanoemulsion; CH-LEO: guava coated with chitosan containing lemon EO.....	43
Figure 4.9: pH value of guava during storage time. CH: guava coated with chitosan; CH-CEO (HPH): guava coated with chitosan containing clove EO nanoemulsion; CH-CEO: guava coated with chitosan containing clove EO; CH-LEO (HPH): guava coated with chitosan containing lemon EO nanoemulsion; CH-LEO: guava coated with chitosan containing lemon EO.	44

LIST OF TABLE

Table 3.1: Formulations of primary emulsion 21

Table 3.2: Treatment on fruit sample..... 23

Table 4.1: Formulation of nanoemulsions 28

Table 4.2: Polydispersity Index (PDI) of essential oil nanoemulsions 30

Table 4.3: Physical appearance of guava during storage time at 25°C 35



LIST OF ABBREVIATION

CEO	Clove Essential Oil
CFU	Colony Forming Unit
CH	Chitosan
EO	Essential Oil
HPH	High Pressure Homogenizer
LEO	Lemon Essential Oil
nm	nanometer
PCA	Plate Count Agar
PDA	Potato Dextrose Agar
SOR	Surfactant-Oil Ratio
TPC	Total Plate Count
YM	Yeast and Mould

CHAPTER 1

INTRODUCTION

1.1. Background

Essential oils (EOs) are a complex mixture of the volatile organic components of fragrant (aromatic oily liquid) plant matter that contribute to the flavour and fragrance of the plant. They can be extracted from any part of the plant (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) by steam distillation, cold pressing, or using organic solvents; the oil yield is affected by environmental conditions and distillation processes. The yield can differ between batches from the same trees and between sites (Price S., 1989). Nowadays, EOs have been used as flavouring additives, as medicines or cosmetics (Dima and Dima, 2015), as insecticidal, antioxidant, anti-inflammatory, anti-allergic, and anticancer agents (Seow et al., 2014). EOs exhibit antimicrobial properties (Guenther, 1948;

Boyle, 1955) which make their application as food preservatives against foodborne pathogens and spoilage bacteria have been increasing recently (Caillet et al., 2006).

The encapsulation of EOs into nanometric delivery systems which are also known as nanoemulsions means a colloidal dispersion formed by combination of two immiscible phases (EOs and water) stabilized by a surfactant, with oil droplets of diameters in the range of 20–200 nm. For the preparation of nanoemulsions, two primary categories of method which are high-energy method and low-energy method have been used in many research works (Gupta, Eral, Hatton, & Doyle, 2016). Both of these methods will result such small droplet size of nanoemulsions that are kinetically stable system since the Brownian motion effects dominate gravitational forces, presenting higher resistance to particle aggregation than conventional emulsions. Therefore, these nanoemulsions can be directly applied to incorporate with the edible coatings.

Edible coatings are thin layers of edible materials applied in liquid form to coat the food products for preservation. In terms of active ingredients that can be incorporated into films and coatings, essential oils (EOs) have received much attention as having potential biological activity. Chitosan, a cationic polysaccharide consisting of (1,4)-linked-2-amino-deoxy-b-D-glucan, is the deacetylated form of chitin. Chitosan and its derivatives have been shown to possess diverse biological activities, including antioxidant, antihypertensive, anticoagulant, antidiabetic, antiobesity, antiallergic, antiinflammatory, antimicrobial, anticancer, neuroprotective and matrix metalloproteinases inhibitory effects (Ngo et al., 2015). In addition, chitosan is considered as an ideal biopolymer for production of active edible films and coatings due to its non-toxicity, biocompatibility, biodegradability and film-

forming ability (Dutta, Tripathi, Mehrotra, & Dutta, 2009). These potential attributes of chitosan-based coating has make it as the suitable edible coating for food especially for fresh fruit which is recently increasing demand from consumers. Chitosan films and coatings are promising systems to be used as active ingredient carriers.

Guavas (*Psidium Guajava*) are one of most popular fruit cultivated about 2,880.0 hectares with production 52,858.6 metric ton in Malaysia (Department of Agriculture, 2015). It is rich with vitamin and minerals, there are ascorbic acid which is Vitamin C, Vitamin A, dietary fiber, pectin, sugar, folic acid, potassium, manganese, copper and certain dietary minerals. It is also used in pharmacological. Guavas also being process and preserved to form jam, pulp , puree , squash, nectar and juice (Sevda et. al , 2012). However, it is estimated that about 20-25% of the harvested fruits are decayed by pathogens during post-harvest handling even in developed countries (Droby 2006, Zhu 2006). Fungal infection on the fruit may occur during the growing season, harvesting, handling, transport and post-harvest storage and marketing conditions, or after purchasing by the consumer. Fruits contain high levels of sugars and nutrients, and their low pH makes them vulnerable to fungal decaying (Singh and Sharma 2007). Therefore, for this study, guavas were chose to investigate the edible coating incorporated with antimicrobial active ingredient; essential oils in nanometric size.

1.2. Problem statement

Nowadays, many researches have been done on the direct incorporation of EOs in biopolymeric matrix as the active agent in edible coating. However, their direct incorporation in food and beverage products is not only a tough challenge due to the high reactivity and hydrophobicity of EOs but also their impact on the sensory perception. Besides, this technique significantly reduces the EOs loading capability in the coating, increases the risk of oiling off of EOs, as well as undermines the mechanical properties of the film or coating. In order for EOs to function efficiently when incorporated in food, they have to be encapsulated in appropriate delivery systems.

The encapsulation of EOs into nanometric delivery systems has shown great potential to transport the EOs in edible coating. Due to the nanometric size, the dosage limitations related to their low solubility in water (Donsi et al., 2011) can be overcome and also increase the physical stability of active substances, protecting them from the interactions with other food ingredients. In addition, the encapsulation of EOs into nanometric delivery systems can enhance their bioactivity, improving the passive mechanisms of cell absorption, enabling the reduction of the doses of essential oils required to ensure antimicrobial activity, thus minimizing the impact on aroma, flavour and taste (Donsi et al., 2011). Between those two methods to produce nanoemulsions which are low energy method and high energy method, high energy method is more favourable in food industry.

1.3. Objectives

This research is an experimental study to prepare essential oil nanoemulsion and to apply on guava as edible coating. The objectives for this experiment are:

- i. To investigate the effect of concentration of EO nanoemulsion produce using high pressure homogenizer on appearance, particle size and stability
- ii. To evaluate the microbial load and physico-chemical characteristic of guava coated with edible coating incorporating essential oil nanoemulsions of guavas during storage time

1.4. Scope of study

This study will be focused on the preparation of nanoemulsion using high pressure homogenizer. Then, the effect of essential oils concentration with coconut oil on partile size of nanoemulsion and its stability also will be studied. The microbiological evaluation and physic-chemical analysis are conducted to evaluate the effectiveness of the edible coating incorporated with nanoemulsified essential oil.

CHAPTER 2

LITERATURE REVIEW

2.1. Essential oils as antimicrobial agent

Several EOs such as oils of garlic, cinnamon, thyme, oregano, clove, basil, coriander, citrus peel, laurel, ginger, rosemary, and peppermint, among others, have been studied as antimicrobial natural products against both bacteria and molds (Edris and Farrag 2003; Novak and others 2003; Arcila-Lozano and others 2004; Jugl-Chizzola and others 2006; Wang 2006; Mukherjee and Datta 2007; Ravi and others 2007; Senhaji and others 2007; Zeller and Rychlik 2007). The inherent aroma and antimicrobial activity of EOs are related commonly with the chemical configuration of the components, the proportions in which they are present and to interactions between them, affecting their bioactive properties (Fisher & Phillips, 2008). Some studies have concluded that whole EOs have a greater antibacterial activity than their major constituents separately (Marino and others 1999, 2001; Chang and others

2008), which suggests that the components at lower concentration are critical to the activity and the entire mixture must have a synergistic effect. Considering that EOs are formed by several constituents is difficult to attribute the antimicrobial efficacy to only one compound, in this context, the mode of action of a given compound could be different compared to others, being reported several targets in the microbial cell (Figure 2.1). It seems that they may cause deterioration of cell wall, damage to cytoplasmic membrane, damage to membrane proteins, leakage of cell contents, coagulation of cytoplasm, depletion of proton motive active sites, inactivation of essential enzymes, and disturbance of genetic material functionality (Burt 2004; Ayala-Zavala and others 2008a; Gutierrez and others 2008).

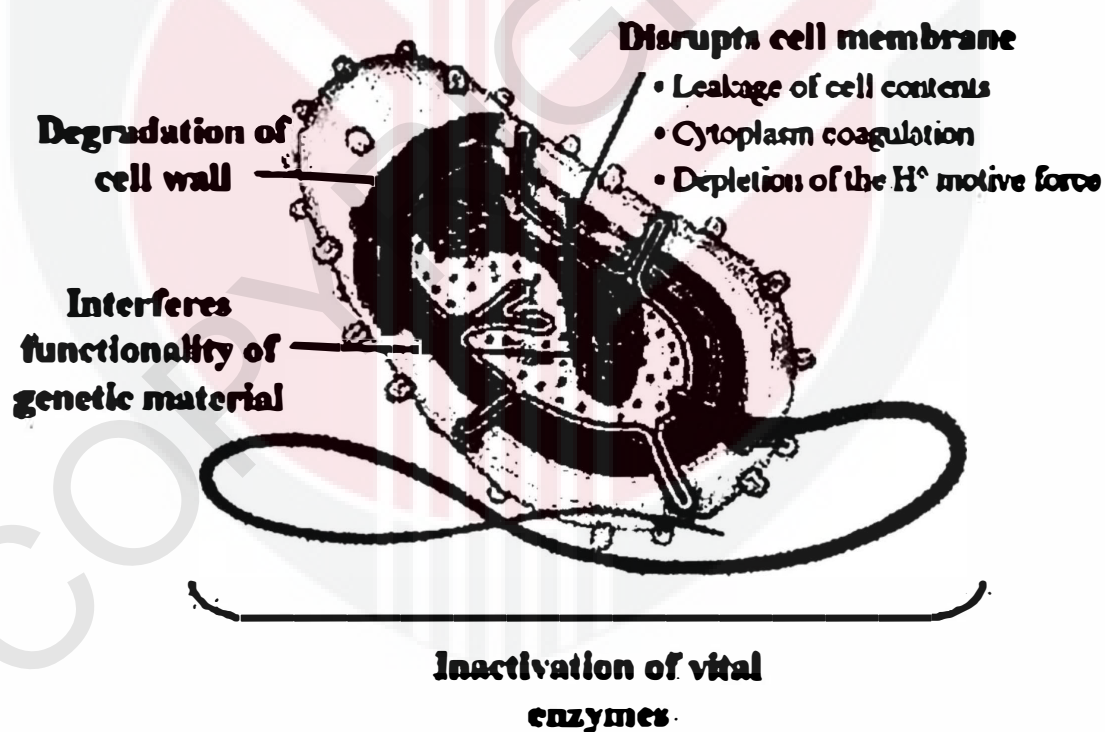


Figure 2.1: Antimicrobial mode of action of EOs against different cell targets of microorganisms

2.1.1. Lemon essential oil

Lemon essential oil is extracted from citrus lemon which has been proved to have antimicrobial agent. The main components found in this essential oil are

limonene (50%), β -pinene (12.4%) and p-Cymene (0.27%) (Moufida & Marzouk, 2003). Limonene is the most abundant among all components shows great potential as the active agent against pathogenic microbes. The citrus oils are widely applied in food and are generally recognised as safe (GRAS) and have been found to be inhibitory both in direct oil and vapour form against a range of both Gram-positive and Gram-negative bacteria (Fisher & Phillips, 2008). The essential oils of lemon showed the capacity to reduce or inhibit the growth of the moulds *P. chrysogenum*, *P. verrucosum*, *A. niger* and *A. flavus* (Vitoratos, Bilalis, Karkanis, & Efthimiadou, 2013). According to Vitoratos et al (2013), essential oils from lemon (*Citrus limon*) exhibited antifungal activity against *Fusarium oxysporum* and *Glomerella cingulate* which can be supported by Chuah, Tan, & Ismail (2010).

2.1.2. Clove essential oil

Clove essential oil is extracted from *Syzygium aromaticum* and has gained many attention because of its properties that can reduce or inhibit pathogenic microbial. The highest component found in clove essential oil is eugenol (85.3 %) (Pinto, Vale-Silva, Cavaleiro, & Salgueiro, 2009). Clove bud essential oil in direct contact method demonstrated antibacterial activity against all the food-associated bacteria which are *E. coli*, *B. cereus*, *S. enteritidis* and *S. aureus* (Dobre, Gagi, & Petru, 2011). Eugenol (4 allyl-2-methoxy phenol; $C_{10}H_{12}O_2$) is a major constituent of clove essential oil; that is an organic phenol compound. It has specification of antipyretic, analgesic, antiinflammatory, and anesthetic effects. Based on latest conducted research, clove oil (eugenol) also has antimicrobial (Manganyi et al., 2015), antioxidant (Avila Farias et al., 2014; Gu"lc, in et al., 2012), antifungal (Pinto et al., 2009), insect repellent (Shapiro, 2012), and anticancer (Dwivedi et al., 2011)

activities. Minimizing eugenol side effects, with low toxicity, and non-metabolized residue, eugenol has wide application in pharmaceuticals (Kong et al., 2014), cosmetics (Prashar et al., 2006), dentistry (Xu et al., 2013), food (Hyldgaard et al., 2012; Vrinda Menon and Garg, 2001), agriculture (Isman, 2006) and pesticides (Isman, 2000; Jiang et al., 2012). The anti-insect activity of eugenol strictly depends on the organic structure of its phenolic ring with specific hydroxyl functional group.

In grain treatment, Huang and his co-workers (Huang et al., 2002) demonstrated that different doses of eugenol were required to exterminate *Tribolium castaneum* and *Sitophilus zeamais*. Pessoa and his team (Pessoa et al., 2002) studied the inhibition of eugenol on small ruminants of *Haemonchus contortus* by ovum hatching test and obtained the highest inhibition rate when concentration was about 0.5%. Machado and his group (Machado et al., 2011) studied the effect of eugenol on the growth, activity, adherence, and ultra-structure of *Giardia lamblia*. Their results showed that eugenol inhibits the adherence of Trophosome after being monitored for 3 h and does not cause cytolysis. They also found that eugenol affected on cell shape and caused the coagulation of cytoplasm and autophagy in the cells. These investigations demonstrated that eugenol can be used as a drug to prevent Giardiasis and Verminosis. An emulsion of clove oil is a kinetically stable system which is obtained by the dispersion of one liquid (dispersant) into another phase; where each liquid is immiscible or poorly miscible in the other, e.g. oil and water (Purwanti et al., 2015).

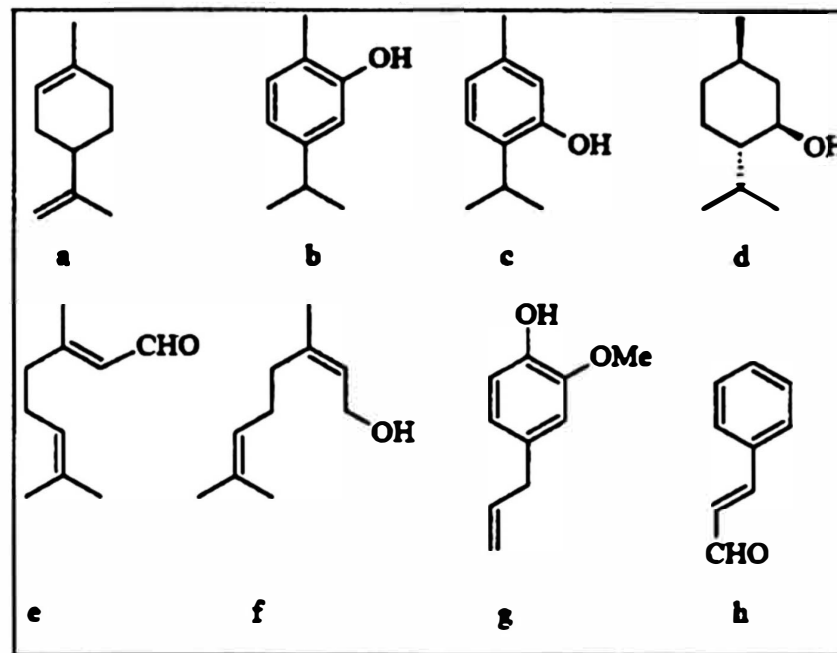


Figure 2.2: Structures of classical phytochemicals derived from EOs with known antimicrobial activity: the terpene a) limonene and the terpenoids b) carvacrol, c) thymol, d) menthol, e) citral, f) geraniol, g) eugenol, h) cinnamaldehyde.

2.2. Essential oil nanoemulsion as edible coating

2.2.1. Nanoemulsion

Emulsions are used to change the texture, flavour, and appearance of foods. Moreover, they are also increasingly used to delivery biologically active components that are lipophilic such as antioxidants, flavours, and nutraceuticals. Gupta et al. (2016) and Donsì and Ferrari (2016) stated that nanoemulsions are emulsions in nanometric sizes which the oil droplets diameters in the range of 20–200 nm stabilized by a surfactant (cited in Acevedo-fani et al., 2017). Nanoemulsions consist of continuous phase, dispersed phase and stabilized by surface active agent (surfactant).

In this nanosystem, the essential oil not only can be protected from oxidized or evaporated but also aid their antimicrobial activity by providing diverse diffusion properties trough biological membranes due to nanoscale particles dimension (Pedro, Santo, Silva, Detoni, & Albuquerque, 2013). In comparison with conventional emulsions, the small size of the particles in nanoemulsions ($r < 100$ nm) make them

to have higher stability to droplet aggregation and gravitational separation, high optical clarity, ability to modulate product texture, and, increased bioavailability of lipophilic components (McClements & Rao, 2011).

2.2.2. Methods of preparation of nanoemulsion

2.2.2.1. Formulation

The formulation of food emulsions is restricted that all components should be edible, food-grade and approved for use by international legislation. Oil-in-water nanoemulsions have been regarded as highly stable systems suitable for delivering vitamins, flavors, antioxidants, preservatives, or nutraceuticals (Acevedo-fani et al., 2017).

To prepare nanoemulsions, there are two primary methods which are by using low-energy method and the other one using high-energy method. High-energy approaches utilize mechanical devices (“homogenizers”) capable of generating intense disruptive forces that are capable of disrupting and intermingling the oil and aqueous phases into tiny oil droplets, e.g., high pressure valve homogenizers, microfluidizers, and sonication methods (Gutierrez et al., 2008; Velikov et al., 2008; Wooster et al., 2008; Leong et al., 2009). At present, high-energy approaches are the most common method used to prepare nanoemulsions in industrial food operations because their utilization in the production of conventional emulsions is already well-established, they are capable of large-scale production, and they can be used to prepare nanoemulsions from a variety of different starting materials (McClements & Rao, 2011). Low-energy approaches rely on the spontaneous formation of tiny oil droplets within mixed oil-water-emulsifier systems when the

solution or environmental conditions are altered, e.g., phase inversion and spontaneous-emulsification methods (Bouchemal et al., 2004; Tadros et al., 2004; Freitas et al., 2005; Chu et al., 2007a; Anton et al., 2008; Yin et al., 2009). The minimum particle size that can be produced using each approach depends on many different factors.

The available evidence suggests that nanoemulsion-based edible coatings can be formed through two approaches. One alternative is preparing coating-forming nanoemulsions in one step, where all the components, including the disperse phase, emulsifiers and the continuous phase containing the biopolymer dissolved, are pre-mixed in a coarse emulsion, and homogenized to decrease the droplet size down to the nano-range. These nanoemulsions can be directly applied on the product as an edible coating. The second approach is based on a two-step procedure where in nanoemulsions are firstly formed without containing the biopolymer in the aqueous phase, and then the coating-forming solutions are obtained by mixing previous nanoemulsions within a biopolymer. In both cases, the objective of encapsulating non-polar substances within edible coatings can be achieved.

2.2.2.1.1. Physicochemical stability – Ostwald ripening

The non-negligible solubility in water of many EOs, such as carvacrol (Donsi et al., 2014, 2012a), triggers a specific coalescence phenomenon, named Ostwald ripening (Suriyarak and Weiss, 2014). Preventing Ostwald ripening is one of the main difficulties associated with the formulation of physically stable EOs nanoemulsions (Chang and McClements, 2014; Ziani et al., 2011). Ostwald ripening can be described as the growth of larger droplets at the expense of the smaller ones,

because of molecular diffusion of oil between droplets through the continuous phase, which is induced by the higher local oil solubility around smaller droplets than larger ones (Wooster et al., 2008). Oils consisting of long-chain triglycerides, which exhibit a negligible aqueous phase solubility, are less prone to Ostwald ripening than oils containing components with an appreciable water solubility (Rao and McClements, 2012). When the use of EOs prone to Ostwald ripening is unavoidable, the rate of Ostwald ripening can be reduced by changing the partitioning of EOs between the lipid droplets and the aqueous phase, for example by mixing with medium or long chain triglycerides oils (Donsì et al., 2014), generating an entropy of mixing effect that counteracts the imbalance of droplet size effect (Chang et al., 2015). Examples of these “ripening inhibitors” oils include highly non-polar substances, such as corn oil (Chang et al., 2012; Ziani et al., 2011), sunflower oil (Donsì et al., 2014, 2012a), MCT, sesame oil (Terjung et al., 2012), as well as canola oil (Majeed et al., 2016a). With different EOs and emulsifiers, the optimal weight ratio between EO and the ripening inhibitor may be different (Donsì et al., 2014): for example, Ostwald ripening was inhibited by mixing in the lipid phase thyme oil with $\geq 60\%$ corn oil (Chang et al., 2012; Ziani et al., 2011), carvacrol with $\geq 50\%$ MCT (Chang et al., 2013) or sunflower oil (Donsì et al., 2014), orange oil with $\geq 40\%$ MCT (Chang and McClements, 2014), or eugenol with $\geq 50\%$ MCT (Ghosh et al., 2014). In alternative, the incorporation of a slightly water-soluble EO, such as d-limonene, into an organogel based nanoemulsion also resulted in an improved physical stability. The organogel structure, obtained by adding an oleogelator agent, such as stearic acid, to the lipid phase, effectively contributed to reducing the polarity of EOs and actively prevented the occurrence of Ostwald ripening (Zahiet al., 2014). Finally, another route reported to inhibit Ostwald ripening is based on the use of

macromolecules, able to bind the EO components, as observed for soybean polysaccharide (Wu et al., 2014), or sodium caseinate and lecithin (Xue and Zhong, 2014) with thyme oil, for sodium caseinate with thymol (Pan et al., 2014), for whey protein-maltodextrin conjugates with eugenol (Shah et al., 2013), and for pectins with mandarin or lemongrass oil (Guerra-Rosaset al., 2016). However, when the interaction between the EOs and the macromolecule is weak, destabilization mechanisms by Ostwald ripening become significant (Guerra-Rosas et al., 2016).

2.2.2.2. High Pressure Homogenizer

High pressure homogenization (HPH) is a continuous process, where a process fluid is compressed at high pressures (50–400 MPa), and forced through a specifically designed, micrometric homogenization chamber. The intense shear and elongational stresses, turbulence, and cavitation developed in the homogenization chamber generate the high-intensity fluid-mechanical stresses, responsible for the disruption of the oil droplets dispersed in the fluid. The emulsification process is affected by the efficiency, with which the energy dissipated in the homogenization chamber is transmitted to the oil droplets, which directly depends on the chamber geometry. In particular, among the different chambers equipping commercial HPH devices, ranging from a simple orifice plate to colliding jets and radial diffuser assemblies (Donsì et al., 2009), the orifice valve appears to be one of the most efficient for emulsification (Donsì et al., 2012b; Stang et al., 2001). Orifice valves enable not only an efficient droplet disruption, with high elongational stresses coupled with elevated turbulence, but also a homogeneous volume of interaction downstream of the valve, where emulsifier adsorption at the droplet interface occurs uniformly, reducing the polydispersity of the emulsion droplets (Donsì et al.,

2012b). Figure 3 shows the examples of different EOs, which have been prepared by HPH treatment, reaching submicrometric mean droplet sizes with surfactant-to-oil (or emulsifier -to-oil) ratios(SOR) often ≤ 1 . It must be remarked that to achieve a uniform, narrow size distribution, often several HPH passes, or number of processing cycles, are recommended, to compensate the uneven distribution of fluid-mechanical stresses in the homogenization chamber (Donsi et al., 2012b). Typical operating pressures for EO nanoemulsions are in the range of 200–300 MPa. However, for high SOR values (≥ 1), significantly lower pressures can be used (Zahiet al., 2014): for example, no additional droplet size reduction was observed when increasing the pressure from 100 to 150 MPa (Lianget al., 2012).

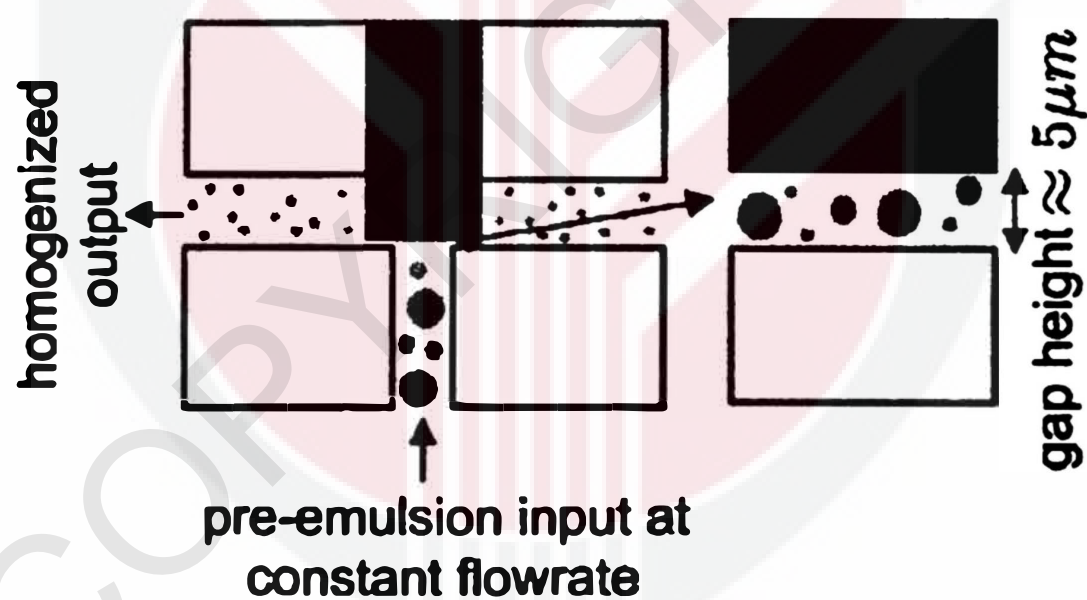


Figure 2.3: Schematic of high pressure homogenizer (HPH) used for making nanoemulsions.

2.2.3. Application of essential oil nanoemulsion as edible coating

The current applications of nanotechnology in the food sector are few, as the science is relatively newly emergent, but they have been on a steady rise in recent years, and are predicted to grow rapidly in the coming years. The current and short-term projected applications of nanotechnologies in the (health) food sector include nano-sized or nano-encapsulated ingredients and additives for food, beverage and health food applications. Table 2.1 shows some food applications of nanoemulsions and the products available.

The pharmaceutical industry is the dominant field where most applications of nanoemulsions are proposed. Extensive research has been conducted on a variety of drug delivery systems with enhanced solubilization of poorly soluble drugs and improved bioavailability following incorporation into nanoemulsions (Shah *et al.*, 2010; Shafiq *et al.*, 2007). In the food industry, many food-derived bioactive compounds demonstrate significant health benefits when consumed in relatively high concentrations (Flanagan and Singh, 2006). Unfortunately, most of these compounds exhibit poor solubility and bioavailability in aqueous-based foods.

Recently the development of nanoemulsions loaded with lipophilic food components has demonstrated the potential of nanoemulsions as a carrier to deliver lipophilic actives in food applications (Garti *et al.*, 2007). Nanotechnology is also being used to create healthier foods that can deliver nutrients and medications to different parts of the human body (Chun, 2009) and can alleviate allergenic properties (Wogan, 2010). And nanotechnology is also likely to enable substantial benefits to food manufacturing and agricultural production. Nanomaterials, for

instance, might be developed to improve the delivery of nutrients and pesticides to crops.

Another unique application of nanoemulsions is non-weighted flavour emulsions. For conventional flavor emulsions, weighing agents (e.g., ester gum) are commonly used to weigh the oil phase and reduce density contrast between oil and aqueous phases. These weighing agents significantly reduce the creaming velocity of droplets, but they have strict usage limits due to their toxicity. Weighing agents can be removed in nanoemulsions due to their high stability against creaming, which provides a cleaner label. These emulsions are also getting more attention in recent years in the beverage industry because they have very small droplet sizes, which improves creaming stability and may facilitate flavour release and perception due to a large specific surface area and are transparent, which is desirable for flavoured clear beverages, mouthwashes and fortified beverages.

2.3. Chitosan as biopolymer matrix

Chitosan, a high molecular weight cationic polysaccharide, is soluble in dilute organic acids, and could theoretically be used as a preservative coating material for fruit (Jiang et al., 2005). Furthermore, chitosan has great potentialities as a biodegradable, edible coating or film in food packing (Arvanitoyannis, 1999), exhibits excellent biocompatibility, nontoxicity (Jayakumar et al., 2005; Prabakaran and Mano, 2006) and also possesses film-forming and barrier properties (Arvanitoyannis et al., 1998), thus making it a potential raw material for edible films or coatings. Previous researches proved that application of chitosan coating had been shown to improve the storability of several perishable fruits, such as strawberry (El

Ghaouth et al., 1991a), tomato (El Ghaouth et al., 1992), litchi (Zhang and Quantick, 1997), longan (Jiang and Li, 2001), peach (Li and Yu, 2001), mango (Kittur et al., 2001) and table grape (Romanazzi et al., 2002). In addition, numerous researches have clearly demonstrated that chitosan can be used as an effective preservative for improvement of quality and shelf life of various fruits (Kittur et al., 2001; Dong et al., 2004; Chien et al., 2007; Meng et al., 2008). For example, chitosan coating could extend postharvest life of longan fruit and maintain their quality (Jiang and Li, 2001), and similar results were obtained in papayas and litchis coated with chitosan (Ali et al., 2011; Lin et al., 2011). Previous studies have indicated that fresh-cut guava coated with chitosan delayed weight loss and increase of soluble solids content (SSC), while it did not have any significant effect on firmness and titratable acidity (TA) reduction compared to the control (Thommohaway et al., 2007).

Chitosan films and coatings are promising systems to be used as active ingredient carriers. In terms of active ingredients that can be incorporated into films and coatings, essential oils (EOs) have received much attention as having potential biological activity. Many EOs, such as clove, oregano, thyme, nutmeg, basil, mustard or cinnamon, are categorized as GRAS by the USFDA (Manso, Pezo, Gomez- Lus, & Nerin, 2014) and are gaining popularity due to their volatile nature, which facilitates the use of small concentrations that are safe for consumption (Sivakumar & Bautista-Banos, 2014). EOs, the secondary metabolites of plants, have been traditionally used to extend food shelf life due to their antimicrobial/antifungal activity. These potential attributes and an increasing demand for natural food additive options have led to an interest in the use of EOs as potential alternative antimicrobials. The antimicrobial properties and their potential application in food

systems have been recently reviewed (Calo, Crandall, O'Bryan, & Ricke, 2015; Vergis, Gokulakrishnan, Agarwal, & Kumar, 2015). In recent years, EOs also have been extensively studied as additives in edible films and coatings.



CHAPTER 3

METHODOLOGY

3.1. Materials

Lemon and clove essential oils were purchased from the brand Naturh. High molecular weight chitosan (CH), with a deacetylation degree of 75.6% (CAS Number 9012-76-4, Sigma–Aldrich, viscosity in 1% (wt) glacial acetic acid solution: 1.406 Pa s), and acetic acid, were used to prepare the coating. Guavas were purchased from the Pasar Borong Selangor and were chosen to ensure uniformity in size and peel colouration.

3.2. Nanoemulsion Fabrication

Two essential oils (lemon and clove essential oils) were screened as natural antimicrobial agents and used in nanoemulsions preparation. In the emulsion fabrication, a mixture of essential oil, virgin coconut oil and Tween 80 (as surfactant) were dispersed in distilled water, using magnetic stirrer at 1500 rpm for 15 min, to form a primary emulsion according to the five formulations

in Table 3.1. Subsequently, the primary emulsions were subjected to a high pressure homogenization (HPH- Avestin Canasa C50) treatment for 20 cycles at 100 MPa, to reach a droplet size distribution in the nanometric range.

Table 3.1: Formulations of primary emulsion

Formulation	Essential oil (%)	Virgin coconut oil (%)	Tween 80 (%)	Water (%)
1	1	4	5	90
2	2	3	5	90
3	3	2	5	90
4	4	1	5	90
5	5	0	5	90



Figure 3.1: High pressure homogenizer (HPH- Avestin Canasa C50)

3.2.1. Particle size analysis

The particle size of the nanoemulsions were measured by using the particle size analyzer (Nanoplus Zeta/ Nano particle Analyzer, Japan). The samples were filled into a cuvette capillary cell to avoid multiple scattering effects. Measurements were performed with an angle of 173° at room temperature (25 ± 0.5 °C). The measurements were carried out in fully automatic mode, with each sample will be analyzed once and each analysis consisting of three replicates (Che Sulaiman et al.,

2016). The polydispersity index (PDI) of particle size also obtained from this equipment.

3.2.2. Stability

The stability study for optimal nanoemulsion was done by recording the particle size of nanoemulsions on day 1, 6, 13, 20 and 28 under storage at room temperature (25 ± 0.5 °C) (Che Sulaiman et al., 2016).

3.3. Preparation of edible coating solution

The coating solutions were prepared dissolving chitosan at 1.0% w/w in aqueous solutions of acetic acid (1.0% v/v), and were stirred for 24 h to ensure total solubility. Subsequently, nanoemulsions (4.0% w/w) were added to the solutions and mixed vigorously using a magnetic stirrer for 5 min.



Figure 3.2 Preparation of edible coating solution

3.4. Application of edible chitosan–nanoemulsified EO coatings to guavas

The application of edible coatings to guavas was performed as described by Azevedo et al. (2014) with several modifications. Guavas were first sanitised by immersion in a 50 ppm sodium hypochlorite solution for 10 min, rinsed in distilled water and dried at room temperature on nylon trays. Guavas were randomly divided into five treatments, as follow (Table 3.2).

Table 3.2: Treatment on fruit sample

Sample	Treatment
1	control
2	chitosan-coated fruits (CH)
3	chitosan-clove oil nanoemulsion (CH-CEO (HPH)) coated fruit
4	chitosan-lemon oil nanoemulsion (CH-LEO (HPH)) coated fruit
5	chitosan-clove oil (CH-CEO) coated fruit
6	chitosan-lemon oil (CH-LEO) coated fruit

A total volume of 200 mL of edible coating solution for each formulation was prepared, and each guava was immersed in the coating solution, using sterile forceps, for 3 min. After this period, the guavas were removed with forceps and the coated samples were allowed to dry for 30 min in a biological safety cabinet (Figure 3.3). The coated guavas were stored in aluminium trays in at 25°C and were subjected to microbiological and physicochemical analysis on Day 0, 3, 7 and 10 of the storage period.



Figure 3.3: Coating application on guava

3.5. Microbiological evaluation

The determination of Plate Count Agar (PCA) and Potato Dextrose Agar (PDA) for guava coated with chitosan-nanoemulsified essential oil was using Food and Drug Administration standard method of Bacteriological Analytical Manual (FDA, 2001). Total plate count (TPC) and yeast and mould count (YM) were determined during 0, 3, 7 and 10 days of storage using spread plate method. PCA

was used to determine total plate count while PDA was used to determine yeast and mold count. Samples (10 g of guava) for microbiological analysis were homogenized with a stomacher for 90 s in a 1:10 dilution with sterile 0.1% peptone water. A series of dilutions (10^{-1} to 10^{-5}) were made adding guava sample in 0.1% peptone water. Then, 0.1 ml of dilution from appropriate dilution series (10^{-3} to 10^{-5}) was pipetted onto the surface of agar plate. Hockey stick was dipped into alcohol and flamed over a Bunsen Burner. The samples were spread evenly over the surface of agar using the sterile hockey stick. The agar plate was incubated at 37 °C for 24h. The log CFU/g value of the sample was calculated using equation [Eq. 1]:

$$\text{CFU/g} = \log ((\text{number of colonies} \times \text{dilution factor of plate}) / \text{aliquot plated}) \text{ [Eq. 1]}$$

3.6. Physico-chemical analysis

3.6.1. Fresh weight loss analysis

Fresh weight loss of each sample was measured every day and calculated based on the comparison of the weight of the control and chitosan-nanoemulsified essential oil at its initial condition and its final condition. The determination of fresh weight loss followed method by Ierna et al. (2017). The percentage of fresh weight loss was calculated using the following formula [Eq. 3]:

$$\% \text{ of fresh weight loss} = ((\text{initial weight} - \text{final weight}) / \text{initial weight}) \times 100 \text{ [Eq. 3]}$$

3.6.2. Firmness determination

According to Bezerra, Aquino, Fitzgerald, Cristina, & Aquino (2015), the uncoated and coated guavas was analysed by a normal test (single compression cycle) using a Texture Analyser equipped with a probe that was 6 mm in diameter

and 35 mm in length. Measurements were performed on the halves of each fruit (central region) that were placed on the texture analyser, where the probe descended at a speed of 1 mm/s, with a penetration of 10 mm, and returned to the home position. Firmness (N) was defined as the maximum force penetrating into the fruit.



Figure 3.4: Texture analyzer for firmness analysis

3.6.3. pH determination

According to Che Sulaiman et al. (2016), the pH measurement of the optimal nanoemulsion was performed at room temperature (25 ± 0.5 °C) by using a FiveEasy pH meter (Mettler-Toledo, China).

3.6.4. Colour parameters

The colour of the guavas were measured using a CR-410 Chroma (Konica Minolta, Japan) according to Min Yap et al. (2017). The colour was recorded using CIE- L^* , a^* , b^* scale where L^* represent lightness, a^* represent on green (-) to red (+) and b^* represent blue (-) to yellow (+).

3.6.5. Physical appearance

The appearance of guavas was recorded using an Iphone 5s of 8 megapixels. The appearance was observed at day 0, 3, 7 and 10.

3.7. Statistical analysis

Data collected were analysed using Minitab version 18. One way analysis of variance (ANOVA) at 95% confidence level was used to determine the differences using method for all responses with significant level of ($p < 0.05$). The significant differences between the samples were determined by using Turkey's test and all the data were reported in mean $\pm$ standard deviation with significant level.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Preparation and characterization of essential oils nanoemulsions

4.1.1. The appearance of essential oils nanoemulsions

Two types of essential oils, clove essential oil and lemon essential oil were used to produce nanoemulsions. The nanoemulsions were formulated according to five formulations (Table 4.1) which varies the composition of essential oil and virgin coconut oil, while the surfactant and water composition were fixed. In the end, there were ten samples of nanoemulsions produced which five samples contained clove essential oil (CEO), virgin coconut oil (VCO), surfactant (Tween 80) and water, and another five samples contained lemon essential oil (LEO), virgin coconut oil (VCO), surfactant (Tween 80) and water.

Table 4.1: Formulation of nanoemulsions

Formulation	Essential oil (%)	Virgin coconut oil (%)	Tween 80 (%)	Water (%)
1	1	4	5	90
2	2	3	5	90
3	3	2	5	90
4	4	1	5	90
5	5	0	5	90

By referring to **Figure 4.1 (a)**, Formulation 1 and 2 for nanoemulsions that contained clove essential oil (CEO) appear as translucent emulsions while the other three formulations that contained CEO appear as milky emulsions. However, for lemon essential oil (LEO) nanoemulsions shows in **Figure 4.1 (b)**, Formulation 1 and 2 appear as milky emulsions and Formulation 3 appears as translucent emulsion. Transparent emulsions obtained for nanoemulsions of Formulation 4 and 5.

Both essential oils showed different characteristics to the emulsions produced especially on the optical properties of nanoemulsion. Different types of essential oils have different polarity. Low polar oil attributed to the more transparent emulsion, compared with high polar or nonpolar oil (Byeong-Gyu Park, 2012). This shows that some components in lemon essential oil make it as low polar oil which can easily attracted with water by the help of surfactant compared to clove essential oil ("Chemical Interactions," n.d.).

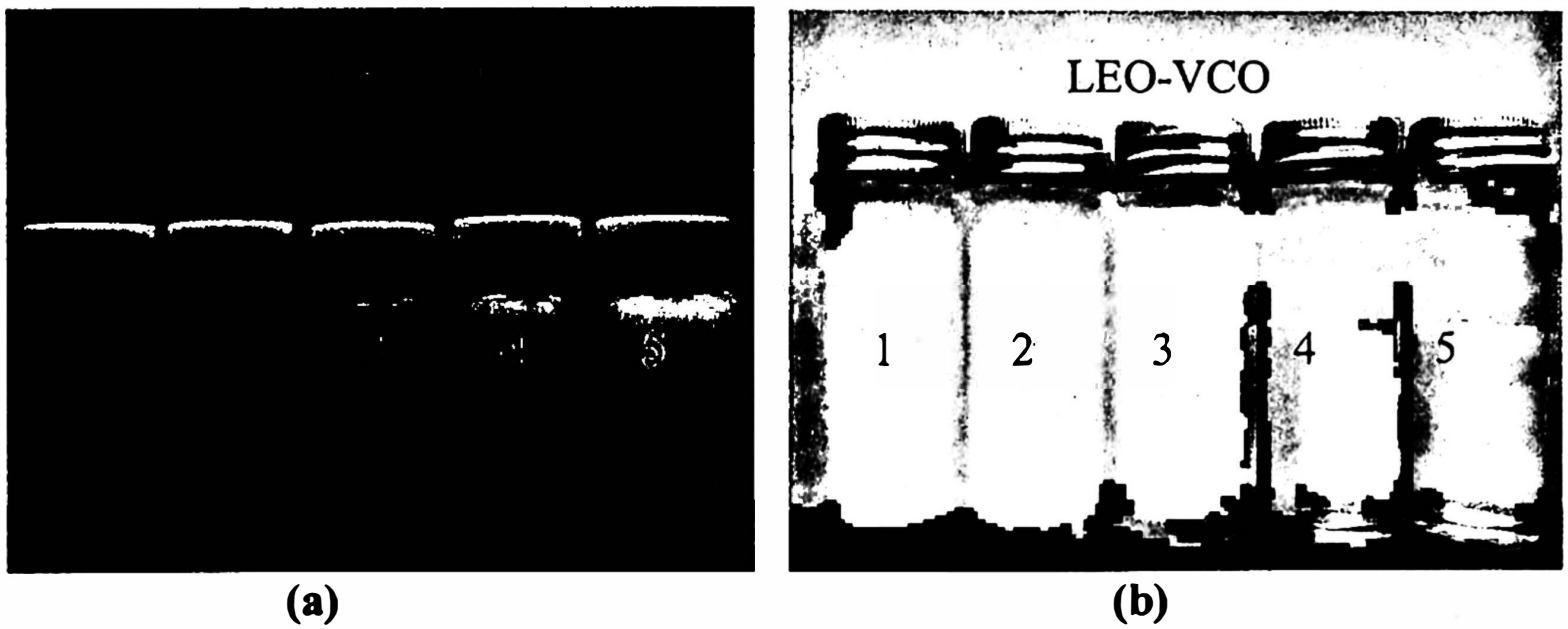


Figure 4.1: Nanoemulsion of five formulation starting with formulation 1 to 5 from left to right. (a) Clove essential oil nanoemulsions, and (b) lemon essential oil nanoemulsions

4.1.2. The effect of oil phase composition on droplet size of nanoemulsions

The nanoemulsions produced were formulated by mixing two types of oil; essential oil (clove essential oil or lemon essential oil) and carrier oil (virgin coconut oil). Size is obtained from the correlation function by using various algorithms, which is can be taken by fit a single exponential to the correlation function to obtain the mean size (z-average diameter) and an estimate of the width of the distribution (polydispersity index).

Polydispersity index (PDI) is a dimensionless measure of the broadness of the size distribution calculated from the cumulants analysis. According to Aida, Mustapha, & Mohamad (2014), a PDI of <0.08 indicates nearly monodisperse sample, while $0.08-0.70$ is midrange value of PDI. It is the range over which the distribution algorithms best operation over. A PDI close to 1 (>0.7) indicates a very broad distribution of droplet size.

From Figure 4.2, the droplets size of nanoemulsions obtained for systems containing clove essential oil and virgin coconut oil are at range of 140.6nm to

67.1nm. Formulation 2 which consists of CEO-VCO 2:3, has the lowest droplets size (67.1nm). Moreover, the PDI for all formulations that contained clove essential oil are in midrange value which is showed in Table 4.2.

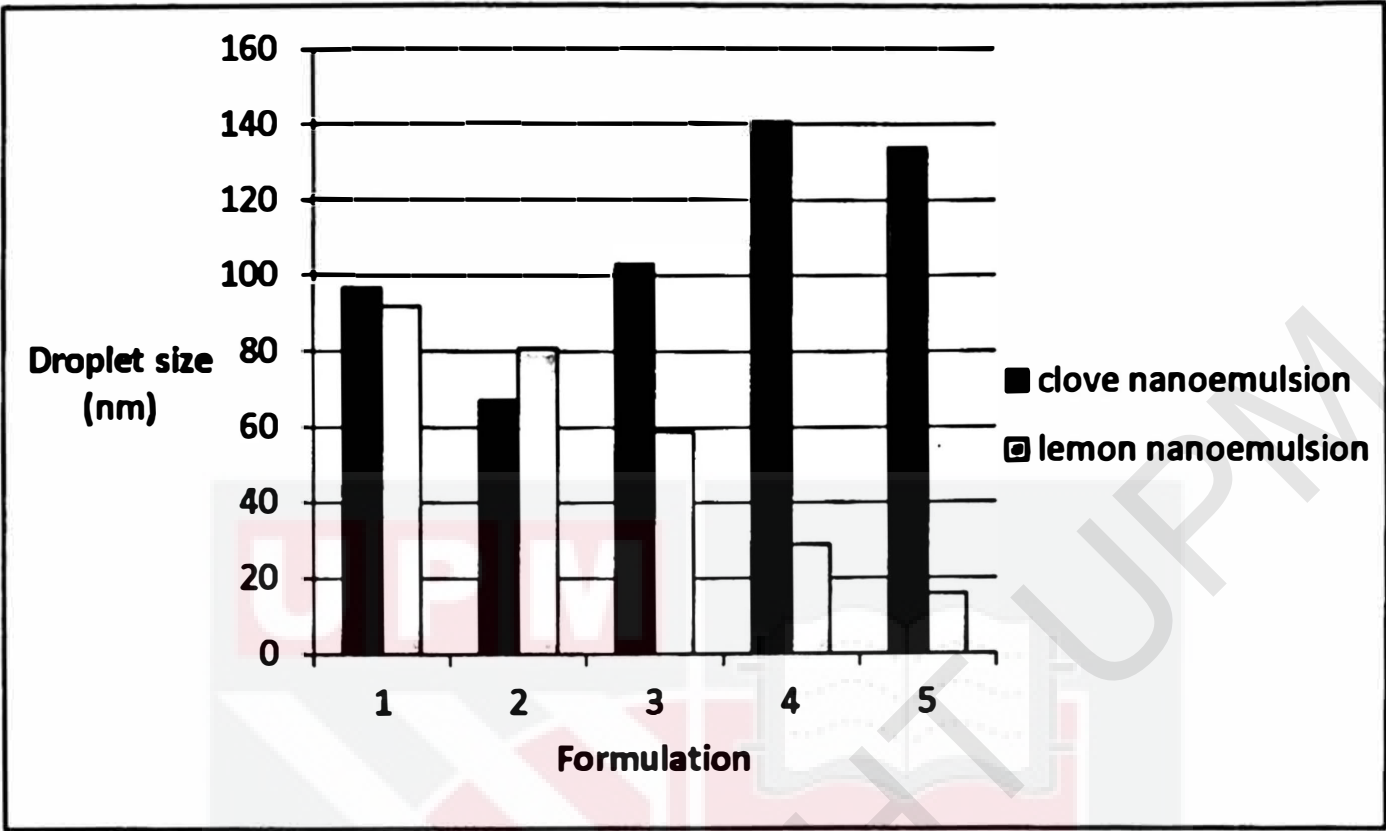


Figure 4.2: Graph of size of clove and lemon essential oil nanoemulsions

Table 4.2: Polydispersity Index (PDI) of essential oil nanoemulsions

Polydispersity Index (PDI)					
Formulation	1	2	3	4	5
CEO	0.319 ± 0.00	0.301 ± 0.01	0.100 ± 0.01	0.124 ± 0.03	0.093 ± 0.01
LEO	0.326 ± 0.01	0.324 ± 0.01	0.310 ± 0.00	0.232 ± 0.01	0.195 ± 0.01

For lemon essential oil nanoemulsions, the droplets size are decreasing from Formulation 1 to Formulation 5 as shown in Figure 4.2. Formulation 1 (LEO-VCO 1:4) has the largest size of nanoemulsion while Formulation 5 (LEO-VCO 5:0) has the smallest size of nanoemulsion which are 91.8nm and 15.8nm respectively. The PDI for all formulations that contained lemon EO are in midrange value which is showed in Table 4.2.

Nevertheless, when using same formulation but different type of essential oils, different ranges of droplets size were obtained. This shows that different types of EOs have their own chemical characteristics that make them behave differently to form nanoemulsion. To the best of our knowledge, there are not available studies dealing with the size comparison of different types of EOs to form nanoemulsions.

4.1.3. The effect of oil phase composition on stability of CEO-VCO emulsions and LEO-VCO emulsions during a month of storage

To form a stable emulsion, the suitable amount of surfactant used in the fabrication of emulsion is important. The function of surfactant is widely known to protect the oil droplets from coalescence. However, low amount of surfactant in the formulation of emulsions can also give a nanometric size of emulsions as proved by Tubtimsri et al., 2018. This condition is the same as the formulations for emulsions in this study where the surfactant used is fixed at 5%. The oil composition is varied between the EOs and VCO to achieve 5% oil composition. Therefore, from the results obtained, it shows that the ratio of EOs to VCO influenced the stability of nanoemulsions as in Figure 4.3 and Figure 4.4.

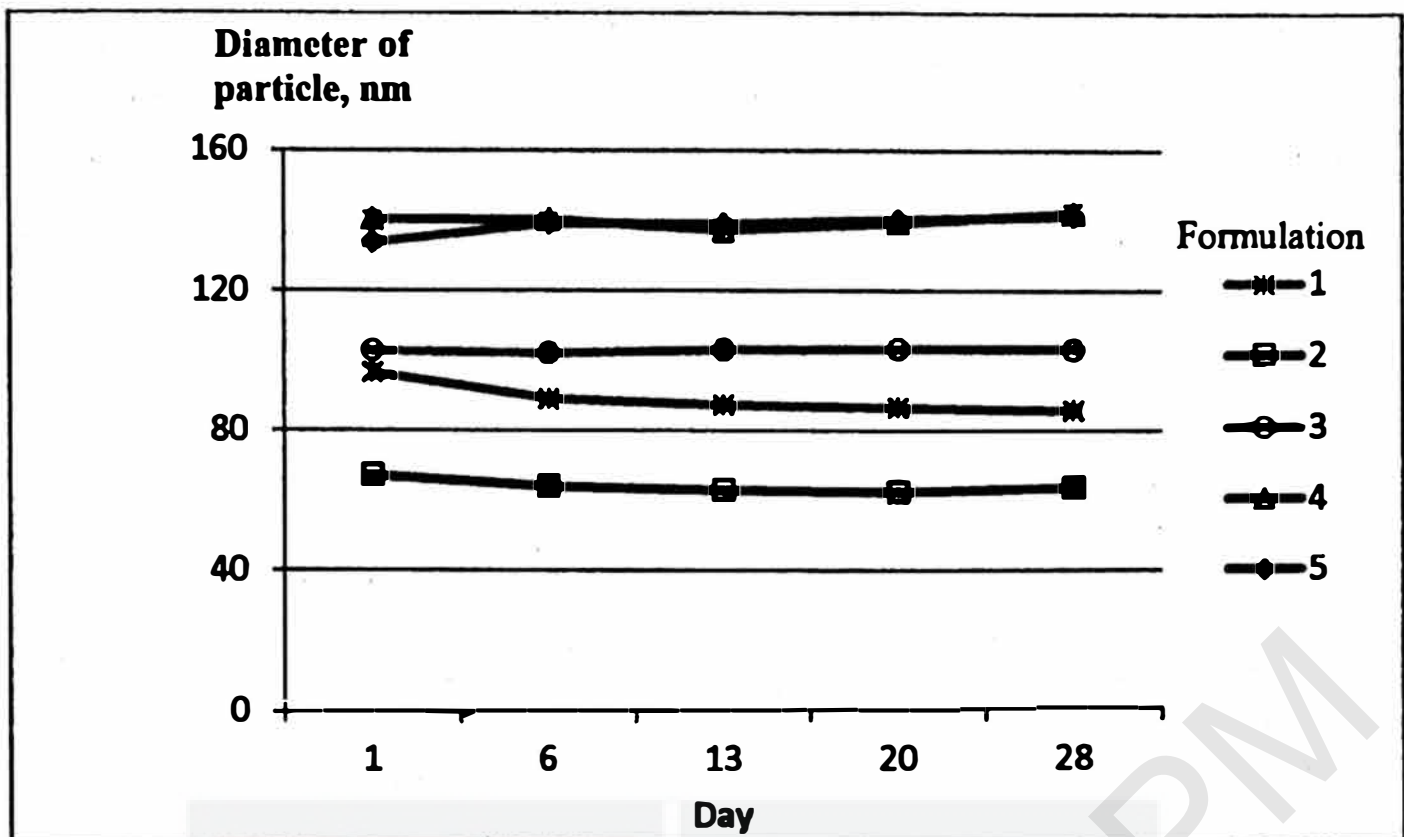


Figure 4.3: Graph of the stability of clove essential oil (CEO) nanoemulsion from Formulation 1 to Formulation 5 during a month

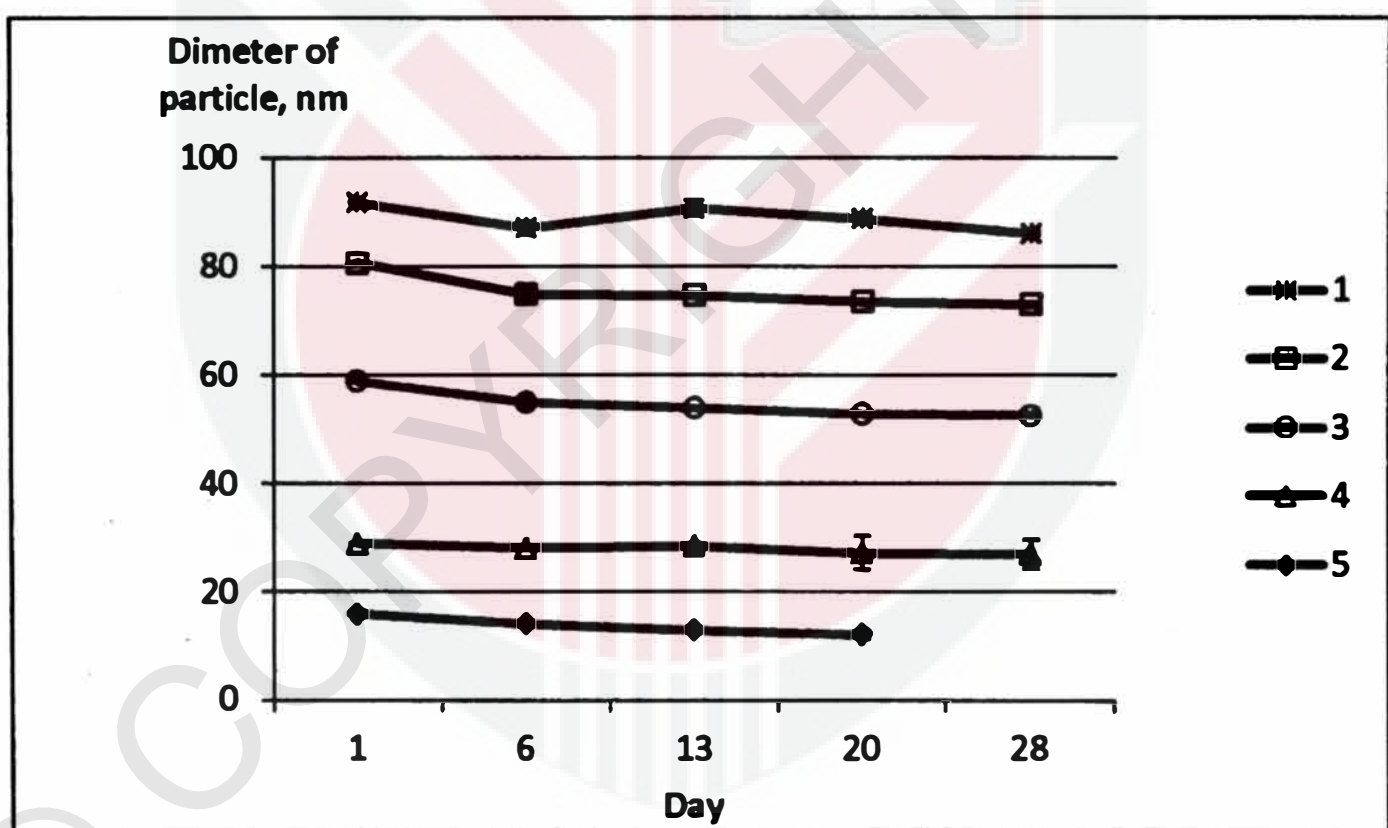


Figure 4.4: Graph of the stability of lemon oil nanoemulsions during a month

Based on Figure 4.3, all formulations of CEO-VCO nanoemulsions show a stable trend. Same goes to all formulations of LEO-VCO nanoemulsions showed in Figure 4.4 that maintain as a stable trend. However, on day 28, Formulation 5 that contained only lemon essential oil as oil phase was having a thin layer of phase separation.

In addition, the constant droplets size and slight increase in the droplets size in some cases are a sign of their physical stability during storage time for duration of 28 days (Hassan, Hosseini, Jahanshahi, Louise, & Najafpour, 2015). This finding has confirmed that the oil phase composition in an emulsion affect the stability of nanoemulsions. Other study also supported this statement as Chang and McClements reported that orange oil alone could not produce stable emulsions while the transparent nanoemulsions were only formed if 30% – 50% w/w orange oil were blended with medium chain triglyceride.

One phenomenon relates with the growth of oil droplets through the continuous phase of nanoemulsion is Ostwald ripening which cause the destabilizing of nanoemulsions. Mostly essential oils contained a small non polar molecule compared to the bigger non polar molecule, traulin in VCO. Therefore, by changing the partitioning of EOs between the lipid droplets and the aqueous phase, for example by mixing with medium or long chain triglycerides oils, generating an entropy of mixing effect that counteracts the imbalance of droplet size effect (Donsi & Ferrari, 2016).

4.1.4. Conclusion for optimal formulations to be choose for application as edible coating

Based on the results that had been discussed about the appearance of nanoemulsions produced, the droplets size of nanoemulsions and the stability of nanoemulsions during a month of storage, the optimal formulation for both essential oil to be used in the application for edible coating can be choose. For clove essential oil, Formulation 2 which consists of 2% CEO, 3% VCO, 5% Tween 80 and 90%

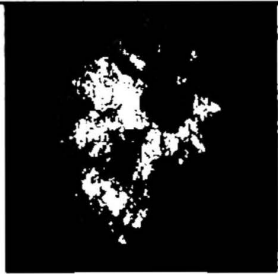
water. This formulation appears as translucent nanoemulsion with droplets size of 67.1nm and maintains its stability during a month of storage. In contrast, for lemon essential oil, Formulation 4 which consists of 4% LEO, 1% VCO, 5% Tween 80 and 90% water. This formulation appears as transparent nanoemulsion with droplets size of 28.9nm and maintains its stability during a month of storage. Even though Formulation 5 for lemon essential oil has the lowest droplets size, it is not choosing because at day 28 of storage, the system was not stable and showed a phase separation.

4.2. The application of essential oil nanoemulsion as edible coating on guava

4.1.1. The physical appearance of guava during storage time at 25°C

The physical appearances of guavas are shown in Table 4.3. For uncoated guava, it was observed to be rotten on day 4 of storage. Guava coated with chitosan containing clove essential oil (CH-CEO) showed the most appearance of black spots at day 7. On the tenth day of storage, guava coated with chitosan containing clove essential oil nanoemulsion (CH-CEO (HPH)) and guava coated with chitosan containing lemon essential oil nanoemulsion (CH-LEO (HPH)) resulted in better appearance when compared to chitosan coating (CH) chitosan containing clove essential oil coating (CH-CEO) and chitosan containing lemon essential oil coating (CH-LEO).

Table 4.3: Physical appearance of guava during storage time at 25°C

Treatment	Storage time (day)			
	0	3	7	10
CH				
CH-CEO (HPH)				
CH-CEO				
CH-LEO (HPH)				
CH-LEO				

4.1.2. Effect of treatments on microbiological properties of guava during storage time at 25°C

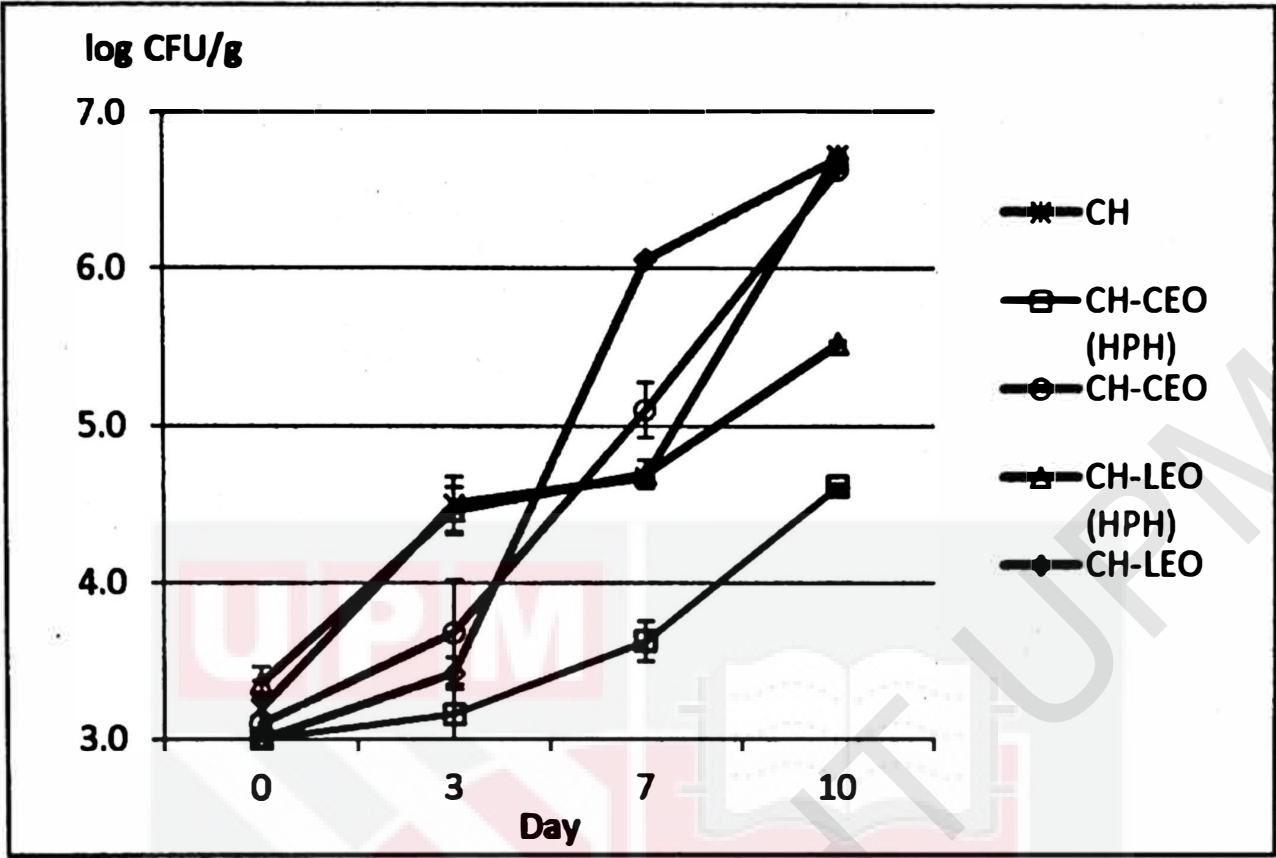
From the result obtained that showed in Figure 4.5, the total plate counts and yeast and mould counts of guava coated with chitosan only (CH), guava coated with chitosan containing clove essential oil (CH-CEO) and guava coated with chitosan containing lemon essential oil (CH-LEO) increase rapidly during the storage period

of ten days. However, guava coated with chitosan containing clove essential oil nanoemulsion (CH-CEO (HPH)) and guava coated with chitosan containing lemon essential oil nanoemulsion (CH-LEO (HPH)) increase gradually during the storage time. On day 7 of storage, the total plate counts and yeast and mould counts for guavas coated with CH-CEO and CH-LEO showed higher log CFU/g value compared to CH-CEO (HPH) and CH-LEO (HPH). This is probably due to the evaporation of essential oils in the coating because they are not encapsulated as nanoemulsions like CH-CEO (HPH) and CH-LEO (HPH).

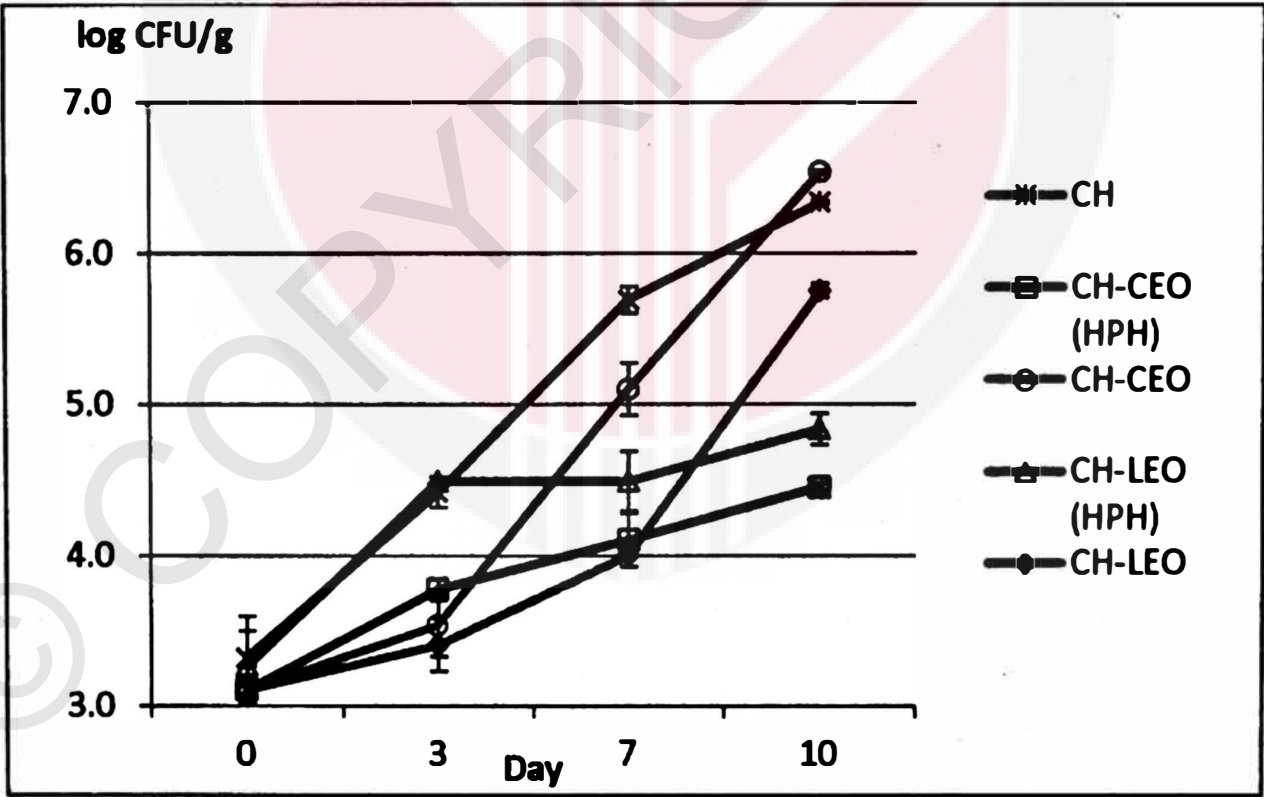
A count of 6 log CFU/g for yeast, mould or total bacteria is considered to be the limit of acceptance for fruit-based products during a study of shelf life (Bezerra, Aquino, Fitzgerald, Cristina, & Aquino, 2015). On the day 10 of storage, guava coated with CH-CEO (HPH) and CH-LEO (HPH) were successful to have log CFU/g value below 6 log CFU/g for total plate counts (4.6 log CFU/g and 5.5 log CFU/g, respectively) and yeast and mould counts (4.5 log CFU/g and 4.85 log CFU/g, respectively). This result shows that when guava coated with chitosan containing nanoemulsified essential oil, the microorganisms in the guava can be reduced to prolong its shelf life.

This finding is proved by Weiss, Takhistov, & McClements (2006), who stated that a decrease in the oil droplets size would enable faster penetration of antimicrobial compounds in the bacterial cell. Moreover, Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso (2015) also reported that bacterial counts of 4.6 log CFU/g were observed at the end of the evaluated storage period for fresh-cut apples coated with nanoemulsions of 0.1% (v/v) while for conventional emulsions

with the same lemongrass essential oil concentration led to microbial concentrations above 5.4 log CFU/g after two weeks of refrigerated storage.



(a)



(b)

Figure 4.5: Log CFU/g graph (a) for total plate count and (b) for mould and yeast count

4.1.3. Weight loss of guava during storage time at 25°C

Weight loss is mainly due to the loss of water caused by transpiration and respiration processes (Zhu et al., 2008). This process affected the weight of guava which caused the guava to lose weight and become shrinking. Figure 4.6 shows that at the end of storage period, guava coated with chitosan containing clove essential oil nanoemulsion (CH-CEO (HPH)) has the lowest weight loss percentage ($p < 0.05$) with the value of 26.53% compared to other samples. However, guava coated with chitosan only (CH) has the highest weight loss percentage which is 33.87%. Weight loss for guava coated with CH is higher than other chitosan coating containing essential oils about 6.5%.

Although, chitosan coating with its filmogenic properties has been used as a water barrier to minimize water and weight loss of fruit during storage (Bourlieu, Guillard, Valles-Pamies, Guilbert, & Gontard, 2009; Vargas, Pastor, Chiralt, McClements, & Gonzalez-Martinez, 2008), the application of chitosan coatings and films to fresh food products has poor water vapour barrier properties. In coatings and films, the water vapour transfer processes depend on the hydrophilic-hydrophobic ratio of the film constituents. The incorporation of hydrophobic EOs into hydrophilic polymer matrices may result in the reduction of their water vapour permeability. Therefore, as showed in Figure 4.6, the chitosan coatings containing EOs with improved water vapour barrier properties showed greater effectiveness for reducing the weight loss of guava at day 10 of storage compared to pure chitosan coatings.

Fruits losing weight when their metabolic rate increases especially when the environment temperature increasing. Since the storage temperature for this

experiment is quite high for fresh fruit which is 25°C, it is also affecting the weight loss of guavas. The high temperature storage leads to physical damage due to tissue breakdown and increase in fruit softening. Thanh (2006) found that temperatures above 20°C could result in abnormal physiological processes in fresh fruit. The major process in this regard is respiration through which water is lost to the immediate surroundings of the produce and hence, a reduction in weight.

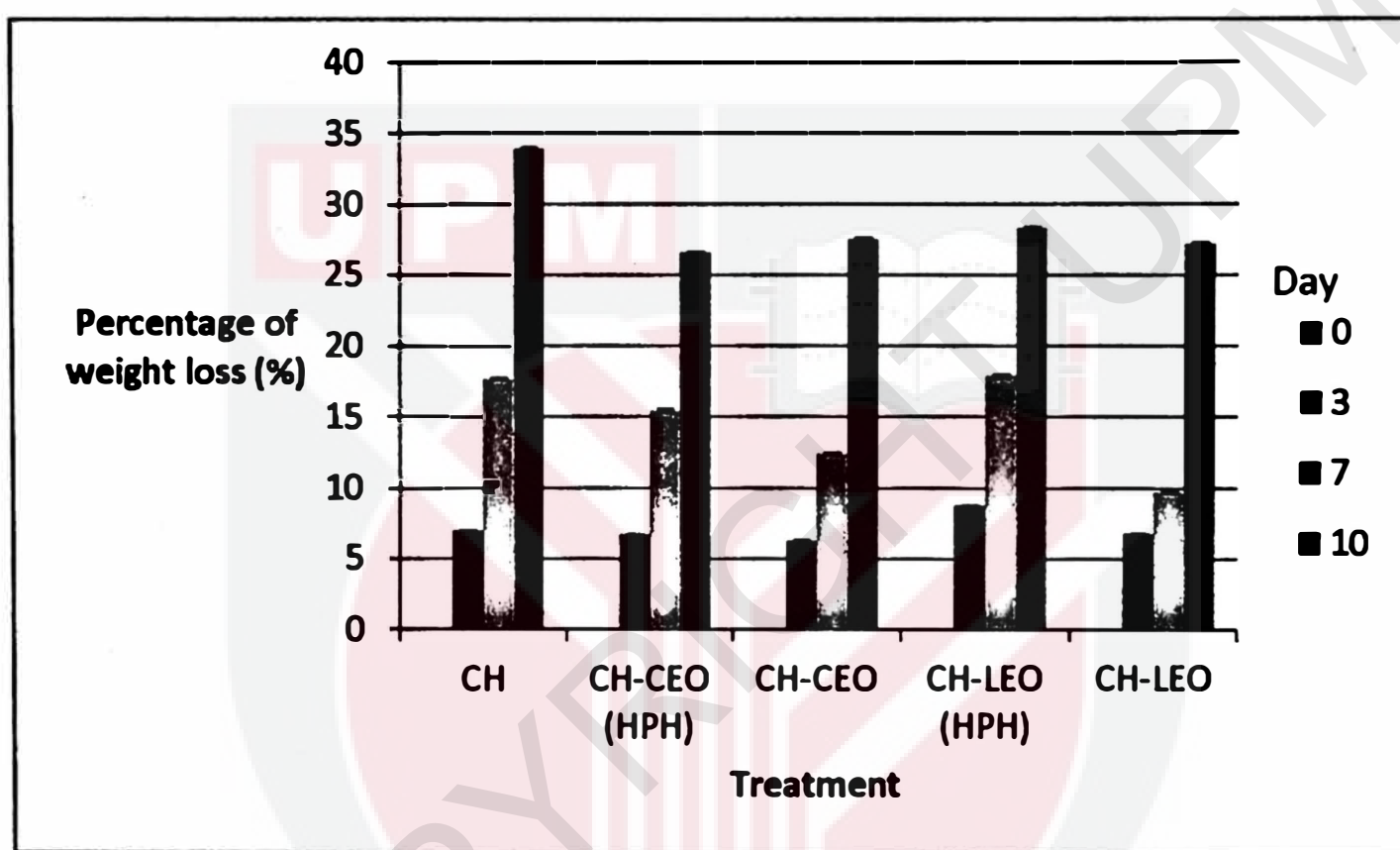


Figure 4.6: Weight loss (%) of guava. CH: guava coated with chitosan; CH-CEO (HPH): guava coated with chitosan containing clove EO nanoemulsion; CH-CEO: guava coated with chitosan containing clove EO; CH-LEO (HPH): guava coated with chitosan containing lemon EO nanoemulsion; CH-LEO: guava coated with chitosan containing lemon EO.

4.1.4. Firmness of guava during storage time at 25°C

Fruit firmness is an important criteria that always been judge by consumer to purchase. During process of deterioration, guava suffers a rapid loss of firmness which contributes greatly to its short postharvest life and susceptibility to fungal contamination. Changes in flesh firmness of the coated fruit samples during 10 days of storage at 25°C are shown in graph of Error! Reference source not found..

From this graph, after the third day of storage, the firmness of all coated fruits started to decrease. It showed that the firmness of CH-, CH-CEO- and CH-LEO-coated fruits decreased rapidly while the firmness of CH-CEO (HPH) - and CH-LEO (HPH) – coated fruits decreased gradually. On the last day of storage, the fruits coated with CH-CEO and CH-CEO (HPH) were found to be firmer than the other samples.

This result is supported by Mohammadi et al. (2015a) and Mohammadi et al. (2016a) that the application of nanochitosan alone or in combination with Zataria multiflora (ZEO) or Cinnamomum seylanicum (CEO) essential oil as a coating material improved the firmness of cucumber. Reduction in respiration rates of coated cucumber could be responsible for delaying softening which resulted in retention of firmness during storage.

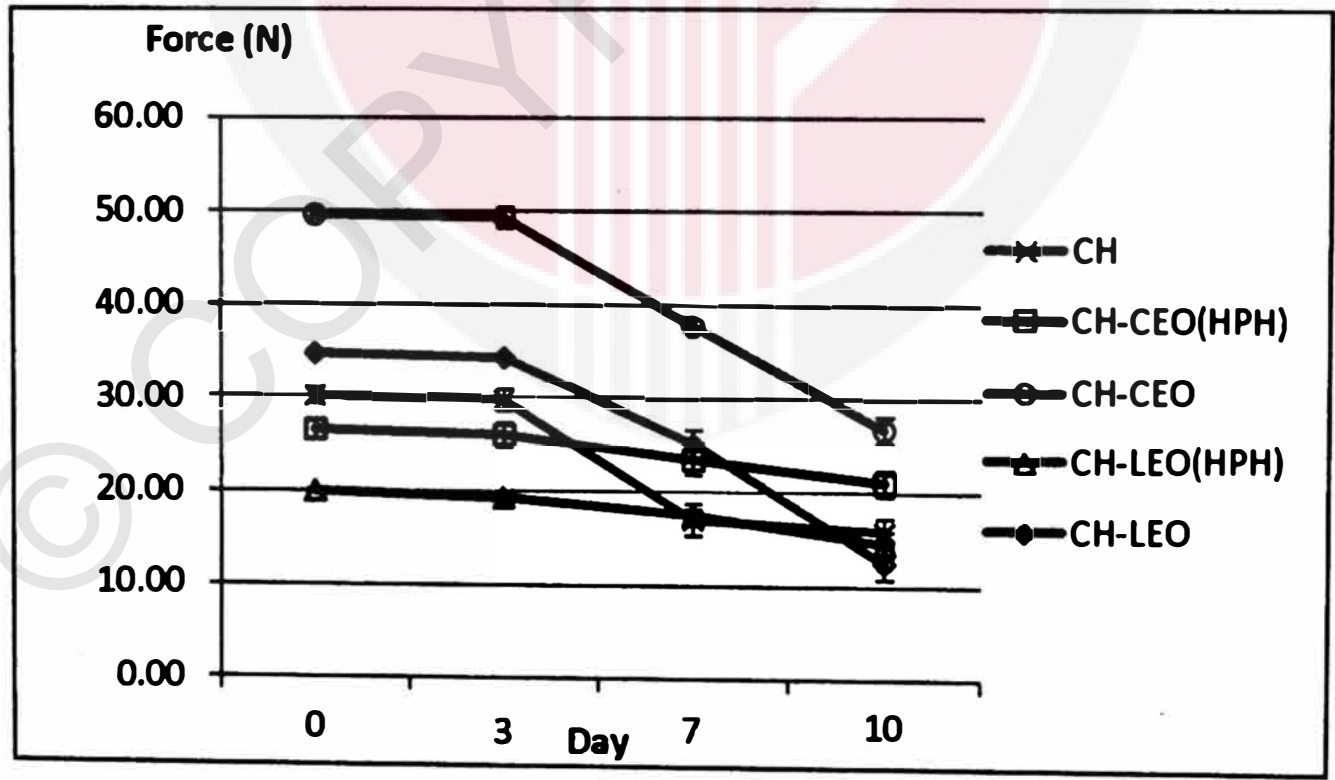
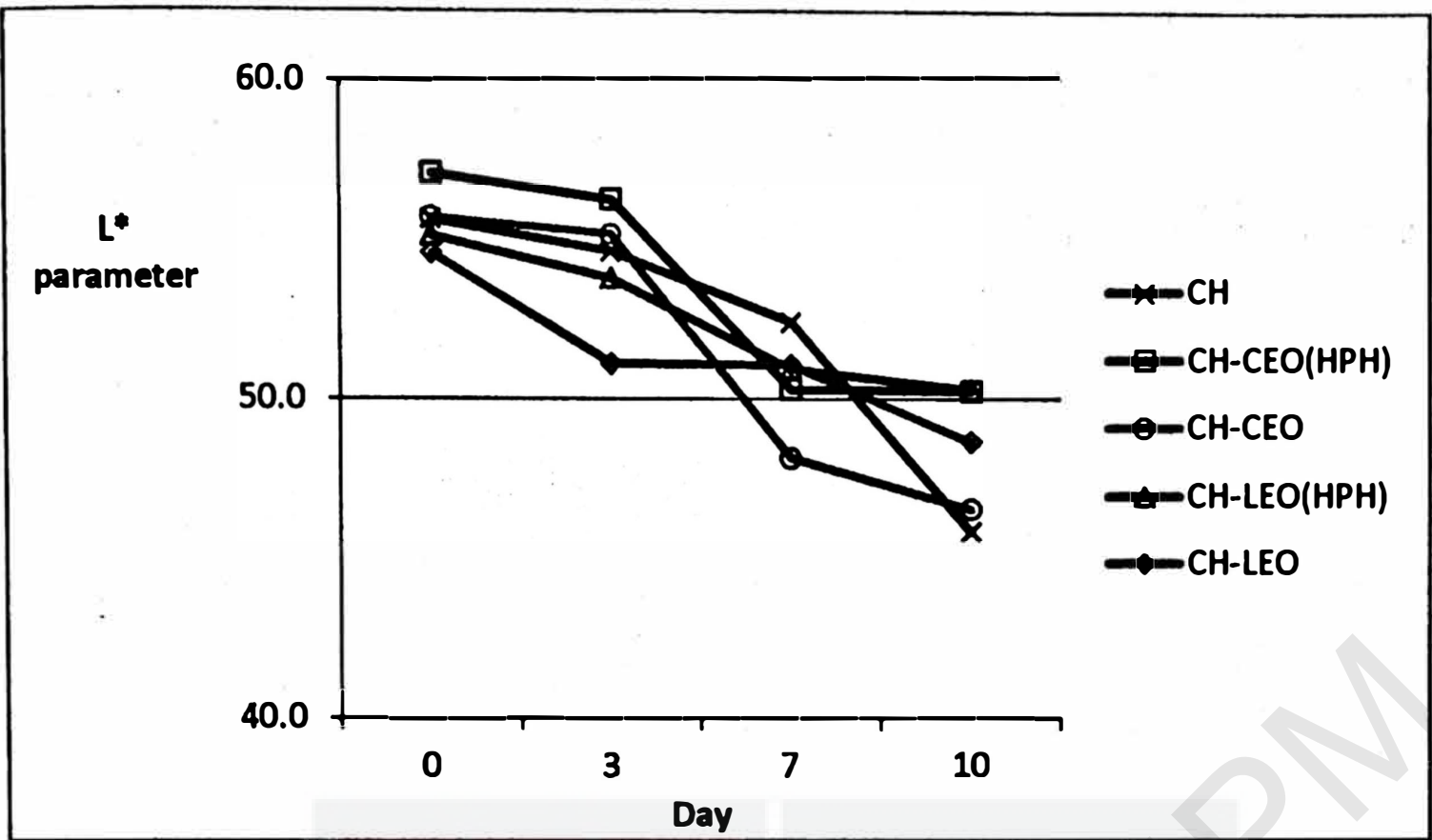


Figure 4.7: Graph of the guava firmness. CH: guava coated with chitosan; CH-CEO (HPH): guava coated with chitosan containing clove EO nanoemulsion; CH-CEO: guava coated with chitosan containing clove EO; CH-LEO (HPH): guava coated with chitosan containing lemon EO nanoemulsion; CH-LEO: guava coated with chitosan containing lemon EO

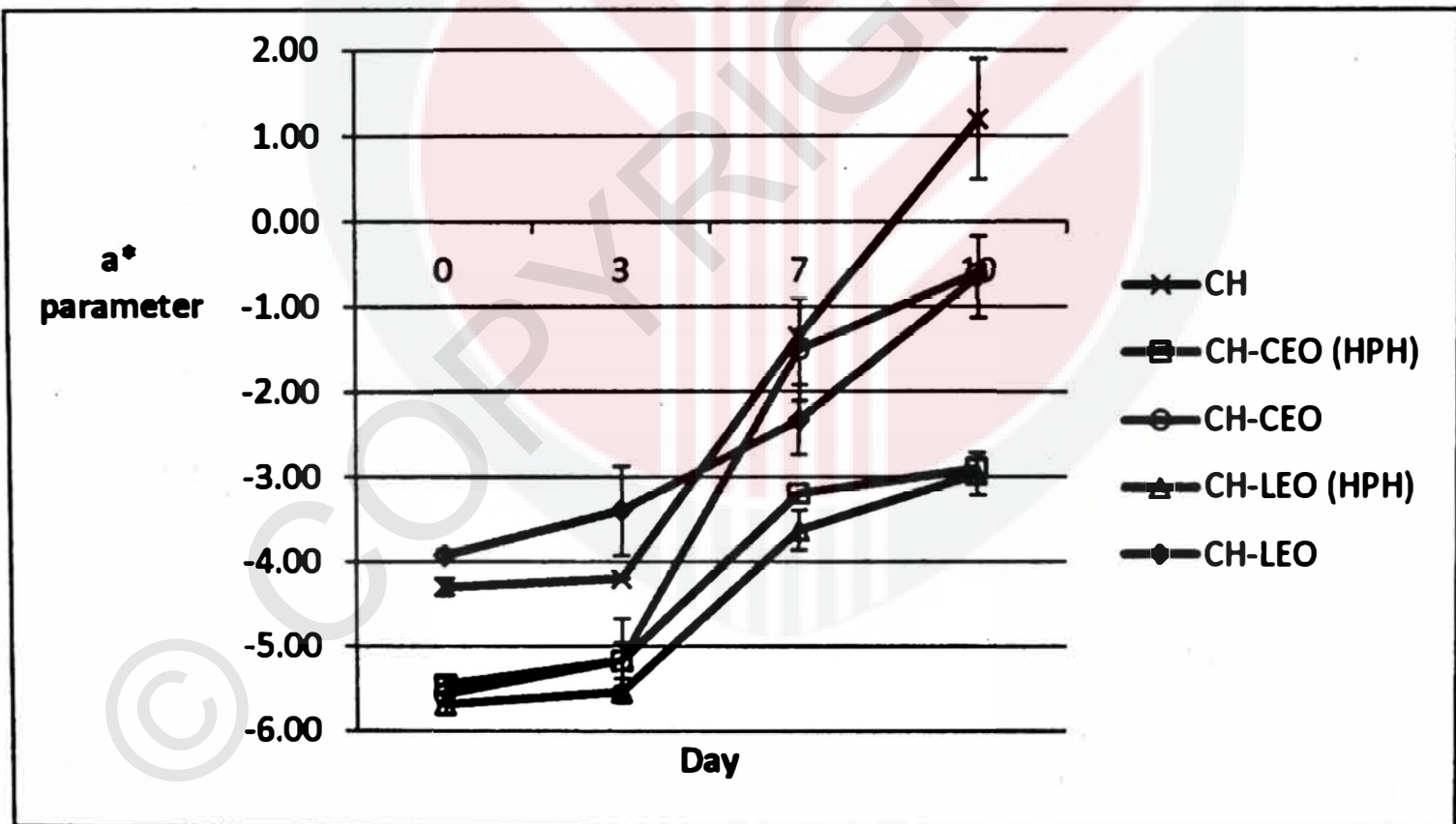
4.1.5. Colour properties of guava during storage at 25°C

For colour properties of guava, there are three parameters to be discuss which are the L^* represent lightness, a^* represent on green (-) to red (+) and b^* represent blue (-) to yellow (+). From **Error! Reference source not found.**, the L parameter ho indicates the lightness of the fruits decreased with increasing storage time. However, a^* and b^* parameter of the fruits increased throughout the storage time. This indicates that the fruits lost their skin greenness since the a^* parameter becoming less negative value and they become more yellowish. Fruits coated with CH-CEO (HPH) and CH-LEO (HPH) had significantly higher value of L parameter and the lower value of a^* and b^* .

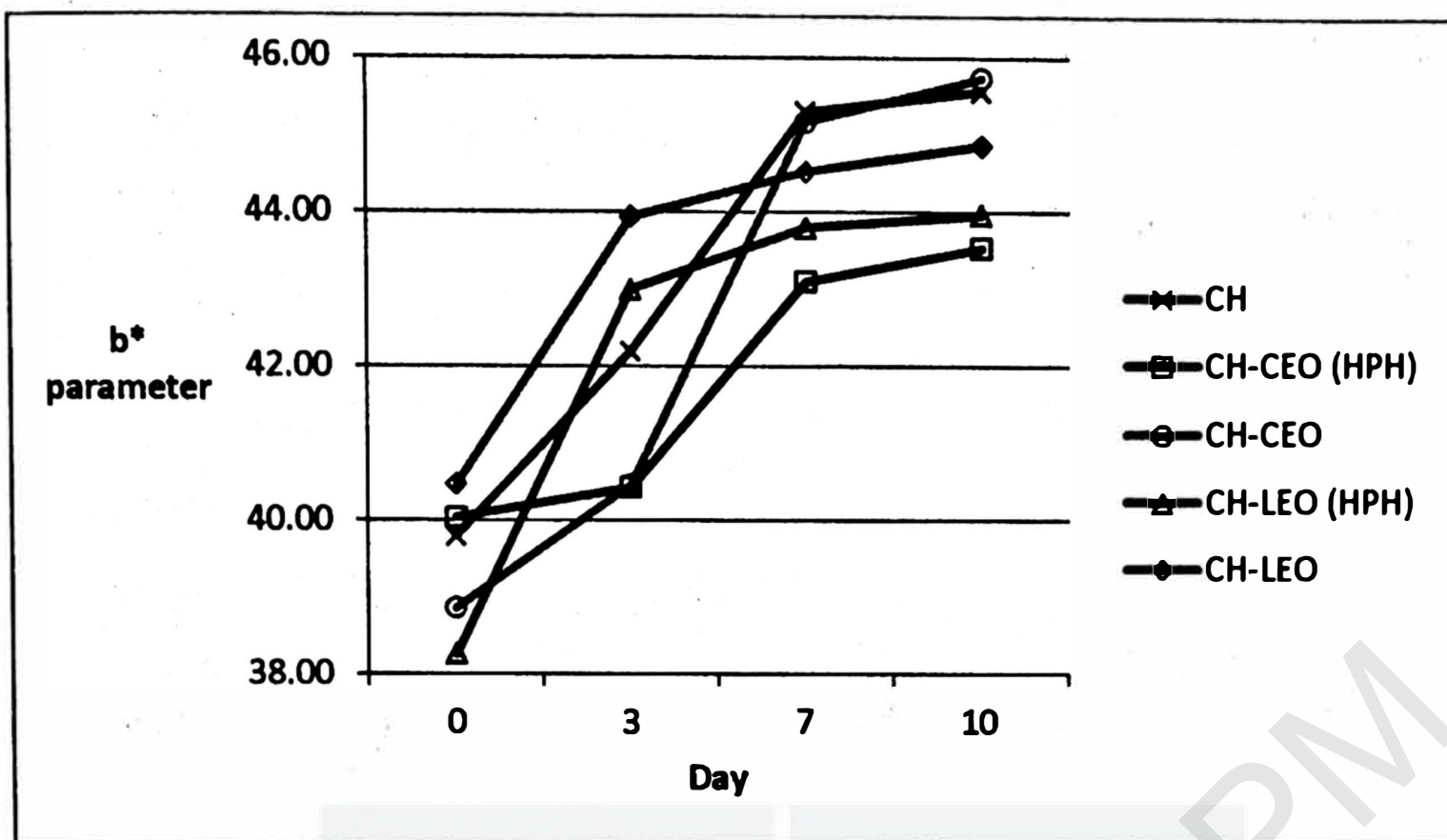
This result also supported by the physical appearance of the fruits during storage at 25°C shows in Table 4.3. Bezerra et al. (2015) reported that the addition of a 3.0% EO mixture was most effective in preventing browning (low reduction of L parameter) in guavas. The decreases of L and a^* value were due to over-ripening and rapid senescence, where the fruits turned dull and yellowish brown in colour. The decrease in negative a^* value (greenness) was accompanied by the increase in yellowness (b^* value). The loss in skin greenness during ripening perhaps may be due to increased activities of chlorophyll degrading enzymes including chlorophyllase, chlorophyll oxidase and peroxidase (Jain et al., 2001).



(a)



(b)



(c)

Figure 4.8: (a) L^* parameter, (b) a^* parameter and (c) b^* parameter of colour properties of guava. CH: guava coated with chitosan; CH-CEO (HPH): guava coated with chitosan containing clove EO nanoemulsion; CH-CEO: guava coated with chitosan containing clove EO; CH-LEO (HPH): guava coated with chitosan containing lemon EO nanoemulsion; CH-LEO: guava coated with chitosan containing lemon EO

4.1.6. The effect of treatment on the pH of guava during storage time at 25°C

The pH did not show significant changes during storage (Figure 4.9), although there were differences among the samples. On the tenth day of storage, guava coated with CH showed slightly greater mean values (4.33) than the other coated guava. However, these can be attributed to the natural variability of the product and not to the treatment. Due to the nature of fruit organic acids, the usual decrease in fruit acidity (Vargas et. al, 2006) did not provoke any notable changes in pH.

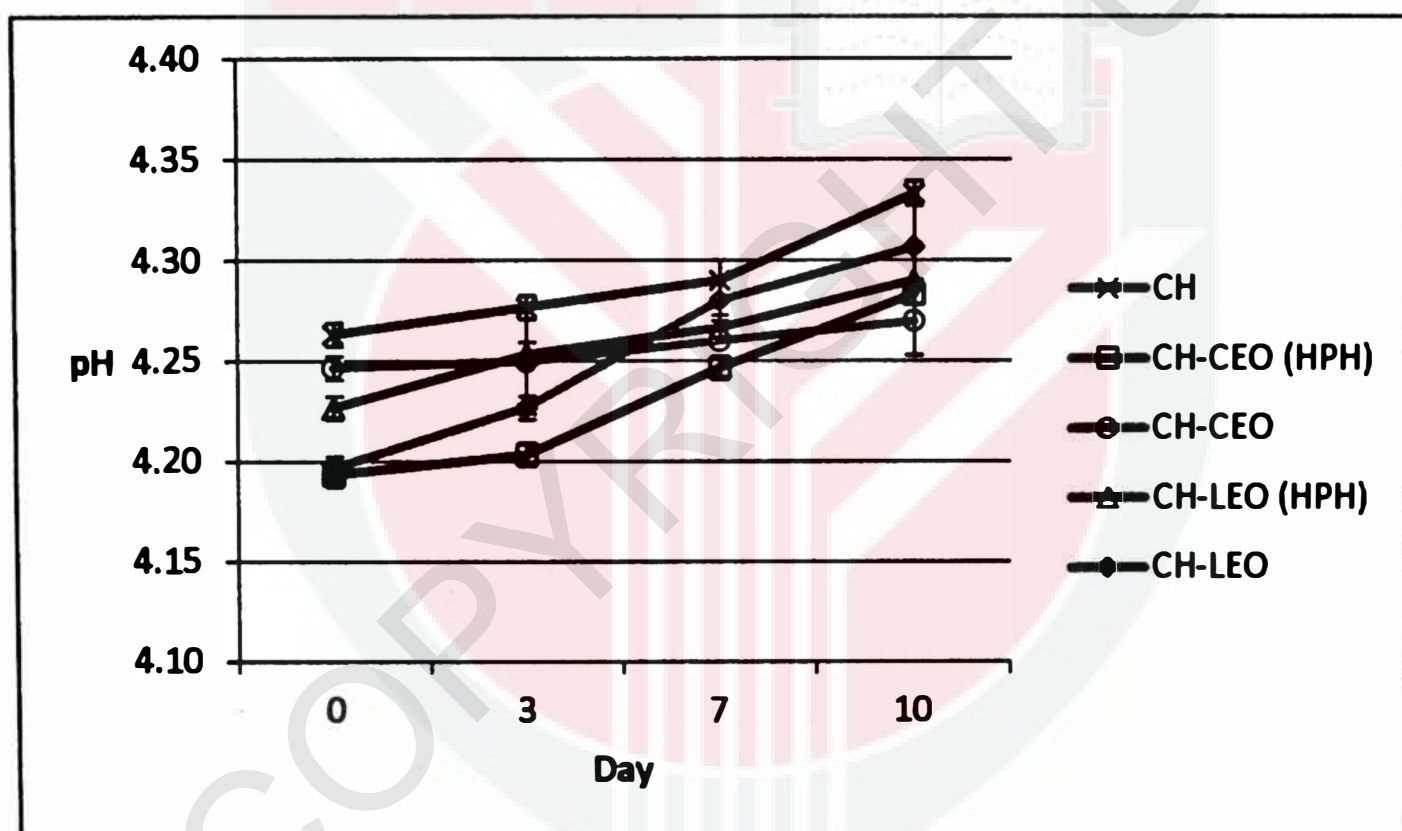


Figure 4.9: pH value of guava during storage time. CH: guava coated with chitosan; CH-CEO (HPH): guava coated with chitosan containing clove EO nanoemulsion; CH-CEO: guava coated with chitosan containing clove EO; CH-LEO (HPH): guava coated with chitosan containing lemon EO nanoemulsion; CH-LEO: guava coated with chitosan containing lemon EO.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

The experiment aimed to investigate the stability of nanoemulsion containing virgin coconut oil as the carrier oil. Formulation containing 4% lemon EO, 1% VCO, 5% Tween 80 and 90% water, and formulation containing 2% clove EO, 3% VCO, 5% Tween 80 and 90% water showed the most stable nanoemulsions and give the transparent or translucent appearance. Furthermore, the stable nanoemulsions were used to incorporate with chitosan as edible coating to study the effect of edible chitosan coatings containing essential oils of clove and lemon on the shelf life of guavas. Guavas coated with CH-CEO (HPH) and CH-LEO (HPH) demonstrated excellent microbiological analysis in term of total aerobic microbial and yeast and moulds. These type of coatings also had shown their capabilities to reduced weight loss and had better physical appearance of guavas at the end of day tenth. The results obtained indicate that edible chitosan enriched with essential oil in nanometric size

has potential as the bioactive packaging of guavas, which are very perishable, in order to extend their shelf life at room temperature.

For further studies, it is recommended to investigate the effectiveness of nanoemulsion on specific microorganisms which is the in-vitro studies. From this analysis, we can compare which formulation of nanoemulsions that has the greater zone of inhibition. Sensory analysis also should be carried out to test in terms of the intensity of each of the sensory parameters; aroma, gloss, surface roughness, flavour, sweetness, acidity and astringency. This analysis is important to know whether the application of coating has affected the sensory quality of guava or not.

REFERENCES

- Aida, W., Mustapha, W., & Mohamad, S. (2014). Characteristics of Cinnamaldehyde Nanoemulsion Prepared using APV-High Pressure Homogenizer and Ultra Turrax, 244. <https://doi.org/10.1063/1.4895203>
- Bezerra, A., Aquino, D., Fitzgerald, A., Cristina, L., & Aquino, L. De. (2015). Impact of edible chitosan – cassava starch coatings enriched with *Lippia gracilis* Schauer genotype mixtures on the shelf life of guavas (*Psidium guajava* L .) during storage at room temperature. *FOOD CHEMISTRY*, 171, 108–116. <https://doi.org/10.1016/j.foodchem.2014.08.077>
- Chemical Interactions. (n.d.). *ClassZone*, 320. Retrieved from http://www.classzone.com/vpg_ebooks/ml_sci_gr8/accessibility/ml_sci_gr8/page_320.pdf
- Donsì, F., & Ferrari, G. (2016). Essential oil nanoemulsions as antimicrobial agents in food. *Journal of Biotechnology*, 233, 106–120. <https://doi.org/10.1016/j.jbiotec.2016.07.005>
- Fisher, K., & Phillips, C. (2008). Potential antimicrobial uses of essential oils in food: is citrus the answer? *Trends in Food Science and Technology*, 19(3), 156–164. <https://doi.org/10.1016/j.tifs.2007.11.006>
- Gupta, A., Eral, H. B., Hatton, T. A., & Doyle, P. S. (2016). Nanoemulsions: formation, properties and applications. *Soft Matter*, 12(11), 2826–2841.

Hassan, M., Hosseini, M., Jahanshahi, M., Louise, R., & Najafpour, G. (2015). Evaluation of critical parameters for preparation of stable clove oil nanoemulsion. *ARABIAN JOURNAL OF CHEMISTRY*.
<https://doi.org/10.1016/j.arabjc.2015.08.024>

Mohammadi, A., Hashemi, M., & Hosseini, S. M. (2015). Chitosan nanoparticles loaded with *Cinnamomum zeylanicum* essential oil enhance the shelf life of cucumber during cold storage. *Postharvest Biology and Technology*, 110, 203–213. <https://doi.org/10.1016/j.postharvbio.2015.08.019>

Mohammadi, A., Hashemi, M., & Hosseini, S. M. (2016). Integration between chitosan and *Zataria multiflora* or *Cinnamomum zeylanicum* essential oil for controlling *Phytophthora drechsleri*, the causal agent of cucumber fruit rot. *LWT - Food Science and Technology*, 65, 349–356.
<https://doi.org/10.1016/j.lwt.2015.08.015>

Salvia-Trujillo, L., Rojas-Graü, M. A., Soliva-Fortuny, R., & Martín-Belloso, O. (2015). Use of antimicrobial nanoemulsions as edible coatings: Impact on safety and quality attributes of fresh-cut fuji apples. *Postharvest Biology and Technology*, 105, 8–16. <https://doi.org/10.1016/j.postharvbio.2015.03.009>

Tubtimsri, S., Limmatvapirat, C., Limsirichaikul, S., Akkaramongkolporn, P., Inoue, Y., & Limmatvapirat, S. (2018). Fabrication and characterization of spearmint oil loaded nanoemulsions as cytotoxic agents against oral cancer cell ☆. *Asian*

<https://doi.org/10.1016/j.ajps.2018.02.003>

Vargas, M., Albors, A., Chiralt, A., & González-Martínez, C. (2006). Quality of cold-stored strawberries as affected by chitosan-oleic acid edible coatings. *Postharvest Biology and Technology*, 41(2), 164–171.
<https://doi.org/10.1016/j.postharvbio.2006.03.016>

Weiss, J., Takhistov, P., & McClements, D. J. (2006). Functional materials in food nanotechnology. *Journal of Food Science*, 71(9), 107–116.
<https://doi.org/10.1111/j.1750-3841.2006.00195.x>

APPENDICES

APPENDIX A: Reading obtained for microbiological analysis

Total Plate Count

Chitosan coating

Reading	Day 0	Dilution factor	Day 3	Dilution factor	Day 7	Dilution factor	Day 10	Dilution factor
1	2	10^{-2}	4	10^{-3}	46	10^{-2}	52	10^{-4}
2	2	10^{-2}	2	10^{-3}	48	10^{-2}	52	10^{-4}
3	1	10^{-2}	4	10^{-3}	48	10^{-2}	54	10^{-4}

Chitosan-CEO (HPH) coating

Reading	Day 0	Dilution factor	Day 3	Dilution factor	Day 7	Dilution factor	Day 10	Dilution factor
1	1	10^{-2}	1	10^{-2}	5	10^{-2}	43	10^{-2}
2	1	10^{-2}	3	10^{-2}	5	10^{-2}	40	10^{-2}
3	1	10^{-2}	1	10^{-2}	3	10^{-2}	40	10^{-2}

Chitosan-CEO coating

Reading	Day 0	Dilution factor	Day 3	Dilution factor	Day 7	Dilution factor	Day 10	Dilution factor
1	1	10^{-2}	2	10^{-2}	1	10^{-4}	43	10^{-4}
2	1	10^{-2}	8	10^{-2}	1	10^{-4}	44	10^{-4}
3	2	10^{-2}	7	10^{-2}	2	10^{-4}	41	10^{-4}

Chitosan-LEO (HPH) coating

Reading	Day 0	Dilution factor	Day 3	Dilution factor	Day 7	Dilution factor	Day 10	Dilution factor
1	3	10^{-2}	4	10^{-3}	5	10^{-3}	34	10^{-3}
2	2	10^{-2}	2	10^{-3}	4	10^{-3}	34	10^{-3}
3	2	10^{-2}	3	10^{-3}	6	10^{-3}	32	10^{-3}

Chitosan-LEO coating

Reading	Day 0	Dilution factor	Day 3	Dilution factor	Day 7	Dilution factor	Day 10	Dilution factor
1	1	10^{-2}	2	10^{-2}	115	10^{-3}	50	10^{-4}
2	1	10^{-2}	3	10^{-2}	112	10^{-3}	50	10^{-4}
3	1	10^{-2}	3	10^{-2}	114	10^{-3}	51	10^{-4}

Yeast and mould counts

Chitosan coating

Reading	Day 0	Dilution factor	Day 3	Dilution factor	Day 7	Dilution factor	Day 10	Dilution factor
1	1	10^{-2}	3	10^{-3}	5	10^{-4}	22	10^{-4}
2	3	10^{-2}	3	10^{-3}	6	10^{-4}	22	10^{-4}
3	3	10^{-2}	2	10^{-3}	4	10^{-4}	21	10^{-4}

Chitosan-CEO (HPH) coating

Reading	Day 0	Dilution factor	Day 3	Dilution factor	Day 7	Dilution factor	Day 10	Dilution factor
1	1	10^{-2}	7	10^{-2}	2	10^{-3}	30	10^{-2}
2	2	10^{-2}	5	10^{-2}	1	10^{-3}	27	10^{-2}
3	1	10^{-2}	6	10^{-2}	1	10^{-3}	28	10^{-2}

Chitosan-CEO coating

Reading	Day 0	Dilution factor	Day 3	Dilution factor	Day 7	Dilution factor	Day 10	Dilution factor
1	2	10^{-2}	5	10^{-2}	1	10^{-4}	35	10^{-4}
2	1	10^{-2}	4	10^{-2}	1	10^{-4}	35	10^{-4}
3	1	10^{-2}	2	10^{-2}	2	10^{-4}	33	10^{-4}

Chitosan-LEO (HPH) coating

Reading	Day 0	Dilution factor	Day 3	Dilution factor	Day 7	Dilution factor	Day 10	Dilution factor
1	3	10^{-2}	30	10^{-2}	3	10^{-3}	6	10^{-3}
2	2	10^{-2}	30	10^{-2}	2	10^{-3}	9	10^{-3}
3	1	10^{-2}	33	10^{-2}	5	10^{-3}	6	10^{-3}

Chitosan-LEO coating

Reading	Day 0	Dilution factor	Day 3	Dilution factor	Day 7	Dilution factor	Day 10	Dilution factor
1	1	10^{-2}	4	10^{-2}	1	10^{-3}	6	10^{-4}
2	1	10^{-2}	2	10^{-2}	1	10^{-3}	5	10^{-4}
3	2	10^{-2}	2	10^{-2}	1	10^{-3}	6	10^{-4}

APPENDIX B: Firmness reading

Day 0

SAMPLE	Chitosan	Chitosan- CEO (HPH)	Chitosan- LEO (HPH)	Chitosan- CEO	Chitosan- LEO
1	29.548	27.59	19.87	50	34.98
2	29.576	26	20.213	49.17	34.45
3	31.008	26	20.004	49.71	34.72

Day 3

SAMPLE	Chitosan	Chitosan- CEO (HPH)	Chitosan- LEO (HPH)	Chitosan- CEO	Chitosan- LEO
1	30.361	25.239	19.699	48.182	34.365
2	28.455	25.239	18.7	50.324	34.32
3	30.362	27.608	19.5	49.578	34.341

Day 7

SAMPLE	Chitosan	Chitosan- CEO (HPH)	Chitosan- LEO (HPH)	Chitosan- CEO	Chitosan- LEO
1	18.004	21.962	17.25	38.325	26.507
2	15.021	23.074	18.25	38.047	25.443
3	18.01	25.03	17.029	37.1	23.58

Day 10

SAMPLE	Chitosan	Chitosan- CEO (HPH)	Chitosan- LEO (HPH)	Chitosan- CEO	Chitosan- LEO
1	17.323	22.516	15.68	25.468	13.679
2	14.68	20.31	14.802	28.28	13.17
3	15.563	20.084	13.021	26.503	10.576

APPENDIX C: colour reading

L* parameter

Chitosan coating

Day	0	3	7	10
Reading 1	55.60	55.10	52.60	45.90
Reading 2	55.48	54.00	52.90	45.90
Reading 3	55.70	54.70	51.70	45.80

Chitosan-CEO (HPH) coating

Day	0	3	7	10
Reading 1	56.80	56.50	50.80	50.39
Reading 2	57.20	55.00	50.20	50.27
Reading 3	57.10	57.10	49.90	50.10

Chitosan-CEO coating

Day	0	3	7	10
Reading 1	55.70	54.90	47.80	47.10
Reading 2	55.59	55.20	48.30	46.90
Reading 3	55.70	55.30	48.40	45.70

Chitosan-LEO (HPH) coating

Day	0	3	7	10
Reading 1	55.90	54.30	51.80	50.40
Reading 2	54.60	53.50	50.80	50.30
Reading 3	54.80	53.50	50.20	50.20

Chitosan-LEO coating

Day	0	3	7	10
Reading 1	54.50	50.80	51.50	48.70
Reading 2	54.80	52.40	50.70	48.60
Reading 3	54.30	50.10	51.00	48.70

a* parameter

Chitosan coating

Day	0	3	7	10
Reading 1	-4.30	-4.20	-1.40	0.90
Reading 2	-4.20	-4.20	-1.30	0.70
Reading 3	-4.40	-4.20	-1.30	2.00

Chitosan-CEO (HPH) coating

Day	0	3	7	10
Reading 1	-5.55	-5.50	-3.20	-2.90
Reading 2	-5.40	-5.40	-3.20	-3.00
Reading 3	-5.40	-4.60	-3.20	-2.80

Chitosan-CEO coating

Day	0	3	7	10
Reading 1	-5.50	-5.00	-0.90	-0.50
Reading 2	-5.70	-5.40	-1.50	-0.60
Reading 3	-5.50	-5.10	-2.10	-0.70

Chitosan-LEO (HPH) coating

Day	0	3	7	10
Reading 1	-5.70	-5.50	-3.90	-2.70
Reading 2	-5.80	-5.60	-3.50	-3.20
Reading 3	-5.55	-5.50	-3.50	-3.00

Chitosan-LEO coating

Day	0	3	7	10
Reading 1	-4.00	-4.00	-2.70	-0.95
Reading 2	-3.90	-3.00	-1.90	-0.90
Reading 3	-3.88	-3.20	-2.40	-0.10

b* parameter

Chitosan coating

Day	0	3	7	10
Reading 1	39.50	42.10	45.70	45.80
Reading 2	39.80	41.80	45.60	45.50
Reading 3	40.10	42.70	44.60	45.40

Chitosan-CEO (HPH) coating

Day	0	3	7	10
Reading 1	40.10	40.40	43.20	43.50
Reading 2	40.10	40.90	43.00	43.60
Reading 3	39.90	40.00	43.10	43.50

Chitosan-CEO coating

Day	0	3	7	10
Reading 1	39.50	39.00	45.80	46.20
Reading 2	39.10	40.70	45.10	45.50
Reading 3	38.00	41.70	44.60	45.50

Chitosan-LEO (HPH) coating

Day	0	3	7	10
Reading 1	38.00	43.70	44.30	43.80
Reading 2	38.00	43.30	43.90	43.91
Reading 3	38.80	42.00	43.20	44.20

Chitosan-LEO coating

Day	0	3	7	10
Reading 1	40.80	44.40	44.52	44.85
Reading 2	40.50	44.20	44.52	44.86
Reading 3	40.10	43.20	44.51	44.86

APPENDIX D: pH reading

Chitosan coating

Day	0	3	7	10
Reading 1	4.26	4.28	4.29	4.34
Reading 2	4.26	4.27	4.30	4.33
Reading 3	4.27	4.28	4.28	4.33

Chitosan-CEO (HPH) coating

Day	0	3	7	10
Reading 1	4.20	4.20	4.24	4.28
Reading 2	4.19	4.21	4.25	4.29
Reading 3	4.19	4.20	4.25	4.28

Chitosan-CEO coating

Day	0	3	7	10
Reading 1	4.25	4.26	4.26	4.26
Reading 2	4.24	4.27	4.26	4.29
Reading 3	4.25	4.22	4.26	4.26

Chitosan-LEO (HPH) coating

Day	0	3	7	10
Reading 1	4.23	4.25	4.27	4.29
Reading 2	4.22	4.25	4.26	4.29
Reading 3	4.23	4.26	4.27	4.29

Chitosan-LEO coating

Day	0	3	7	10
Reading 1	4.2	4.23	4.27	4.29
Reading 2	4.19	4.23	4.28	4.33
Reading 3	4.2	4.22	4.29	4.30