Encapsulation of Essential Oils in Food Grade Materials

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

Publications from the thesis

A patent application has been made the novel approach for encapsulation. Currently a number of manuscripts have been prepared for publications once the patent has been filed

However, the following manuscripts have been prepared and ready for possible publication in various high impact journals:

1- **The physiochemical properties of thyme essential oils**. Based on the results from the chemical analysis and physical properties of thyme essential oils and its components (Chapter 3).

2- **Natural delivery systems for bioactive compounds**. Based on results of thyme essential oil in skimmed milk (Chapter 4).

3- **Enhancing the Antimicrobial and bioavailability of essential oils using a novel encapsulation technique.** Based on the results obtained from microbiological tests (Chapter 5).

4- **Using the ultrasound techniques to determine the solubility of powders.** Based on findings from powder dissolution using "Ultrasound Velocity Metre-UVM) as a rapid and novel technique (Chapter 4).

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To Ghada and Serena

Terminology

Colony forming unit (CFU): Is the number of colonies formed by the viable cells after incubation.

Controlled release: The process of the core material release at a given time and location.

Core: Any active ingredients that locates inside the capsules during encapsulations such flavour, EOs or pharmaceuticals.

Critical micelle concentration (CMC): The concentration of the surfactant at which the monomers tend to aggregate to form micelles.

Delivery: The system of loading the active ingredients to transfer them to the interested site such as mouth if flavour encapsulated and bacteria cell wall if an antimicrobial is encapsulated.

Encapsulant: The material used to encapsulate active ingredients.

Encapsulation efficiency: The amount of retained core material from the initial amount during encapsulation.

Encapsulation: The incorporation and entrapping the active material as a core material in the wall matrix.

Essential oils (EOs): The mixture of different compounds extracted from plants such thyme and oregano.

Feed: The emulsion or solution prepared for spray drying.

Hurdle technology: It the food preservation approach which uses more than one method to produce safe food.

Minimum inhibitory concentration (MIC): Is the lowest concentration required of any antimicrobial agents which prevents the growth of a specific suspension of microorganisms.

Payload: The amount of core encapsulated by wall material.

Retention: The ability of the particle to retain the core material during storage until release.

Surface oil: The amount or concentration of oils which transferred onto the surface of the powder particles during spray drying.

Ultrasound velocity meter (UVM): The instrument which uses ultrasound to investigate the solution properties such as dissolution of powders.

Wall materials: Are materials used to protect the core materials during capsule preparation and storage such as biopolymers.

Zeta potential: It is the magnitude of charges on the shear plane of the particles.

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Abstract

The objective of this study was firstly to assess the ability of liquid skimmed milk in combination with different concentrations of maltodextrin (MD) to emulsify 5% w/w thyme essential oils (EOs). Secondly, to analyse the chemical composition of the used thyme EOs and further examine the physical properties of some EOs components such as solubility, surface activity and partitioning coefficient. Moreover, assessing the antibacterial activity of free and encapsulated EOs *in vivo* and *in vitro* mediums. From the results of thyme EOs analysis, 29 compounds were identified that containing different terpenes. The results show that skimmed milk with MD performed as an excellent delivery system for emulsifying thyme EOs with high stability for 60 days and formation of small size droplets < 234 nm. The produced powders obtained by spray drying the initial emulsions were analysed, and the results showed that increasing the concentration of MD from zero to 3 % w/w decreased surface oil, retained higher amounts of EOs with the encapsulation efficiency reaching up to 97.4 %. The antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* was assessed by using free and encapsulated thyme EOs in the form of the solution or direct addition to the inoculum or food products. The minimum inhibitory concentration (MIC) was lowest 0.3 mg/mL against both *Escherichia coli* and *Staphylococcus aureus,* when encapsulated EOs was directly added to the inoculum and prolonged shelf-life of both skimmed and unpasteurised raw milk were attained with the same formulation. Furthermore, the food matrices had a great role in decreasing the antibacterial activity of EOs by decreasing the availability of the active components and preserving the bacteria cells from them. The encapsulation of EOs increased the bioactivity of thyme EOs. The combination of EOs and low temperatures had a significant role in increasing the shelf-life of whole unpasteurised milk.

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Chapter 1 Introduction

1.1 Background

For many centuries, spices, herbs and their extracts were used in food as flavours and aroma enhancers and as a remedies for many diseases in folk medicaments. Essential oils prove to have the potential to act as antibacterial and antioxidant when applied to food or cosmetic products. Food industry requires supplying safe food to consumers with possible longer shelf-life without altering the quality. Many foodborne microorganisms are transferred to consumers resulting in different severe health consequences. Perishable foods such as dairy products, ready-to-eat foods and salads are the most often contaminated with these types of microorganisms during preparation, transportation and storage, which require preservation and maintain safety for the desired storage period. According to WHO (2015) organisation, food contamination is responsible for foodborne illness of 1 in 10 people every year which results in 420,000 deaths, among them 150,000 children under 5 years old. Diarrhoea is responsible for around 550 million illness cases, and 230,000 of these cases lead to death. The consumption of raw milk and dairy products made with them, uncooked or raw meat and egg are the main causes of these incidents. Due to the possible existences of harmful microorganisms such as *Escherichia coli*, *Campylobacter*, norovirus and non-typhoid *Salmonella* in these types of foods.

Preserving food by synthetic chemicals is associated with many health problems. For example, adding salt to foods increase the risk cardiovascular disease, using nitrate and nitrite are related to negative health consequences. Therefore, there is a possibility to replace a part of salt or nitrate with EOs in order to maintain food safety. Using natural additives or preservatives is highly demanded by consumers (Holley and Patel, 2005; Tajkarimi et al., 2010). Most of the essential oils (EOs) extracted from herbs and spices are classified as “generally recognised as safe (GRAS)” with excellent antibacterial, antioxidant, anticancer and anti-inflammatory effects. According to federal regulation, code 21CFR using thyme, nutmeg, basil, lemon grass, clove and cinnamon, in addition to other types of oil, are allowed in food products in the USA (FDA, 2015). The European Commission also permitted some bioactive compounds to be used in food products such as carvacrol, menthol, linalool, limonene, thymol, vanillin, eugenol, carvone and citral which are available in EOs (Hyldgaard et al., 2012; Tajkarimi et al., 2010).The EOs possess antibacterial activity against a wide range of microorganisms. Therefore their application in food products is beneficial to improve the quality of food and the consumer’s health. The use of EOs as an antimicrobial agent is well reviewed by many

researchers including (Lanciotti et al., 2004; Burt, 2004; Fisher and Phillips, 2008; Gyawali and Ibrahim, 2014). The presence of the hydrophobic and phenolic compounds with hydroxyl group (-OH) in EOs are responsible for antibacterial and antioxidant activities (Dorman and Deans, 2000; Lai and Roy, 2004; Stojković et al., 2013) which are able to disrupt the cell membrane of the bacteria (Xue et al., 2013).

The factors influencing the selection of EOs for addition when added into food products, include the sensory properties if used at high concentrations, cost, safety, their activity and stability (Holley and Patel, 2005) and their compatibility with the food products. However, adding free oil into food products is not practically feasible due to many reasons including changing the sensory and consumer acceptability for the foods, reduction of their activity due to binding with different food components, loss due to volatility of EOs during preparation, transportation and oxidation of EOs components (Prakash et al., 2015). To overcome these problems, the encapsulation of the EOs as a novel approach which can reduce these deficiencies and potentially increase their bioavailability. The encapsulation may mask the undesirable taste of EOs when applied into food products at high concentration. The encapsulation also preserve the encapsulated EOs from undesirable changes during handling and processing. However, EOs are successfully applied to some food products without altering the organoleptic properties. For example Tsigarida et al. (2000) used oregano to treat beef fillets and found that the meat remained acceptable by the consumers at the concentration of 0.8% v/w after cooking. The flavour and odour of meat treated with 1% v/w oregano oil was also found to be better (Skandamis et al., 2001).

Dairy products contain a variety of nutrients, which are considered essential for human body’s growth and maintenance. They are an excellent source of essential amino acids, essential fatty acids, minerals and vitamins. Hence, they are an excellent media for the growth of microorganism because it contains nutrients used by them as they require for their growth and reproduction. Many types of bacteria are presented in milk, some of them come from the surrounding environment and others transferred from the animals themselves. Some microorganisms are pathogenic and harmful, causing serious health problems for consumers, if not pasteurised properly. Selling unpasteurised raw milk is allowed in many countries due to the growing consumer demands including the UK but under restricted sanitation regulations. However, sellers should practice effective hygiene and sanitation practice. Many types of soft cheeses are available on the market that are

produced from raw milk which may cause fatal illnesses. The probability of the disease by raw milk is 150 times higher than that from pasteurised milk (FDA, 2012). Raw milk can carry a variety of harmful bacteria such as *Salmonella, Escherichia coli, and Listeria monocytogenes.* These microorganisms can affect the health of consumers who consume raw milk or foods made from it; for instance diarrhoea, listeriosis, typhoid fever, tuberculosis, diphtheria, and brucellosis are the most prevalent, all over the world, as food-borne illnesses (Tajkarimi et al., 2010). Cheeses made from raw milk such as Brie and Camembert, Queso Fresco Panela, Asadero, and Queso Blanco, Meadow Creek Grayson, Bardwell Manchester can transfer these foodborne illnesses to consumers (FDA, 2012).

The aims of the current study were to develop an effective encapsulation system to deliver the active components of thyme EOs into the food products such as milk and juice, and evaluate their antimicrobial activities.

In recent years, many attempts have been made to encapsulate various essential oils and flavours to be used in food and cosmetic products. However, the techniques used were relatively complex, required different procedures to prepare the capsules, some wall materials required a long time to disperse before they could be used in encapsulation. In addition to the high price of some wall materials and some procedures required special equipment that limited their applications in the food industry. Moreover, some of these techniques were less efficient in retaining the flavours and volatiles inside capsules, which resulted in producing expensive capsules. Furthermore, some compounds added to the encapsulation systems are not GRAS. The aim of the current study was to produce capsules of EOs using cost-effective materials such as skimmed milk and maltodextrin which are easily available and can be used in the food industry. The novel encapsulation approach does not require special equipment and procedure. The milk proteins are already dissolved and dispersed in the milk in contrast to commercially available products such as sodium caseinate or whey proteins isolate which require dispersion for at least 24 hours are prone to deterioration by microorganisms. Additionally, currently used process requires pH adjustment by using buffers during preparation which adds more work and cost to the process.

In many studies thyme EOs have been encapsulated such as a study by Martins et al. (2009) who found that only 65% of EOs retained in polylactide (PLA) and dimethylformamide (DMF) with a particle size of 40 μm by a coacervation process.

Moreover, in other studies small molecule surfactants have been used to emulsify EOs, such as Tween-80 which has been found to neutralise the phenolic compounds and decrease their antibacterial efficiency (Juven et al., 1994). It was also concluded that the surfactant protects some bacteria such *Listeria monocytogenes* from EOs (Cressy et al., 2003) and increase the turbidity of the inoculum which makes it difficult to assess the bacterial growth visually in the nutrient broth (Carson and Riley, 1995). There are many factors which are limited the use of some wall materials for encapsulation of flavours and bioactive compounds such as the availability, high cost and solubility. The disadvantages of some wall materials is reported by Estevinho et al. (2013) such as the cost and availability of gum arabic, low solubility of ethyl cellulose in the gastrointestinal system, difficulty in solubilisation of k-carrageenan which requires high temperature (60-80 ºC) and increased feed viscosity when starch is used in addition to low rates of retention and stability. In other studies, low retention of the volatiles were obtained despite using expensive wall materials which was not useful for industrial applications. For example, in an attempt to encapsulate cardamom oleoresin in gum arabic, MD and modified starch, it was found that the recovery was less than 62% for all wall types (Krishnan et al., 2005). Whereas, the combination of MD and modified starch (Hi-cap 100) increased the encapsulation efficiency of flax seed oil to 95.7% while decreased to 62.3% when WPC and MD used. In another attempt by (Carmona et al., 2013) orange EOs retention encapsulated in whey protein and MD and at the best experimental conditions with higher total solids the EE % reached 72 %. As mentioned earlier, some of these compounds are too expensive to be used in the food products. Therefore, searching for alternative materials is crucial for the food industry to produce new food products using food-grade ingredients with low cost and high quality with small particle size and high retention of the active ingredients.

Biopolymers, such as proteins and polysaccharides, are able to create nano and micro scale colloidal particles (Dickinson, 2003; Norton and Frith, 2001; Burey et al., 2008b). Many attempts have been made to understand the protein-polysaccharide interactions and their ability to form colloidal particles (Tolstoguzov and McClements, 2007; van der Goot and Manski, 2007). Biopolymers do not adversely affect the properties of food products, and they also can enhance the texture, sensory, appearance and the flavour of the foods (van den Berg et al., 2008; Macku et al., 2008). Therefore, in the current study

maltodextrin and different proteins in skimmed milk were utilised to encapsulate thyme EOs.

1.2 The hypothesis

The encapsulation enhances the solubility and the dispersibility of the hydrophobic essential oils which may improve their antimicrobial activity against possible microorganisms in the food products. By means of encapsulation, lower concentrations of EOs can possess higher activity. Therefore, ideally food preserves its organoleptic properties by masking the undesirable flavour in addition to cost effectiveness.

This study seeks to answer the following research questions.

- What are the compounds in thyme EOs used in this study? What are the physical and chemical properties of these components? How do these properties affect the delivery system and the bioactivity when added to food products?
- Is it possible to use a natural food product such as skimmed milk to deliver thyme EOs to another food product? What is the lowest concentration of EOs that can be emulsified by it? This is because milk contains different proteins as natural emulsifiers in addition to carbohydrate such as lactose which may help the retention of the EOs during spray drying by forming amorphous shell around powder particles.
- Can maltodextrin improve the emulsifying properties of skimmed milk? At what concentration is it more efficient? Maltodextrin has been used in combination with other emulsifiers in other studies and showed to enhance the emulsifying properties of the systems.
- Is skimmed milk able to emulsify all types of oil similarly? Thyme EOs is reported to contain diverse compounds with different hydrophobic properties in contrast to vegetable oils which are formed from fatty acids.
- What types of emulsions can be obtained when skimmed milk is used and for how long is the emulsion stable?
- What types of particles will form when spray drying the emulsion? What is the encapsulation efficiency? There is a possibility of producing different particle morphology depending on the formation of the hard crust around particles during spray drying.
- At what rate does the powder dissolute when introduced into an aqueous phase? Does the powder help the improvement in EOs dispersibility and availability of the active components in the food product? This is because rapid dissolution is required in the current study.

- Is there any reduction in EOs activity by different food components such as protein or fat? It is reported in many publications that the presence of these components reduce the activity of the EOs when added in the form of free oil. The encapsulation may enhance the dispersity and reduce the effect of these components.
- Is the activity of the encapsulated EOs against both gram-negative and gram-positive bacteria the same? Many reports showed that the EOs are more efficient against the former compared to the later; the encapsulation may reduce that difference and increase the activity against both types.
- Is it possible to extend the shelf-life of food products by applying hurdle technology? Using more than one hurdle to prolong the shelf-life of foodstuffs is well illustrated in many studies. The combination of temperature and EOs may result in better quality and safer food products.

1.3 The outline of the thesis

Chapter two reviews the definition and the applications of essential oils EOs in various products. The chemistry of the various components in the EOs is described, and the synthesis of terpenes, monoterpenes, sesquiterpenes and other components in the plants is the main focus. In this section, the extraction methods of essential oils is also highlighted. The potential use of EOs in food products and previous findings are described in detail.

In a later section of chapter two, encapsulation as a novel approach of introducing bioactive components into food products is explained. The aim and importance of encapsulation as a novel delivery systems, the different coating materials used for encapsulation and their applications are described in detail. The potential use of biopolymers such as proteins and polysaccharides as food-grade materials to form colloidal particles, their interactions and roles in stabilising colloidal suspension is clarified. The use of spray drying as a novel approach for drying thyme oil emulsions is explained. The effect of various stages of drying on the formed powder in relation to the retention of encapsulated active materials and characterisation of particles are described. The factors affecting the encapsulation efficiency such as particle size, total solids, stability of the feed are discussed in details.

In chapter three a brief introduction to thyme EOs and their possible components is given. The experimental section contains different methodologies used to evaluate the

chemical and physical characterisation of thyme essential oils used in this study. The chemical analysis of thyme essential oils using gas chromatography-mass spectrometry (GC-MS), in particular solid phase microextraction (SPME) technique, is highlighted. Various methodologies which are used to evaluate the physical characteristics of EOs components are explained, such as the solubility of monoterpenoids and their partitioning between water and octanol phases, the self-association and surface activity are discussed in details. In the later sections results from the different experiments on thyme essential oils and explanations for every phenomenon are given in details.

In chapter four in addition to an introduction to encapsulation and delivery systems, the details of methodology on preparing thyme oil emulsions and their characterisation, stability and the spray dried powder of these emulsions are highlighted. In the results and discussion section (4.4), the composition of skimmed milk used as a developed delivery system is presented. The characterisation of different emulsions including the stability, particle size distributions, zeta potential, viscosity and emulsion capacity are presented. In addition, various methods used for characterising the spray dried powder particles such as scanning electron microscopy, light microscopy are described and the Clevenger apparatus to recover encapsulated oil from the capsules to evaluate encapsulation efficiency and the dissolution of the powder using ultrasound as a novel technique are also highlighted.

In chapter five the bioactivity of EOs *in vitro* and *in vivo* are highlighted in addition to the differences between gram-negative and gram-positive bacteria susceptibility to EOs. The challenges and limitations of using EOs in food products in the previous studies are also outlined. In experimental section, the methods to assess the antibacterial activity of different formulation of EOs *in vivo* and *in vitro* are also explained. The mode of action of different components of EOs on bacterial cell are emphasised. In every chapter the results are compared with previous studies and critically analysed with conclusion drawn from the findings. Finally, there is a general conclusion from the results of this study and recommendations for future work are given at the end of the thesis based on the findings of this research work.

Chapter 2 Literature Review

2.1 Essential oils

Essential oils (EOs) are a complex mixture of hundreds of chemical compounds produced in some types of plant with strong aroma (Burt, 2004; Bakkali et al., 2008; Tajkarimi et al., 2010; Cueva et al., 2010; Negi, 2012). EOs and their components do not contribute to plant development and growth directly. Most of the EOs roles in plants are to act as anti-herbivores or to catch the attention of insects to transfer their pollens to other plants or to protect them from harmful insects and other competitors. However, some terpenes play a defensive role when the plant is attacked by insects or fungi; other attract pollinators such as linalool, cineole and myrcene (Figueiredo et al., 2008; Tajkarimi et al., 2010). EOs are liquid, have a strong aroma, soluble in organic solvents, fats and oils, alcohols and low soluble in water, and they have low molecular weight and are volatile at room temperature. Therefore, they need to be prevented from exposure to air and light during storage. They are available in special plant cells such as resin ducts, cavities, secretory cells and glands which can store and secrete them (Carson and Hammer, 2011; Nazzaro et al., 2013). The amount of EOs in the plant is less than 1% of fresh plant's weight (Pengelly, 2004). Additionally, there are many other aromatic compounds such as alcohols, acids, esters, ketones, amines and aliphatic aldehydes within essential oils (Berger, 2007, Kemal et al., 2007, Bakkali et al., 2008). There are many plants able to produce EOs such as oregano, coriander seeds (*Coriandrum sativum)*, rosemary (Rosmarinus officinalis), thyme (*Thymus vulgaris*), marjoram (*Marjorana hortensis*-Moench), clove (*Syzygium aromaticum*), peppermint, cumin seeds (*Nigella sativa*), ginger (Zingiber officinalis), clove (Syzygium aromaticum*)*, eucalyptus(*Eucalyptus* spp.), orange, lime, fennel seed (*Foeniculum vulgare* L) basil lemon, cinnamon (Cinnamon zeylanicum), lemongrass (*Cymbopogon citratus*), carrot Juice (*Daucus carrot* L.), bergamot (*Citrus bergamia)* and many more. (Baratta et al., 1998; Nabiha et al., 2010; Elaissi et al., 2012; Naveed et al., 2013; Teixeira et al., 2013; Roby et al., 2013; Singh et al., 2014; Ma et al., 2015).

Historically, the healing and flavour enhancing properties of plants containing essential oils make them useful as remedies for many diseases and spices in foods, in addition to their use in religious ceremonies (López et al., 2005; Bilia et al., 2014; Karl-Heinz, 2015). In 1881 De La Croix was the first who tested EOs vapour as an antibacterial agent (Burt, 2004). Further studies revealed that EOs possess activity against fungi and viruses (Silva et al., 2011), insects (Kim et al., 2003), parasites (George et al., 2009),

yeasts (Tserennadmid et al., 2011). For example oregano, cinnamon, thyme, basil and many other plants were used in past in folk medicine for treatment of indigestion and relieve of colic or bowel pain (Nadkarni, 2001). Essential oils are chemically different from other types of oils, which are commonly made from esterified fatty acids, while EOs are formed from terpenes and are volatile at room temperature (Kemal et al., 2007).

The biological activity of the essential oils is associated with the presence of volatile compounds such as mono-, sesqui-, and diterpenes, benzenoids, phenylpropanoids, alcohols, esters, aldehydes and ketones (Mahmoud and Croteau, 2002; Bakkali et al., 2008). Due to their beneficial chemical and biological effects, they have been used in food, pharmacy, sanitary, cosmetic, agricultural industries and perfume industry (Bakkali et al., 2008; Baser and Gerhard, 2009). The bioactivity, world consumption and main industrial applications of essential oils are illustrated in (Figure 2.1 and Table 2.1). However, there is a lack of data on global consumption of thyme essential oils in this table and in others literatures.

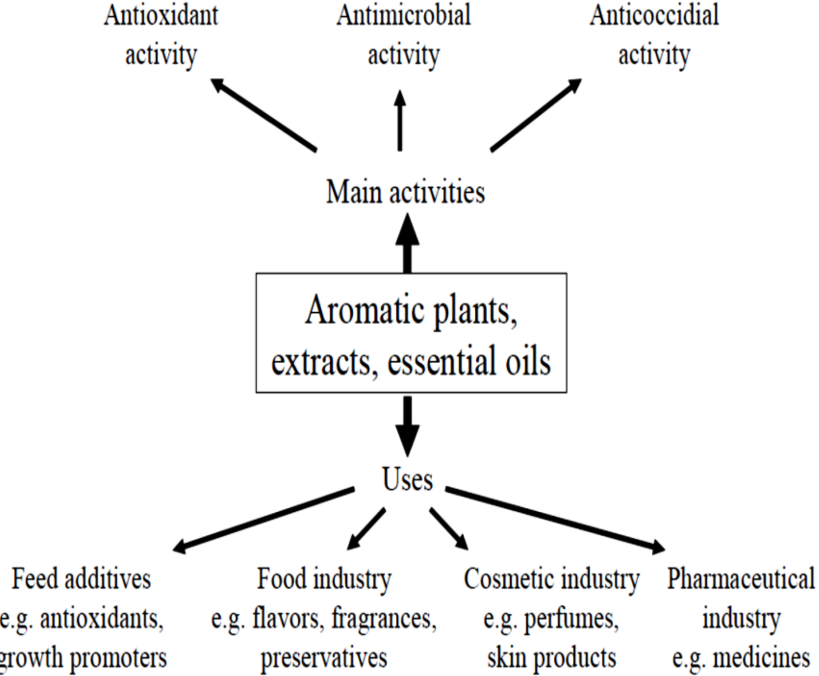

Figure 2.1: The uses of essential oils (Christaki et al., 2012).

Table 2.1: The world consumption and main application of the different EOs (Brud, 2009)**.**

Oil Name	Consumption (tons)	Approximate Value (€million)	Major Applications
Orange	50,000	275	Soft drinks, sweets, fragrances
Cornmint (*Mentha arvensis*)	25,000	265	Oral care, chewing gum, confectionery, fragrances, menthol crystals
Peppermint	4500	120	Oral care, chewing gum, confectionery, liquors, tobacco, fragrances
Eucalyptus (*Eucalyptus globulus*)	4000	22	Oral care, chewing gum, confectionery, pharmaceuticals, fragrances
Lemon	3500	21	Soft drinks, sweets, diary, fragrances, household chemicals
Citronella	3000	33	Perfumery, toiletries, household chemicals
Eucalyptus (*Eucalyptus citriodora*)	2100	10	Confectionery, oral care, chewing gum, pharmaceuticals, fragrances
Clove leaf	2000	24	Condiments, sweets, pharmaceuticals, tobacco, toiletries, household chemicals
Spearmint (*Mentha spicata*)	2000	46	Oral care, chewing gum, confectionery
Cedarwood (*Virginia*)	1500	22	Perfumery, toiletries, household chemicals
Lime	1500	66	Soft drinks, sweets, diary, fragrances
Lavandin	1000	15	Perfumery, cosmetics, toiletries
Litsea cubeba	1000	20	Citral for soft drinks, fragrances
Cedarwood (China)	800	11	Perfumery, toiletries, household chemicals
Camphor	700	3	Pharmaceuticals
Coriander	700	40	Condiments, pickles, processed food, fragrances
Grapefruit	700	9	Soft drinks, fragrances
Star anise	700	7	Liquors, sweets, bakery, household chemicals
Patchouli	600	69	Perfumery, cosmetics, toiletries
Basil	500	12	Condiments, processed food, perfumery, toiletries
Mandarine	500	30	Soft drinks, sweets, liquors, perfumery, toiletries

2.1.1 Extraction methods of essential oils

The first people who distilled essential oils from aromatic plants lived in East India and Persia two thousand years ago, then the Arabs enhanced the methodology by the 9th century (Güenther, 1972). Essential oils can usually be extracted from roots, leaves, flowers, buds, stems, and seeds of fresh, partially dehydrated or dried plants (Rahman and Gray, 2002; Zhu et al., 2004; Erasto et al., 2004).

Different techniques can be used to extract EOs from plants including microwave assisted hydro-distillation, supercritical fluid extraction, steam and water distillation, cold press and solvent extraction. Mainly steam distillation and hydro-distillation are the widely used commercial methods for extracting EOs in large scale (Burt, 2004; Shannon et al., 2011; Solórzano and Miranda, 2012; Barbarad et al., 2015).

In hydro-distillation, the plant is chopped, then added to water and boiled in a vessel. While in steam distillation the chopped plant placed in a basket or perforated trays to allow steam passage between chopped plant parts. The steam is generated in the separate boiler and transferred by pipes from the bottom of the vessel. To prepare EOs evaporation followed by cooling and condensation is required. When the steam heats the oil, the movement of the oil molecules is increased. Moreover, the air pressure around the plant parts decreases. Thus, the oil diffuses out of plant cells by osmosis (Figure 2.2). The mixture of evaporated water and vaporised essential oils transfers to the condensation unit in the cooling chamber, where condensation takes place then they condense to form two layers of water (bottom) and essential oil (upper) in the collecting vessel (Erich, 2009; Karl-Heinz, 2015).

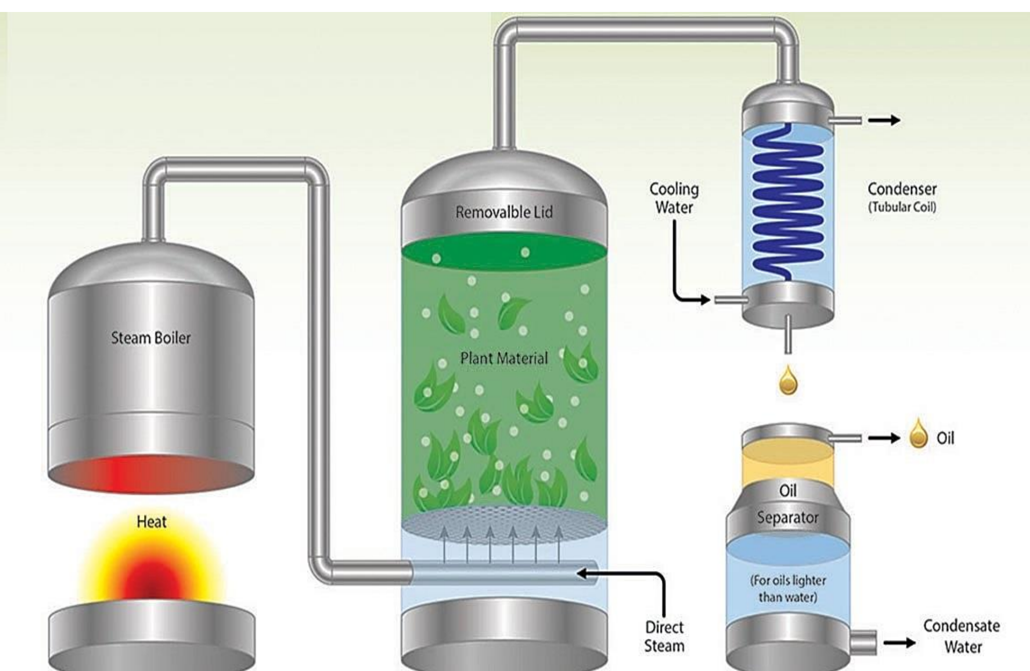

Figure 2.2: Steam distillation apparatus (doTerra, 2016).

The quantity and quality of different compounds in essential oils depend on the genetics, quality of soil, water stress and deficiency, insects and most importantly, the method of extraction (Erich, 2009). Steam distillation is commonly preferred over solvent

extraction of EOs as some chemical changes may take place when solvents are used for extraction (Grigore et al., 2010).

2.1.2 The chemistry of essential oils

Essential oils are comprised of different oxygenated and non-oxygenated hydrocarbons and some compounds contain sulphur or nitrogen (Bakkali et al., 2008). Some essential oils may contain up to 100 different chemical compounds (Pengelly, 2004). Among the different components, terpenes form the major proportion of the EOs, the rest are phenylpropanoids and other chemicals are present in traces (Friedrich, 1975). The main chemical compounds in the EOs are as described in the following sections.

There are more than 30000 structures of terpenes in nature which are composed of different units of isoprene (Breitmaier, 2006; Hüsnü et al., 2007). Three secondary metabolite pathways of the major components such as carbohydrates, lipids and proteins are the main sources of isoprene, the building block of the active compounds. The terpenes are unsaturated hydrocarbons, made from head to tail combination of two or more isoprenes C_5H_8 ($CH_2=C(CH_3)-CH=CH_2$) units (Figure 2.3). These units together comprise aromatic or aliphatic compounds, which form volatile compounds such as hemiterpenes (1 isoprene - C_5H_8), monoterpenes (2 isoprene - $C_{10}H_{16}$), sesquiterpenes (3 isoprene-$C_{15}H_{24}$) and diterpenes (4 isoprenes-$C_{20}H_{32}$) which are made through different pathways.

The synthesis of terpenes occur in highly special cells in plants such as the glandular trichome, leaves secretory cavities and flower petals glands. These special structures are able to store and emit terpinene when it is necessary for the plant such attracting insects for pollination. Terpenes represent 90% of EOs composites and mainly are monoterpenes and sesquiterpenes in less proportion. Limonene, p-cymene, sabinene, terpinene, β-pinene, thymol, carvacrol, geraniol, to name but a few, are examples of different terpenes. Terpenes which contain oxygen are called terpenoid or isoprenoids (Iscan et al., 2005; Carson et al., 2006; Bakkali et al., 2008; Charles, 2009; Nazzaro et al., 2013).

There are around 1500 monoterpenoids in nature formed by isomerisation or cyclisation of the isoprenes (Breitmaier, 2006). If isoprene units join linearly, they produce acyclic monoterpenes such as linalool and geraniol. Alternatively, they link together to form rings of cyclic monoterpenes such as p-cymene which is available in

many EOs as an aromatic monoterpene. Attaching hydroxyl group to the benzene ring in p-cymene produces phenolic compounds such as thymol and carvacrol which are considered as monocyclic monoterpenes (Shapiro and Guggenheim, 1998).

Figure 2.3: Different possible conformations of isoprene (Charles, 2009).

After monoterpenes, **sesquiterpenes** are the second common compounds in EOs. There are 120 different skeleton arrangements of sesquiterpenes such as linear, cyclic and branched. Examples of acyclic sesquiterpenes are nerolidol in *Citrus aurantium* and farnesol in rose flower EOs. Monocyclic sesquiterpenes such as bisabolol and abscisic acid, bicyclic sesquiterpenes such as azulene, β- caryophyllene, widdrol and guaiol (Ganzera et al., 2006).

Diterpenes are a group of compounds found in EOs in smaller quantities than mono- and sesquiterpenes. Because they have heavier molecular weight ($C_{20}H_{32}$), they require more steam distillation time for extracting them from plants (Hüsnü et al., 2007). There are also cyclic, and acyclic diterpenes such as phytol in all green plant leave that are attached to chlorophyll (Croteau et al., 2000), manool and manoyl (Gökdil et al., 1997) and phyllocladene is also found in EOs (Hüsnü et al., 2007).

Norterpenes occur in some EOs of some plants as a result of breaking carotenoids (C_{40}) to C_{13}. They are responsible for flavour and aroma of some EOs (Auldridge et al., 2006). β-damascone and β-ionone which are found in *Boronia megastigma and Rosa damascena* respectively are examples of nor terpenoids (Plummer et al., 1996; Babu et al., 2002).

In addition to the mentioned types of terpenes, there are phenylpropanoids which are made from phenylalanine and tyrosine such as eugenol in clove (Herrmann and Weaver, 1999; Bakkali et al., 2008; Ferrer et al., 2008). Moreover, nitrogen and sulphur

compounds also found in some EOs in minor extents such as allyl isothiocyanate in mustard and allicin in *Allium* spp. (Krest et al., 2000).

Many types of compounds derived from monoterpenes which present prevalent plant chemotypes such as thymol, carvacrol, linalool, p-cymene, geraniol, α-pinene and many other chemicals are present in traces (Thompson et al., 2003).

The composition and chemotype of the essential oils depend on different factors such as the season of cultivation, location, stress, watering, the part of the plant where the oil is extracted from and the method of drying (sun, shade or oven) (Theis and Lerdau, 2003; Novak et al., 2005; Olawore et al., 2005; Sefidkon et al., 2006; Rebey et al., 2012). Naghdi Badi et al. (2004) conducted a study to determine the effect of agronomical factors such as time of harvest and spacing between the cultivated plants on thyme (*Thymus vulgaris* L.) and found that at the beginning of the plant blooming stage the EOs contain the highest yield of EOs with high thymol proportion. Figure 2.4 shows different compounds present in essential oils.

2.1.3 The bioactivity of essential oils

In plant essential oils are produced from secondary plant metabolites products, therefore they will not participate in plant growth and development, but they are performing some biological functions in the plant (Pichersky et al., 2006; Gershenzon and Dudareva, 2007; Vickers et al., 2009). Due to their biological activity, essential oils have been exploited by humans throughout history. Recently essential oils are applied in healthcare products, foods, agriculture, and cosmetics. In food, many studies have been conducted to evaluate the EOs ability as natural preservatives to extend the shelf-life and provide safer food products (Fisher and Phillips, 2008; Gyawali and Ibrahim, 2014; Prakash et al., 2015). The consequences of using chemical preservatives in food to control pathogenic and spoilage microorganisms emphasised the importance of using natural preservatives (Faleiro, 2011). Natural preservatives are those extracted from plant or derived from animals or microorganisms without any alteration to their composition or properties (Li et al., 2011).

Figure 2.4: The chemical structure of different essential oils constitutes (Hyldgaard et al., 2012).

Usually, natural antimicrobial compounds derived from plant sources are already used as part of the human diet and are Generally Recognised as Safe (GRAS) (Chalova et al., 2010; Callaway et al., 2011). Furthermore, essential oils are considered effective as antioxidant, antimicrobial, anticancer, anti-inflammatory and analgesic. They also have the ability to extend the shelf-life of agricultural products due to their fungicidal, bactericidal and insecticidal activities. In the food industry, essential oils or the plants containing EOs are used to enhance the flavour of the food products (Ballabeni et al., 2004; Tognolini et al., 2007; Huang et al., 2007).

2.1.3.1 The antimicrobial activity

To fight the infectious diseases which are caused by bacteria, viruses, fungi and protozoa special antimicrobial compounds can be used. Plants with antimicrobial activity have been used for a long time by ancient people as medicine even before they understood the role of microorganisms in diseases. The use of these plants as traditional medicine declined in the twentieth century after the chemical antibacterial and antibiotics such as sulphur containing drugs and β-lactams were invented. The vulnerability and selectivity of the modern antibiotics against different microorganisms is advantageous and became the main bacterial infection treatment. Recently the use of plant-based medicine has become interesting again since safer natural sources with lower toxicity to human is demanded by the consumer (Gyawali and Ibrahim, 2014; Prakash et al., 2015). Moreover, many pathogenic bacteria showed antibiotic resistance against some of these antibiotics by producing enzymes such as β-lactamase; as a result, essential oils may be used as alternative antimicrobials (Chaves et al., 2008; Carson and Hammer, 2011; Callaway et al., 2011).

Many reports showed the antimicrobial activity of different plant essential oils such as the review by Pascual et al. (2001) who revised the chemical composition and medicinal properties of various types of a genus (*Lippia* spp.) Cavanagh and Wilkinson (2002) reviewed the cosmetic and therapeutic use of different kinds of *Lavandula* spp. Also, Chaieb et al. (2007) went over the studies on using clove as pain relief, flavour and fragrance. Essential oils are likely to be active against bacteria, virus, fungi, and protozoa. Generally, terpenoids, sesquiterpenes and phenylpropanoid, non-phenolic compounds and some aldehydes in essential oils are the compounds recognised as antimicrobial agents, and among them the phenolic compounds considered the most active compounds in EOs (Carson and Hammer, 2011; Hyldgaard et al., 2012).

2.1.3.2 The structure of bacteria cell

Bacteria is a single cell microorganism, and it is prokaryote available in nature in different shapes including rods, spheres, and spirals their size is in micrometre scale (800-2000nm). They are available everywhere on the earth such as in water, soil, air, human body and food. Some are essential for the human body or in food processing, and some are pathogenic. There are many classifications of bacteria according to their requirement

to oxygen, resistance or preferring to different temperatures, or their resistance to salt. The estimated number of bacteria on the earth is around 4-6$\times 10^{30}$ (Whitman et al., 1998).

The bacterial cell structure consists of the nucleoid and cytoplasm surrounded by cytoplasmic membrane, cell wall and capsule if present (Figure 2.5). The bacterial cell wall is rigid and responsible for covering the cell content such as protein, DNA and other components and keeping the cell in intact shape. The cytoplasmic membrane is a barrier between external and interior of the cell it also selectively permeable to permit certain molecules passage between inside and outside of the cell. The main component of cytoplasmic membrane is a lipid bilayer with proteins. Understanding how different parts help the bacterial cell survive and grow is important in choosing the specific antibiotic according to various target sites on the cell (Nester et al., 2009).

The plasma membrane is a highly ordered structure, thin, containing lipid, and protein; some bacteria contain two plasma membrane such as gram-negative bacteria separated by periplasm. The two main components that make up the cell membranes are lipid and phosphate groups forming phospholipid bilayer. The head group of choline and phosphate group usually located outside to make a hydrophilic head group and the hydrophobic tail integrates together to form the phospholipid bilayer membranes (Figure 2.6). The lipids in the membrane have different functions, including giving structure to the bacterial cell, barrier, protection, activation of biological material such as enzymes and substrates. The lipids in a bacterial cell are crystal-like liquid which give the cells motion and fluidity. The change in lipid structure or disruption of the phospholipid bilayer would lead to death as the cell loses fluidity. Some proteins are embedded in the plasma membrane and are responsible for cell communication with the surroundings and other bacteria using chemical signals as well as acting as selective gates. Cytoplasmic membrane is a semi-permeable membrane which allows the passage of certain molecules including water, gases, and hydrophobic molecules using simple diffusion. The cytoplasm of bacteria contains a variety of components making it a viscous suspension such as enzymes, water, carbohydrates, lipids, plasmids, ribosomes and nucleoid (Seltmann and Holst, 2002; Nester et al., 2009).

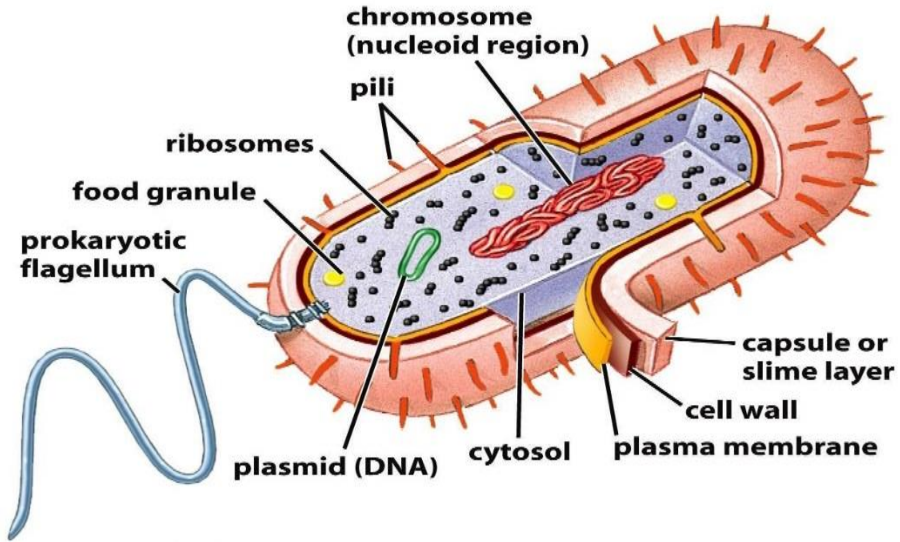

Figure 2.5: Prokaryotic cell structure (Pearson, 2008).

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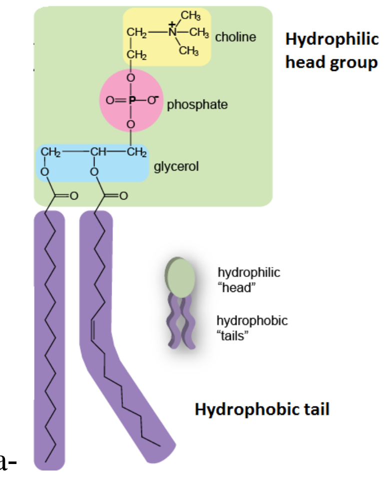

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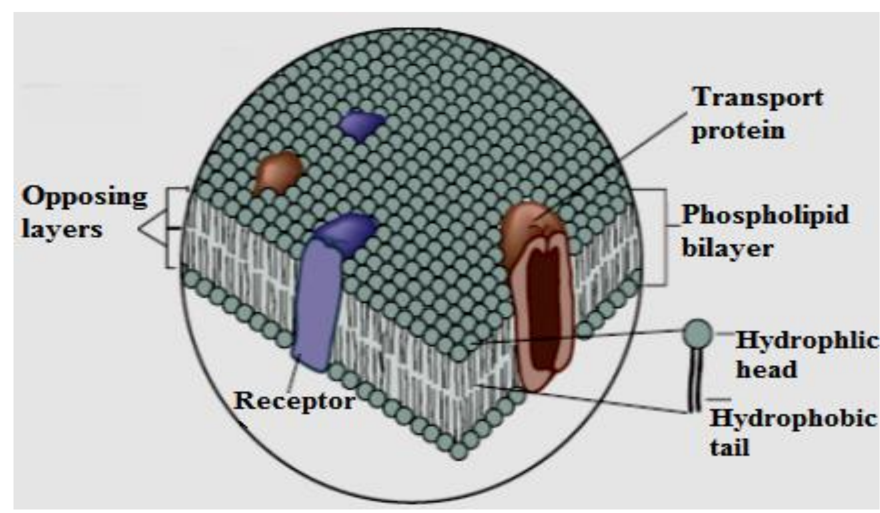


Figure 2.6: (a) Phospholipid (b) phospholipid bilayer in bacteria plasma membrane (Nester et al., 2009).

The bacteria can be classified into two main groups according to gram staining, which was first discovered by a Danish scientist Hans Christian Gram in 1884, into gram-positive and gram-negative bacteria. The cell wall of gram-negative bacteria is more complex than cell wall of gram-positive bacteria. There are a cytoplasmic membrane and outer membrane in gram-negative bacteria with a thin layer of peptidoglycan connecting them. Whereas the peptidoglycan consists a large part of gram-positive bacteria cell wall (Figure 2.7) (Seltmann and Holst, 2002). The permeability barrier of the outer membrane of gram-negative is due to the presence of lipopolysaccharides (LPS), phospholipids, and porins and it is thicker than in the gram-positive bacteria. The LPS contains lipids A which consist of disaccharide and fatty acids; inner core contains sugars and oligosaccharides, and O-antigen consists of glycan polymers. LPS is responsible for repealing and tolerating foreign compounds such as hydrophobic essential oils (Kim et al., 2011; Ravichandran et al., 2011; Nazzaro et al., 2013; Bajpai et al., 2013). Both types of bacteria are widely used as a model in most investigations on antibacterial activity of the EOs.

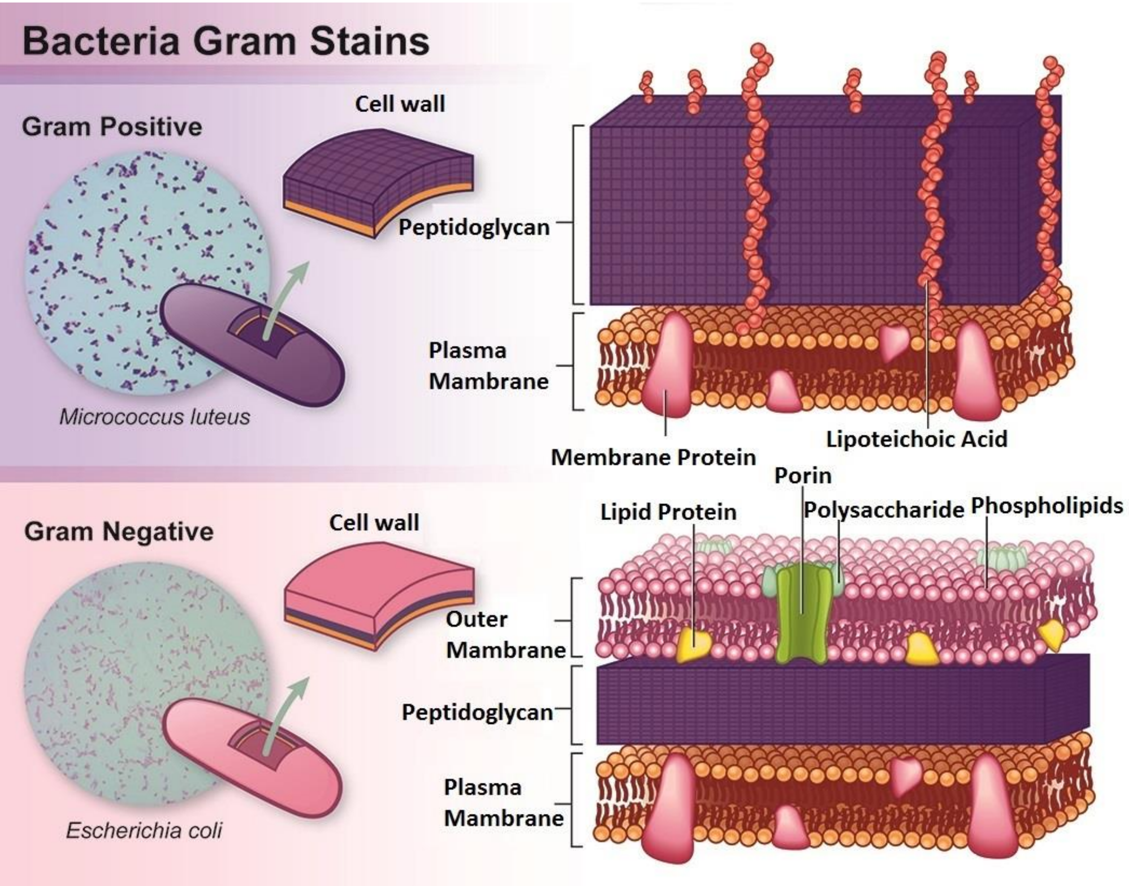


Figure 2.7: Gram-positive and gram-negative cell wall (Cartherine, 2015).

There are different methods which can be used to kill or inhibit bacteria in food. Physical methods including using high temperature, high pressure, radiation and chemical methods also used in broad spectrum such as alcohols, aldehydes, ozone, and heavy metals. These chemicals are divided into organic and inorganic agents. Organic agents considered more active as they target specific sites on the microorganism such as the disruption of peptidoglycan in gram-positive bacteria by penicillin (Nester et al., 2009).

2.1.3.3 The mode of action of EOs

Despite the use of essential oils as antimicrobial compounds for many years, the mechanisms of their action are not understood completely (Calo et al., 2015). However, recently the possible mechanisms of action of EOs on microorganisms is more evident by using different techniques including confocal laser scanning microscopy, atomic force microscopy (AFM), scanning electron microscopy (SEM), ATP efflux measurements (firefly luciferase assay), K^+ and H^+ efflux measurement and membrane potential ($\Delta\psi$) measurements (Hyldgaard et al., 2012).

Different antibacterial agents act differently, and more importantly the concentration of EOs and their active components is crucial regarding their activity. For example, when growth inhibition of bacteria occur they are called bacteriostatic agents this usually happen when a lower concentration of EOs is used, whereas they kill bacteria directly at higher concentrations and then are called bactericidal agents. The responsible enzymes of energy production are affected by low concentration. However, precipitation of proteins occurs at higher levels of EOs. Moreover, it is revealed that EOs affects the enzymes responsible for the synthesis of components which are involved in cell structure and energy production. Furthermore, the cellular membrane such as plasma membrane is another site which is susceptible to EOs that increases their permeability. The impairment of cell membrane affects the proton motive force through pH gradient and the membrane electric potential ($\Delta\psi$) (Peter, 2012).

Antimicrobial compounds act on specific targets in the cell. There are different sites on bacteria cell that antibacterial agents may interfere such as with proteins, the synthesis of peptidoglycan, the cytoplasm, nucleic acid and metabolic pathways. For instance, the cell will burst if the chemical compounds interfere with the synthesis of peptidoglycan which is responsible for cell wall rigidity. Lysozyme and penicillin are examples of the chemical antibacterials that targets peptidoglycan in prokaryote cells. The mechanisms of

action of the essential oils differ because of the presence of various components. Thus, they work on different parts of the microorganisms (Skandamis et al., 2001; Carson et al., 2002; Bajpai et al., 2013). Some researchers suggested that the essential oil components can penetrate into the bacterial membrane due to the hydrophobic nature of EOs and deactivate vital interior organelles affecting the lipophilic parts of bacteria (Guinoiseau et al., 2010; Bajpai et al., 2012).

The porin protein on the outer membrane of gram-negative bacteria allow hydrophilic solute passage from outside into cell but prevent hydrophobic compounds to enter the cell (Nazzaro et al., 2013). The sensitivity of gram-negative and gram-positive bacteria found to be different when carvacrol and thymol used against them (Dorman and Deans, 2000). The ATP release raised as a result of cytoplasmic membrane permeability increased when carvacrol and thymol used against gram-negative bacteria due to breaking down the wall membrane which formed from phospholipid bilayers (Burt, 2004).

The main action of essential oils and their components manifest themselves by interactions with the microorganism's biological membranes due to the solubility of essential oils in cell membranes (Kalemba and Kunicka, 2003). Phenolic compounds in essential oils can disrupt the functionality of the cell membrane and promote leakage (Bajpai et al., 2012). Ultee et al. (2002); Lambert et al. (2001); Carson et al. (2002) and Friedly et al. (2009) all revealed that the partitioning of the hydrophobic essential oil components in both cell membrane and cytoplasm play a major role in increasing their action. When cells are exposed to EOs for an extended period, they may alter the permeability of the cell by disrupting and damaging the cell membrane causing cell contents and vital macromolecule leakage which ultimately leads to bacteria death (Bajpai et al., 2013).

Interestingly, nowadays more accurate and precise mechanisms have been explained subsequently. Terpenes which are hydrocarbons, are unable to pose antibacterial activity. In a study by Dorman and Deans (2000) limited or no activity of α-terpinene, limonene, (+) sabinene, β-pinene and δ-carene against 25 types of bacteria was reported. Rao et al. (2010) used p-cymene and γ-terpinene as an antifungal agent, and they found that they were inefficient against *Saccharomyces cerevisiae*. Furthermore, p-cymene did not show any antibacterial activity against *Shigella* Spp. at 85700 µg/mL (Bagamboula et al., 2004). These studies suggested that using single terpene is unable to be effective against microorganisms (Hyldgaard et al., 2012). However, some terpenes found to potentiate

another compounds activity such as p-cymene, which are found to enhance the inhibitory activity of carvacrol against *Vibrio cholerae* (Rattanachaikunsopon and Phumkhachorn, 2010). It is also demonstrated by Ultee et al. (2002) and Cristani et al. (2007) that p-cymene diffuse into cell membrane without changing the permeability of the cell wall. They can expand the membranes, change in membrane potential, and decrease the melting point of the membranes.

Terpenoids are formed from terpenes by enzymatic reactions which attach oxygen molecules to the terpenes. There are many types of terpenoids such as phenols, alcohols, esters, and ketones in EOs. Thymol, carvacrol, menthol, terpenen-4-ol, borneol, geraniol, linalool are examples of terpenoids. The activity of the different essential oil components depends on their chemical structures. It was found that phenolic compounds are more efficient, and among them carvacrol and thymol found to be the most effective antibacterial constituents apart from carvacrol methyl ester and borneol (Dorman and Deans, 2000). The presence of hydroxyl group on carvacrol and on similar phenolic compounds, which have a delocalised electron, are responsible for the antimicrobial activity of these compounds (Dorman and Deans, 2000, Ultee et al., 2002). Furthermore, the location of the hydroxyl group does not substantially affect the antibacterial activity of the phenolic compounds, since both carvacrol and thymol found to offer the same activity against *B. cereus, Staphylococcus aureus* and *Pseudomonas aeruginosa* as demonstrated by (Lambert et al., 2001, Ultee et al., 2002). Both thymol and carvacrol found to disintegrate the bacterial outer membrane, deteriorating cytoplasmic membrane and they are capable of attaching to different proteins in the membrane. In a study by Turina et al. (2006) alteration of the bilayer model membranes occurred as a result of thymol integration with the polar head group regions, while at lower concentrations the membranes adjusted themselves by decreasing the permeability to preserve their structure and functionality.

Thicker liposomal membranes were observed when non-hydroxylated compounds such as cymene was used, due to their preference to liposomal membranes, in comparison to the hydroxylated compounds. On the other hand, due to the presence of both hydroxyl group and delocalized electron, compounds such as carvacrol, thymol, and menthol are able to destabilise the cytoplasmic membranes, exchange proton and change the membrane potential ($\Delta\psi$), reduce pH gradient, deplete the ATP pool leading to the death

of the microbial cell. Nevertheless non-hydroxylated compounds are unable to perform the same action (Ultee et al., 2002, Xu et al., 2008, Li et al., 2011, Bajpai et al., 2012).

Most of the foodborne pathogens can be destroyed using essential oils with high concentrations of the phenolic compounds such thymol, carvacrol and eugenol (Juliano et al., 2000, Dorman and Deans, 2000, Lambert et al., 2001). Moreover, Helander et al. (1998b) found that the thymol and carvacrol ruptured the outer membranes of *E.coli* cell resulted in releasing the internal membrane materials to outside. When the difference of ATP was measured, they found that the internal ATP pool decreased and the ATP outside the cells increased which verified disruption of the cytoplasmic membrane. Similar results also demonstrated by Ultee et al. (1999), Gill and Holley (2006) and Xu et al. (2008). They described the mechanism of action of carvacrol and thymol on the bacterial cell; they found that carvacrol increased the permeability of cell membranes to cations (H^+ and K^+) and also depletion of internal ATP.

Juven et al. (1994) noticed that hydrogen bonding between phenolic compounds and membrane protein leads to complexation reaction and altering the permeability of the cell membranes. When phenolic compounds accumulate on the bacterial cell wall, the disruption of cytoplasmic cell and separation of proton motivate force occur. They tend to alter the electron flow, affect the membrane proteins and active transport mechanism of the cell then coagulation of internal cell contents (Sikkema et al., 1995; Davidson et al., 2013). Phenolic compounds affect the enzymes and functional proteins in bacteria. It was observed by Burt et al. (2007) that *Escherichia coli* O157: H7 lost their mobility when 1 mM carvacrol added to the overnight inoculum due to inhibition of the flagellin (the protein required for flagella formation). However, the flagella were formed when p-cymene used at concentrations of 1 mM and 10 mM. Another monoterpene, terpinen-4-ol the main component of tea tree essential oil was found to disorder the permeability and lead to the loss of chemiosmosis of microorganism cell membranes releasing K+ and nucleotides (Cox et al., 2000, Carson et al., 2002). Different mechanisms and site of action on the bacterial cell are shown in (Figure 2.8).

The loss of integrity of bacterial plasma membrane occurs, once EOs components reach it. As a result, the permeability of the membrane increases to ions and protons (Cox et al., 1998). Internal cell materials start to move to the outside; this was confirmed in many studies by measuring the potassium ion, ATP, amino acids and nucleic acids (Cox et al., 2000; Burt, 2004; Bajpai et al., 2013). Additionally, when EOs components

penetrate the cell, it may disrupt the synthesis of polysaccharides, lipids, proteins, DNA and RNA. Protein denaturation, cell membrane damage, the leakage of cytoplasm, lysis of cell and death may occur when EOs reach the lipid structure of bacteria (Denyer, 1990).

Some studies found that there is a difference between phenolic compounds regarding their activity. Dorman and Deans (2000) concluded that thymol showed higher antibacterial activity against both gram-positive and gram-negative bacteria. In addition, Bassolé et al. (2010) found that 300 µg/mL carvacrol was capable of inhibiting the growth of different bacteria when testing the MIC, while thymol performed the same action at 800 µg/mL.

Phenylpropenes in essential oil such as cinnamaldehyde was found to prevent the bacterial cell from division by inhibiting the FtsZ protein that regulates cell division (Domadia et al., 2007). Cinnamaldehyde also disrupts the virulence factor which is used by bacteria to spread infection in the body (Niu et al., 2006; Brackman et al., 2008). The phenylpropene eugenol was found to inhibit the growth of 24 strains of bacteria from 25 (Dorman and Deans, 2000). It was also illustrated by Zemek et al. (1987) that both eugenol and isoeugenol were more efficient on gram-negative bacteria than gram-positive bacteria. Vanillin showed an inhibitory action against bacteria, moulds and yeasts (Rupasinghe et al., 2006). Mint essential oils which contains menthol and menthone affected the production of enterotoxin B when added to *Staphylococcus aureus* inoculum (Tassou et al., 2000). Eugenol was found to reduce the production of listeriolysin O. by 80-100%, which is produced by *Listeria monocytogenes* (Filgueiras and Dantas Vanetti, 2006).

Understanding the mechanisms of action of the different essential oils and their components helped researchers to choose appropriate techniques to control the growth of various microorganisms and the diseases they cause. The hydroxyl group on terpenoids play a great role in their ability to bind with proteins, enzymes and disrupt the membrane functionality in bacteria cells.

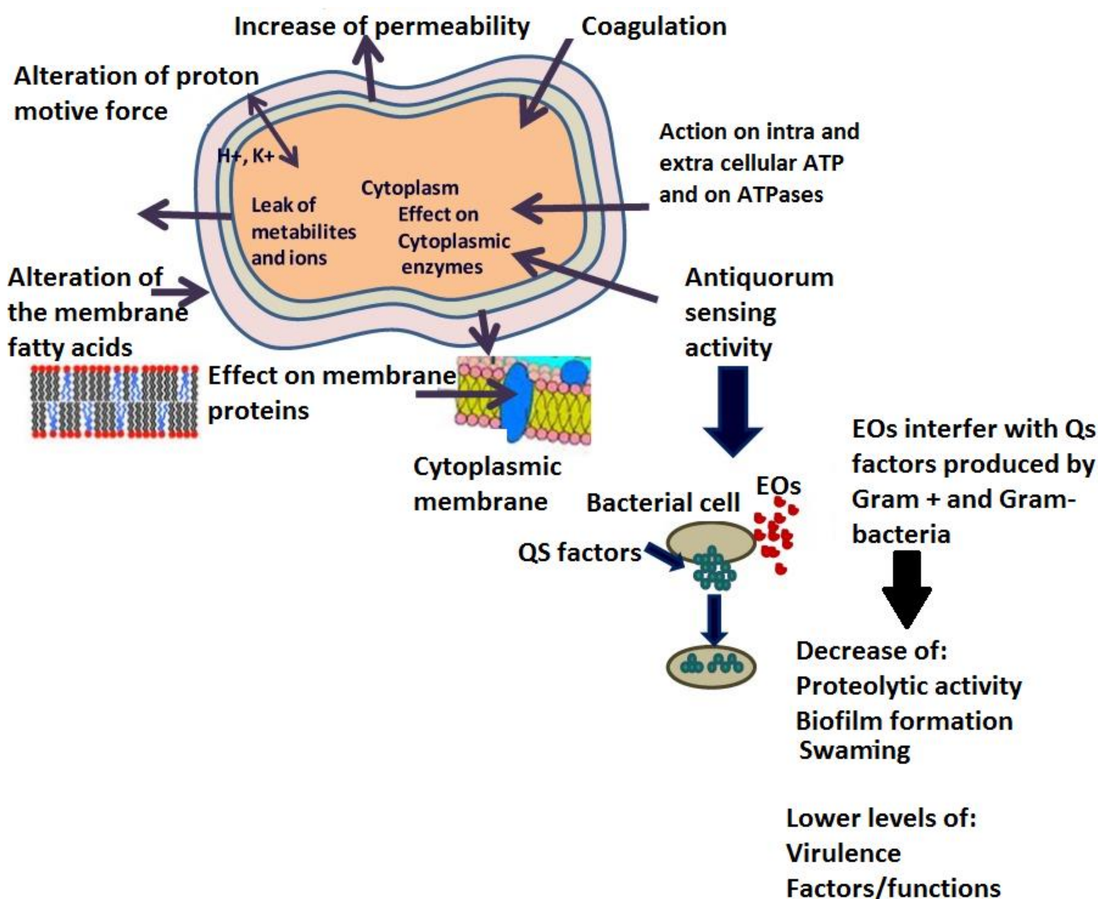


Figure 2.8: Different mechanisms of essential oils on the bacterial cell (Nazzaro et al., 2013).

2.1.4 The application of essential oils as food preservatives

Any process used to produce safe food should prevent microbial contamination, inhibit or inactivate the microorganisms when they are present in the food product, and finally, they should be able to control and inhibit the growth of dormant microorganisms (Gould, 2012).

As mentioned in the above sections essential oils have been tested in non-food mediums and showed antimicrobial activity. However, food contains different chemical matrices such as protein, fat, water, starch, enzymes and various degrees of acidity that may affect the EOs activity. The bioactivity of the EOs in food can be enhanced by different procedures including storing at low temperature, decreasing oxygen within the package, increasing salt concentration and lowering the pH which improves the stability

and solubility of the EOs (Burt, 2004; Friedly et al., 2009). In food systems, higher concentrations of EOs is required to perform an antimicrobial activity (Sivropoulou et al., 1996). In a study, cilantro oil showed antibacterial activity at 0.018% when tested *in vitro,* but no antimicrobial activity observed for the oil even at 6% when applied to ham. The deficiency in EOs activity is due to many factors including the availability of an essential nutrients in food systems in greater quantities compared to laboratory media, which can be used by microorganisms for fast repairing of damaged cell (Gill et al., 2002). Therefore, it is worthwhile to test the *in vitro* activity of EOs in model food media by using known concentrations of food matrices to understand the mechanisms of interactions (Gutierrez et al., 2009). The lower amount of water in food systems hinder the approach of EOs to reach bacteria cells (Smith-Palmer et al., 2001). In comparison, Tassou et al. (1995) found a significant activity of mint oil against *L. monocytogenes* and *Salmonella enteritidis* in cucumber and yoghurt salad which have low fat and low pH, while limited activity was observed when the same oil was used in pate and fish salad which characterised by containing a high amount of fat. They also stated that the availability of a large amounts of water and salt enhanced the antibacterial activity of EOs. Moreover, Smith-Palmer et al. (1998) found that foods with higher lipid content required higher concentrations of EOs to act as antibacterial agents. On the other hand, carbohydrates were found not to hamper the antibacterial action of EOs (Shelef et al., 1984). The structure of the food also plays a great role in limiting the activity of EOs as antibacterial agents. In gel foods, it was found that the structure of the gel limited the availability of the EOs on bacteria target sites (Skandamis and Nychas, 2000).

Another limitation of using EOs as food preservatives is due strong odour which may change the taste of food and acceptability of the consumers (Friedly et al., 2009; Bajpai et al., 2012; Solórzano and Miranda, 2012). As mentioned earlier, hurdle technology may be used to preserve food and extend the shelf-life by combining different physical and chemical methods such as low storage temperature, low water activity, and pH with adding chemical preservatives or natural extracts (Ganesh et al., 2010). Because all food products are prone to contamination and spoilage, it is therefore possible to treat different foods with EOs as a natural antimicrobial. The following sections explain the potential of using EOs in vegetable and fruits, meat and meat products and finally milk and dairy products.

2.1.4.1 The application of essential oils in vegetables, fruits and juices

Vegetables and fruits can be a good carrier for foodborne pathogens which may be contaminated in different stages of preparation from the field and even after reaching our kitchens. Recently EOs vapour was used as natural preservatives to preserve the perishable food products from deterioration and controlling foodborne pathogens (López et al., 2005). To maintain the freshness, safety and quality of foodstuffs its more preferable to use natural antimicrobials than synthetic preservatives (Bajpai and Baek, 2016). Several studies are available on using EOs as antimicrobial agents in different food products. Cinnamaldehyde and sporan used by Yossa et al. (2012) to preserve spinach leaves stored at 4 °C for 14 days. The number of *E.coli* and *Salmonella* on the treated samples decreased by 3 logs (CFU/g) in comparison with controlled specimens. Different EOs used to evaluate their efficacy in preserving lettuce, carrot, and ready-to-eat vegetables. Thyme EOs found to be the most effective on different bacteria when used in the study by Gutierrez et al. (2008). However, the acceptability of the EOs on the vegetables was vary, basil, marjoram, oregano, and thyme EOs were acceptable on carrot while only marjoram and oregano EOs were organoleptically acceptable with lettuce. *Salmonella* number reduced significantly on tomato and lettuce when treated with myrtle EOs (Gündüz et al., 2009). Citron EOs was able to extend the shelf-life of ready-to-eat salad syrups stored at 9 ºC (Belletti et al., 2008). The CFU counts decreased in kiwi fruit when cinnamaldehyde and carvacrol used, while they were least efficient in honeydew melon (Roller and Seedhar, 2002). The foodborne pathogenic bacteria in juices of apple, pear and melon inhibited by using the EOs of lemon grass and geraniol (Raybaudi-Massilia et al., 2006).

Strawberries decay could be controlled by coatings with thymol, eugenol, and menthol. The preservative activity of thymol against decay and tissue deterioration found to be more than other compounds (Wang et al., 2007). Different EOs and some terpenoids showed antibacterial activity against *E. coli* when tested in apple juice. It was found that carvacrol, eugenol, geraniol in compounds and the EOs of oregano, cinnamon, and lemon in plants at different concentrations 0.018-0.0936 % were effective. In addition, *Salmonella enterica* was more susceptible to carvacrol, linalool, terpineol, geraniol, citral compounds and EOs of melissa, oregano, lemon, lemongrass and cinnamon leaf at concentrations of 0.0044-0.011% (Friedman et al., 2004). Grape tomatoes washed in thymol solution and stored for 16 days at 4 ºC and 22 ºC, showed no change in sensory

properties during the storage period. Moreover, the number of the inoculated *Salmonella* reduced in the treated samples by 7.5 logs compared to the control samples. Lu et al. (2014) stated that EOs were more active in fruit and vegetables compared to other foods due to their low-fat content.

2.1.4.2 Application of essential oils in meat and meat products

Meat and meat product are considered as an excellent medium for microorganisms due to the availability of nutrient required by microorganisms and suitable pH for growth. Meat is more prone to spoilage and oxidation which may decrease the consumer acceptability. Chemical preservatives such as nitrate and nitrite are related to potential health risk to the consumers. Therefore, EOs as alternative preservatives may preserve and extend the shelf-life of the meat and its products (Holley and Patel, 2005; Bajpai and Baek, 2016). Modified atmosphere packaging (MAP) is one of the effective methods to control different types of foods from microbial spoilage and extending the shelf-life. Meat is an excellent vehicle for foodborne pathogens such as *Salmonella enteritis*, the presence of this bacteria in meat and minced beef and sheep have been reported many times. Expelling or reducing oxygen within packaging prevented aerobic microorganism growth and preserved the meat from oxidative colour change. A combination between natural antimicrobials and MAP along with refrigerated storage enhanced the packed meat quality and safety (Govaris et al., 2011).

In their investigation, Karabagias et al. (2011) found that the shelf-life of fresh lamb meat increased from 7 days for controlled samples to 21-22 days for treated samples with a combination of MAP (80% CO_2 and 20% N_2) and 0.1% thyme EOs. Oregano and thyme EOs incorporated into soy protein edible film was used to inhibit spoilage and pathogens bacteria on fresh ground beef stored at 4 ºC and showed a high degree of antibacterial efficacy at concentrations of 5% for both EOs (Emiroğlu et al., 2010). The combination of nisin (500 or 1000 IU/g) and oregano EOs (0.6%) was found to be active against *Salmonella enteritidis* in minced sheep meat stored at 4 ºC in comparison with bulk nisin or oregano EOs (Govaris et al., 2010).

Essential oils of Tunisian sage and Peruvian pepper tree were used to inhibit the growth of *Salmonella* genus in minced beef meat which stored at 4 -7 ˚C for 15 days. The EOs had bacteriostatic effect at concentrations of 1 % and below, but the bactericidal effect was clearly evident at concentrations of 3.0 % and above. However, using high

levels of 1.5% and 2% of Tunisian sage and Peruvian pepper tree EOs respectively altered the taste and flavour of the minced beef (Hayouni et al., 2008). In an investigation on the antibacterial activity of Valencia orange oil, Nannapaneni et al. (2009) found that the naturally occurring *Campylobacter jejuni* and *Campylobacter coli* isolated from whole chicken and CFU reduced by 1.5-2 log, when the oil was used in comparison to the control. In another study, pork meat was treated with the EOs of Satureja horvatii to investigate the EOs antimicrobial activity against *L. monocytogenes*. The treatment improved both colour and flavour of the stored meat after four days as a result of the antioxidant activity of the EOs, in addition to successful inhibition of the decaying microorganisms (Bukvički et al., 2014).

In a study by da Silveira et al. (2014), which aimed to increase the shelf-life of Tuscan sausages stored at 7 °C by treating it with bay leaf EOs. Concentrations of 0.05 and 0.1g /100g were able to extend the shelf-life by two days. At these two concentrations, the sausage remained acceptable by consumers. In ready to eat barbequed chicken, oregano and nutmeg EOs were used at a concentration between 1-3 µl/g which was higher than the *in vitro* concentration by 5-15 times to inhibit bacterial growth, which was organoleptically unacceptable (Firouzi et al., 2007). The fresh chicken shelf-life extend by using a combination of EOs and MAP technique. Different concentration of oregano EOs (0.1 % - 1 % w/w) with MAP packaging was used to preserve breast chicken by Chouliara et al. (2007) and the results showed that when the 0.1% oregano EOs was used, the shelf-life was extended for 3 - 4 days. Less shelf-life extension period (2-3 days) was observed when only the MAP was used, while an increase of 5-6 days to the breast chicken shelf-life was observed when a combination of 0.1% oregano EOs and MAP (30% N_2 + 70% CO_2) was used, which verified the synergetic effect of the combination. Moreover, the shelf-life of chicken meat liver was 15 days when a combination of oregano oil, MAP and EDTA was used. While a shorter shelf-life of only 3 days was observed in the samples stored in non-modified atmosphere packaging (Hasapidou and Savvaidis, 2011). According to these studies, the combination of MAP and EOs had an additive effect and can be used in a broad spectrum of perishable food products.

2.1.4.3 Application of essential oils in milk and dairy products

Milk contains a variety of food nutrients that are essential for microorganism's growth and maintenance. They are also found to protect the microorganisms from

antimicrobials such as milk fat and proteins. It was found by many researchers that the fat content in milk has a negative impact on EOs efficiency as antibacterial (Burt, 2004). Smith-Palmer et al. (2001) studied the effect of fat content on the activity of different plants EOs. They found that the antibacterial effect of the EOs decreased when high fat soft cheese was tested in comparison with low-fat soft cheese. The presence of protein was also found to limit the activity of some EOs constitutes such as carvacrol. Pol et al. (2001) stated that the antibacterial of carvacrol against *Bacillus cereus* decreased in dairy products due to the possible reaction of carvacrol with milk proteins.

However, the combination of EOs with other hurdles appeared to be promising for inhibiting microorganisms in dairy products. In their study on yoghurt drink (Ayran) Evrendilek and Balasubramaniam (2011) found that adding mint EOs along with using high-pressure processing (HPP) decreased the *L.monocytogenes* CFU/mL by 6 logs. Thyme EOs 0.1 mL/100g and oregano EOs 0.1 and 0.2 mL/100g EOs combined with MAP packaging (50% CO_2 and 50% N_2) were able to reduce the surviving rate of both *Escherichia coli O157: H7* and *Listeria monocytogenes* in feta cheese separately. Moreover, a combination of nisin and cone (Metasequoia glyptostroboides) EOs was used against *L.monocytogenes,* that were reported to survive pasteurisation process, and it may grow again in milk when stored at refrigerated temperature. In this study, it was revealed that concentrations of cone 1% and 2% with different concentrations of nisin (62.5-500 IU/mL) inhibited *L. monocytogenes* in all three different milk types (whole, low fat and skim) during 14 days of storage (Yoon et al., 2011).

2.2 Delivery strategies of EOs

EOs can be applied to food products via different strategies to increase their activity and reduce the concentration of the EOs to avoid the organoleptic change of the food and reduce the risk on the consumers. In the next sections different possible methods are described.

2.2.1 Hurdle technology

As mentioned earlier, the use of EOs in food as a preservative is often insufficient below permitted concentrations. Moreover, increasing the EOs concentrations to the effective level has additional consequences, both on the organoleptic properties of the

food and safety of the consumers. Alternative techniques such as hurdle technology may help overcome this problem.

The hurdle technology is defined as using a combination of preservative methods to keep the food safe from microorganisms and keeping the food fresh without changing its sensory and quality attributes (Leistner and Gorris, 1995). The combination of the chemical and physical process can be used to achieve hurdles that inhibit microorganism's growth in food. In multiple-hurdle technology a combination of different processes is used including organic acids, nitrite, sulphite, water activity (a_w) control, pH, temperature, increasing salt concentrations, using EOs, chlorine dioxide, and competitive bacteria (Leistner and Gorris, 1995; Leistner, 2000; Friedly et al., 2009). Using different hurdles such as slight heat at 54 $^{\circ}$C, hydrostatic pressure and low concentrations of EOs (0.2 µl/mL) showed inactivation of foodborne microorganisms (Cherrat et al., 2014).

However, other approaches such as encapsulation can combined with other hurdles which may increase the bioactivity of EOs, thus less concentrations are required to show activity against microorganism without changing the organoleptic properties of food.

2.2.2 Other techniques of delivering EOs into food products

EOs have the potential to be used in active packaging instead of adding them directly to food products to preserve the organoleptic properties of the food. Mushrooms are likely to spoil rapidly due to enzymatic browning and high respiration rate. Fumigation of mushrooms with EOs of thyme, cinnamaldehyde and clove, inhibited the senescence, decreased the browning index, cap opening, and microorganisms (Gao et al., 2014). Paraffin incorporated with cinnamon oil was used as covering paper for PET trays and in emulsion to inhibit oxidation and enhance the quality parameters of packed mushrooms. The result showed significant improvement in in weight loss and browning and the better results were observed when the EOs were used in the form of an emulsion (Echegoyen and Nerin, 2015).

In food coating technology there were successful attempts to incorporate EOs into coating materials. Citral and quercetin have been integrated into sorghum protein (kafirin) to produce a film which was used as antimicrobial and antioxidant agent against different microorganisms (Giteru et al., 2015). The shelf-life and quality parameters of strawberry

fruit enhanced by releasing carvacrol and methyl cinnamate from the edible coating (Peretto et al., 2014).

Nanoemulsion could be another strategy that can be used to encapsulate and deliver EOs which are able to minimise the organoleptic changes in food products. Moreover, by using nanoemulsion, the antimicrobial activity of EOs improves by increasing the passive cellular uptake, reducing interaction with food components and by enhancing the stability of the emulsion (Donsì et al., 2012). The size of colloidal particles containing EOs also play a role in their antibacterial efficacy. When a group of bacteria form a biofilm and the EOs droplets are large, they cannot reach the bacteria which are located inside the biofilm, however when the EOs droplets are small they are able to reach the bacteria inside the biofilm.

2.2.3 Encapsulation

Encapsulation is the technology of trapping active compounds, enzymes, antioxidants, polyphenols, flavours, cells or micronutrients inside a shell or coating material by forming a homogeneous or heterogeneous matrix from the particles; thereafter releasing the content in the later stage by the influence of different physical and chemical triggers such as changing temperature, enzyme activity, shear or changing pH (Madene et al., 2006; Champagne and Fustier, 2007; Jafari et al., 2008; Sobel et al., 2014a; Kwak, 2014). The encapsulated components can be in the form of gas, liquid or solid which is called the internal phase or core materials, surrounded by wall material, shell or coat, which is insoluble in the internal phase. Wall materials should retain the core during processing, handling, transport and storage until the release at a required time and location. Encapsulation techniques have numerous beneficial applications in many industries such as cosmetics, pharmaceuticals and foods (Gouin, 2004; Lee et al., 2013).

Different capsule morphologies can be formed according to the distribution of core in the wall material. For example, when the cores are distributed in the shell material (multiple cores obtained) it is referred to as a matrix and the capsules called microspheres, while where a single core is surrounded by the wall material, it is then called microcapsules (Figure 2.9). The size and morphology of the formed particles depend on the techniques used to prepare the capsules. Several techniques are available for encapsulation. For instance, spray drying is used to generate small microspheres; a jet homogeniser is used for preparing small liquid-filled core capsules, while fluid bed dryer

is used for coating solid particles and coacervation to large liquid-filled capsules (Champagne and Fustier, 2007; Gibbs et al., 1999; Oxley, 2014; Wang et al., 2015). There is a classification of the formed particles according to their sizes. If the size of the particle is more than 5000 µm it is called macro-capsules and those with particle size between 1-5000µm referred as microcapsules, while nano-spheres are particles smaller than 1 µm and the technology used to prepare them is called nanoencapsulation technology. The small size of these particles is beneficial regarding delivery of active materials due to their high surface area to volume ratio, better stability and rapid diffusion of encapsulated materials (Lopez-Rubio et al., 2006; Davidov-Pardo et al., 2015; Jafari et al., 2008; Wang et al., 2015). The capsules formed with these techniques are very useful in the food industry with desirable features (Shahidi and Han, 1993; Jackson and Lee, 1991) as shown in (Table 2.2).

The food characteristics such as texture, taste and colour are enhanced using nanotechnology and this is because the nanoparticles are easier to disperse and cannot be seen by consumers when added to the food products. In this sense, the new food product is developed and the bioavailability, solubility and stability of the active compounds enhanced (McClements et al., 2009; Acosta, 2009).

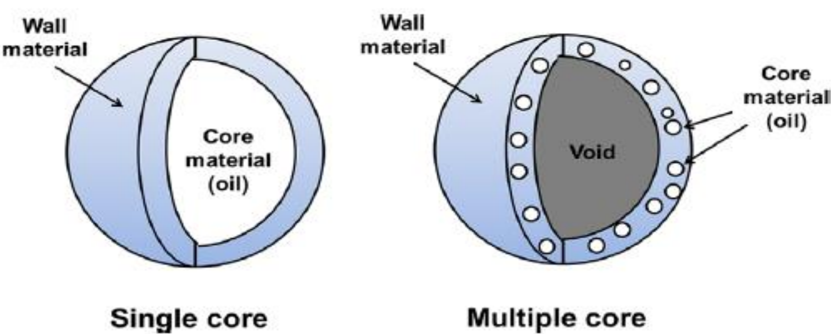


Figure 2.9: The distribution of core materials in the capsules: microcapsule on the left and microsphere on the right (Wang et al., 2015).

There has been an increase in functional foods in the marketplace owing to their perceived health benefits. However upon delivery, most of these bioactive compounds are unable to reach the target site in the body, especially lipophilic compounds. The encapsulation of functional compounds has been shown to enhance the bioavailability and improve target delivery of these compounds (Chen et al., 2006).

Table 2.2: Application of encapsulation techniques in manufacturing some food products, adopted from (Zuidam and Shimoni, 2010).

The reason of encapsulation	Ingredients	Food application
Slow release during manufacture	Organic Acids (Lactic, citric)	Meat-sausages
Slow release during cooking	Flavours	Microwavable foods
Slow release in mouth	Sugars, flavours or acids	Chewing gum, candy coatings
Slow release in GI system	Probiotics	Nutraceuticals
Protection against high temperature and oxidation	Ascorbic acid	Bread-dough
Increased stability during cooking	Vitamins	Pasta and prepared foods
Colour masking	Vitamins A and minerals	MSG table salts
Provides a powder from oils	Flavour and essential oils	Prepared foods
Taste masking	Vitamins, minerals, oils (omega-3)	Dry blends
Increased retention	Enzymes	Cheese ripening

The formation of capsules requires the formation of a core prior to or during encapsulation. Granulation, milling and grinding are essential to reach the required particle size before encapsulation when solid particles are coated. When the core is soluble in the shell material, no prior processing is required before encapsulation. On the other hand, if the core material is liquid, emulsification is required to prepare droplets with incorporated core materials by using surfactants, viscosity modifiers, or biopolymers such as proteins and/or polysaccharides.

In the case where the emulsion technique is used the wall material should be in a liquid state to facilitate enrobing the core. In order to wet the core material by wall material, both the interfacial tension and viscosity of the emulsion system must be low; to increase the flow of the wall material around the core material during droplet formation.

Once the wall material has solidified the retention of core materials increase. There are many methods to solidify the wall materials around the core, for instance, the evaporation of the solvent, chemical reactions, freezing and gelation (Oxley, 2014; Madene et al., 2006).

2.2.4 The delivery system

Active materials can be delivered via utilising the self-assembly behaviour of the surfactant based systems such as liposomes, swollen micelles and/or biopolymer based systems such as proteins and polysaccharides. The use of biopolymer as food-grade materials to encapsulate active materials was the subject of many studies (Jones and McClements, 2010; Burey et al., 2008a; Matalanis et al., 2011). The colloidal particles can be formed by using bottom-up and/or top-down approaches.

However, selecting the carrier materials depends on a number of factors including: (a) the state of core material (solid or liquid), (b) the solubility of one material in another, (c) interfacial tension, (d) melting and boiling points, (e) glass -transition temperature (T_g), (f) the loading capacity, (g) safety when used in food, (h) inconsistency with the core material, (i) economically manufactured, (j) should not affect the quality of food, (k) prevent chemical degradation, (l) has the capacity for holding the active material until release when reconstituted and used in the food medium, (m) able to expel solvent during drying, (n) has a good emulsion stabilisation and high degree of release when dispersed at the desired time and location, (o) capable of preserving the bioavailability of the active compound (Trubiano and Lacourse, 1988; Alan and David, 2005; Chen et al., 2006; Madene et al., 2006; Jafari et al., 2008; Zuidam and Shimoni, 2010; Garti and McClements, 2012; Oxley, 2014; Sobel et al., 2014b; Davidov-Pardo et al., 2015).

When choosing a material as a coating material account should be taken of rheological properties and emulsifying ability. Rheological properties affect the ease of work of encapsulation, especially at high concentrations. Emulsifying ability affects emulsion formation and the subsequent stability of the emulsion. Moreover, the coating materials should not react with the embedded active ingredients; it should preserve the

core during and after encapsulation processing and release the content when it is triggered. It must be soluble in water and ethanol as they are widely used in food products. It should also be able to form a thin film around the core material. Due to the lack of all properties mentioned above in a single wall material alternatively the combination of different materials is more practicable and favourable (Trubiano and Lacourse, 1988; Ré, 1998; Desai and Park, 2005; Jafari et al., 2008).

The composition of the delivery system

Different ingredients such as proteins, water, carbohydrates, surfactants, lipids, minerals and phospholipids can be used to fabricate food-grade particles in the colloidal delivery system (Jones and McClements, 2010; Matalanis and McClements, 2013). It is important to select the ingredients to be fabricated as a delivery system which is available at low cost with desirable functional attributes as required by the food industry. The selection of the ingredients also determines the labelling of the final product such as vegetarian, Halal, allergy, Kosher, vegan and milk proteins. For example, it is not possible to label foods as "all natural" if it contains Tween-80, vegan foods should not contain milk protein, and the labelled vegetarian foods should not contain gelatin. The location and the trigger of the release also determines the composition of the colloidal particles for instance by lipases; proteases amylases enzymes are utilised to release the active compounds from the particles in the upper part of gastrointestinal tract. Thus, proteins, starches, triacylglycerol are selected as delivery systems. In contrast, if the target location is the lower gastrointestinal tract, dietary fibre is chosen, since they subjected to fermentation and degradation by microorganisms resulting in a bioactive release (McClements, 2014).

The two main types of emulsions are usually used as delivery systems are oil- in-water (O/W) and water-in-oil (W/O) emulsions. O/W emulsions are produced by dispersing oil droplets in an aqueous phase in the presence of emulsifiers which are able to form a thin layer around the oil droplets forming colloidal particles (McClements, 2004b; Friberg et al., 2003). The stability, formation and physiochemical properties of the emulsion systems depend on the concentration and various properties of the ingredients contributing to the colloidal particle formation. The size of emulsion droplets determines the functional and physicochemical properties of the emulsions, therefore, sorting emulsions according to the particles sizes is crucial (McClements and Rao, 2011;

McClements, 2011). Thus, particles with radius less than 100 nm are referred to as nanoemulsions, while when the radius lies between 100 and 1000 nm it is considered as mini-emulsions or conventional emulsions and finally macroemulsions have radius more than 1000 nm (McClements and Rao, 2011). The type of the emulsifier used in the system determines the thickness of the interfacial layer around the oil droplets. Polysaccharides are able to form a thick layer followed by proteins and then surfactants which are able to form thinner layers; the range of layer thickness is naturally between 2-20 nm (Dickinson, 1992).

O/W emulsions can be utilised to encapsulate and deliver a wide variety of hydrophobic ingredients such as ω-3 fatty acid into yoghurt, ice cream, meat patty and milk (McClements and Decker, 2000; Sharma, 2005; Chee et al., 2007). These emulsions are also used as a delivery system for lutein and β-carotene (Santipanichwong and Suphantharika, 2007), lycopene (Ribeiro et al., 2006) and oil-soluble vitamins (Saberi et al., 2013). It is possible to convert the liquid emulsion to powder by spray drying to obtain more stable formulation with longer shelf life, easier to handle and use (Soottitantawat et al., 2003; Soottitantawat et al., 2005a).

In this study, the focus will be on biopolymer-based delivery systems. The building blocks of the biopolymer as delivery systems are explained in the next sections. Usually, proteins and polysaccharides are used as food-grade biopolymers for delivering active ingredients.

Proteins

Proteins are biopolymers composed of hundereds of amino acids linked together via peptide bonds. In nature there are 20 amino acids which are characterised by different side chains. Therefore, the molecular characterisation of the proteins such as hydrophilicity, charge, reactivity, molecular weight, flexibility and their interactions depend on the number, type, location, type of the bonds, and sequence of the amino acids presented in the protein chain (McClements, 2014). The different structures of the proteins of secondary, tertiary and quaternary are governed by the sequence of the amino acids along the protein chain. Changes to the protein structure can be utilised to modify protein interaction, configuration and film formation behaviour (John, 2002). The prevalent structures of proteins are a rigid rod, random coil or globular which can be affected by exposing to different pH, temperature, solvent and ionic composition during

extraction or isolation which may appreciably alter their functional properties of the proteins (Damodaran, 2007).

The properties of the amino acid side groups are determined largely by their nature: aliphatic, polar, aromatic and positive or negatively charged. There are a variety of interactions including covalent, steric, hydrogen bond, van der Waals, electrostatic and hydrophobic between protein-protein and proteins with other molecules in the system. The type of interaction in food affects their functionality and physiochemical properties such as solubility, interaction with water, film-forming around hydrophobic compounds and stabilisation of emulsion droplets giving them good characteristics as wall materials (Walstra, 2009; Bylaitë et al., 2001; McClements, 2014).

Gelatin from animal, caseins, whey protein concentrate (WPC), β-lactoglobulin and lactoferrin from milk, skimmed milk powder (SMP), ovalbumin, lysozyme and phosvitin from egg, zein from corn and soy protein isolates are examples of the proteins that have potential to be used as wall materials in encapsulating hydrophobic materials in food sector. As encapsulation agents, they have the ability to preserve the core, such as flavours, by binding with them via hydrophobic and van der Waals interactions (Hemar et al., 2011; Acharya et al., 2013).

When oil droplets are formed in the emulsion system, the proteins change their structure; they tend to unfold and adsorb on the interface, the polar parts of the amino acids remain in the aqueous phase; while the non-polar parts protrude into the oil phase, which stabilises the oil droplets suppressing coalescence due to repulsive forces between the protein layers on the oil droplets (Dickinson, 2001; Jafari et al., 2008). The functional properties of the proteins are governed by temperature, pH and ionic strength of the emulsion system. For instance, if the pH of the system reaches the isoelectric point (PI) of the protein, then it affetcs the stability due to loss in the emulsifying properties of the protein (Jafari et al., 2008).

Casein and whey proteins

Milk proteins (whey & casein) are widely used in the preparation of emulsions including spray dried emulsions. Casein outperforms whey protein (WP) regarding emulsification due to its resistance to denaturation by heat, its ability to decrease the concentration of oil on the surface and ability to form smaller droplets. Whey protein and lactose have been used as a successful encapsulation agent, it is thought that this

combination may prevent a polar diffusion through the wall they form, due to the hydrophilic behaviour of the lactose that prevents the hydrophobic core from diffusing through the wall (Gharsallaoui et al., 2007; Sobel et al., 2014a). Research showed that proteins can be used to encapsulate essential oils (Bylaitë et al., 2001), anhydrous milk fat by whey protein concentrate (WPC) (Young et al., 1993) and fish oil and fatty acids by sodium caseinate (SC) (Hogan et al., 2003). Additionally, Fäldt and Bergenståhl (1996a) found that WPC was less efficient for the encapsulation of soybean oil when compared with SC.

Caseins are made from different proteins of αs1-casein, αs2-casein and β-casein which form the internal part of casein micelles; and κ-casein forms the outside of the molecule stabilising the core by steric repulsion (Kaya-Celiker and Mallikarjunan, 2012). Caseins are characterised by having a flexible structure when presented in aqueous suspension. αs1-casein consists of 199 amino acids, it has three main hyrophobic regions, the first area located at the begninig (1-40 amino acids) of the protein chain and the rest situated at the middle (90-110 amino acids) and at the end of the chain molecules (150-199). They play a role in adsorbing the proteins onto the oil droplet surfaces when they are present in the emulsion system. Different hydrophobic and hydrophilic amino acids can be observed alongside their backbones of a casein molecule chain. The behaviour of the casein molecule is also affected by the degree of phosphorylation due to presence of phosphorine residues (8-10) located between 41-80 region on the αs1-casein. These residues are responsible of the net charge of the proteins above isoelectric point which dermines the electrostatic stability of the proteins (Swaisgood, 2008). Prolines are the main amino acids forming αs1-casein and no cystiene are present in their chain. Therefore, the molecules are randomly distributed in the solution with low ability to form coil-like structre and less ability to form secondry or tertiary srtuctures.

On the other hand, β-casein is very hydrophobic on C-terminus due to the presence of 158 amino acids with no charge mostly comprised of proline, and the rest of 50 amino acids are negatively charged such as phosphorylated serine and comprises the hydrophilic part of the protein on the N-terminus, therefore, they are amphiphilic (Swaisgood, 1982; Nylander and Wahlgren, 1994). β-caseins are not able to form structures of α-helix or β-sheets due to the absence of cystiene residues on their molecules. Caseins are able to adsorb on hydrophobic interfaces as shown in (Figure 2.10).

When a significant amount of Ca+2 (cations) are available, casein will precipitate from a solution; as a result of the sequestration of calcium ions with serine-phosphate (8-10 in αs-casein and 5 in β-casein) via hydrophobic interaction. However, κ-casein consists of insoluble peptides and soluble glycopeptides. It contains only one phosphoserine group which make it stable in excess concentrations of Ca+2; it is also stabilises other caseins and their micelles from precipitation.

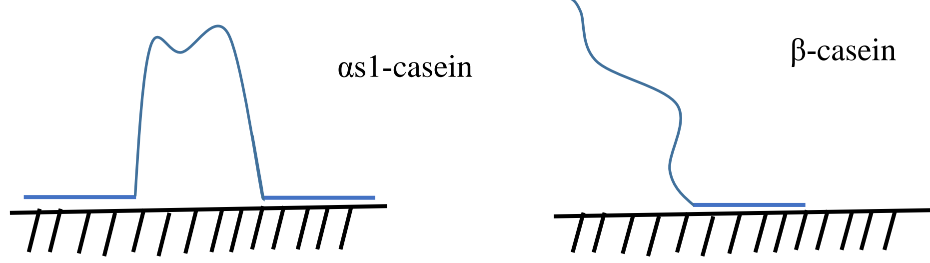


Figure 2.10: Schematic diagram showing the adsorbed casein onto hydrophobic interface (Herley, 1999).

The proteins in milk interact with calcium phosphate to form large colloidal particles which are called casein micelles (CM) (Figure 2.11). These can be utlised as carriers for other food components into food products. Calcium, phosphate, magnesium and citrate are constituents of colloidal calcium phosphate (CCP) this represents 6% of the micelles on a dry basis, and the remaining of 94% is protein. The diameter of the micelles ranges between 50 and 500nm with an average of 150 nm. (Phadungath, 2005; Livney, 2010).

Every millilitre of milk contains about 10^{14}-10^{16} micelles. The milk proteins are self-assembled to form micelles, κ-casein is in the hairy structure surrounding the micelles preventing them from flocculation. Nano-cluster bonds between serine-phosphate from the caseins, by calcium-phosphate and hydrophobic interactions, the disulphide bonds between the two cysteines in κ-casein are cover about 95% of the forces holding the casein micelles together in spherical structures. This behaviour is utilised by researchers to use the micelles as a carrier for other compounds by a mechanism which is called co-assembly. Where the ligand is bound to the casein and minerals are added to restructure the casein micelles, similarly, as occurring in milk, the process formulates nano-vehicles for hydrophobic compound delivery (Semo et al., 2007; Livney, 2010).

Micelles are able to solubilise and deliver the hydrophobic ingredients which can be utilised to encapsulate carotenoids, curcumin, essential oils and vitamins (Benzaria et al., 2013; Sáiz-Abajo et al., 2013).

On the other hand, whey proteins are comprised of different globular proteins with different fractions β-lactoglobulin consists ~55% of the total, followed by α-lactalbumin, serum albumin and immunoglobulins by the fraction of (~24%), (~5%) and (~15%), respectively. They characterised by high solubility in water. However, their use is limited due to cost and their sensitivity to heat. Heating whey proteins causes the exposure of the sulfhydryl groups to each other, and as the result, they induce the aggregation of the colloidal particles (McClements, 2014). The use of whey protein is disccused elsewhere in detail (Vega and Roos, 2006).

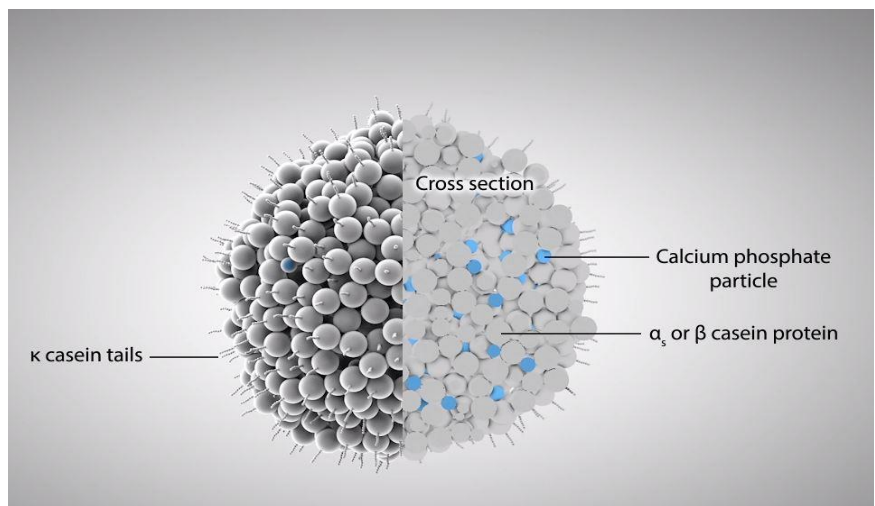


Figure 2.11: Cross section of casein micelle structure (Harlod, 2012).

The interactions of milk proteins

Van der Waals, hydrogen bonding and hydrophobic interaction and interaction are the main mechanisms for binding of hydrophobic compounds by milk proteins. Whey protein isolate (WPI) more efficiently binds flavours than sodium caseinate. Moreover, milk proteins have the ability to decrease the surface tension between water and oil interface because of their amphiphilic structure this can be utilised in emulsion stabilisation. Temperature, pH and ionic strength affect the structure of milk proteins and the emulsions made from them (Livney, 2010).

Heat, pH or rennet play a role in milk protein gelation. By decreasing the pH caseins are gelatinised as they reach their isoelectric point. The hairy layer of κ-casein, which is hydrophilic, is cleaved by rennet enzyme leading to accumulation of casein micelles and aggregation. The gels have been used for probiotics entrapment, when they are prepared by enzymes and transglutaminase crosslinking, this heat-free process does not affect the probiotics efficacy (Heidebach et al., 2009b; Heidebach et al., 2009a). The probiotic drugs have been trapped in gels prepared using genipin from gardenia fruits this has the ability to form crosslinking bonds. Because κ-casein is able to form disulphide bonds and due to its location on the outer surface of the micelle structure it improves and enhances the gel structure. Swelling of the micelles and release of the core materials occur when pH is far away from the isoelectric point (PI), this effect can be utilised as a pH triggered release (Song et al., 2009).

Milk proteins are able to interact with other food compounds as polymers by covalent conjugation or noncovalent interaction to form enhanced delivery systems for bioactive compounds. In the first mechanism, the Maillard reaction takes place between amino acids on the protein and carbonyl residue on the reducing sugars. In this reaction the hydrophobic domain of the protein and the hydrophilic group on the polysaccharide will conjugate to form a block copolymer which acts as a surfactant and has the ability to locate at themselves at interface between water and oil droplets. This conjugation stabilises the emulsion by forming a hairy layer by steric exclusion, electrostatic repulsion and increasing the viscosity of the continuous phase. This technique has been used in many studies; casein-glucose has been used for fish oil encapsulation by (Kosaraju et al., 2009); β-carotene as a hydrophobic material have been encapsulated (sizes between 175-300nm) with the casein-graft-dextran copolymer (Pan et al., 2007).

The stability of emulsions of globular protein can be increased by adding polysaccharide. Polysaccharides attach to the protein when the pH is not close to proteins isoelectric point. Attachment of the polysaccharides leads to an increase in steric repulsion, and/or electrostatic repulsion and prevent hydrophobic attraction at ambient temperature (Joye et al., 2015). The second mechanism of interaction utilises the differences in charged copolymers. The coating of bioactive compounds is formed through the layer-by-layer attraction between proteins and anionic or cationic biopolymers. For example chitosan and β-Ig, heated to form a hydrogel and found to be useful carriers for various compounds (Hong and McClements, 2007). Milk proteins have

the ability to form a shell around the bioactive compounds and protect them from oxidising agents. Because of their ready availability and safety of use, many bioactive agents have been delivered using milk proteins; for example, olive oil, fish oil, sunflower oil, vitamins, polyphenols and flavour compounds. Sodium caseinate has been used for many years as an encapsulation material due to its open structure and ability to bridge between calcium and phosphate; improved result are obtained when carbohydrates are mixed with the casein (Livney, 2010).

Polysaccharides

Other biopolymers which are widely used in delivery system's fabrications are polysaccharides which are composed by covalent bonds between monosaccharides (Rinaudo, 2008). Starch and its by products, pectin, maltodextrin cellulose and their derivatives, gum Arabic, gum karaya, dextrin, corn syrup from plant source, alginate and carrageenan from marines, and galactomannans from microbial sources are used as encapsulating agents due to their excellent physiochemical properties including low viscosity and high solubility in water. Moreover, some polysaccharides have a gelling agent ability which can be used as emulsion stabilisers. The surface active properties of some modified starches make them ideal to encapsulate of food products (Gharsallaoui et al., 2007). Szente and Szejtli (1988) and Risch and Reineccius (1988) explained the complexation reaction between cyclodextrin and volatile molecules which may enhance the stability of the essential oils against environmental factors, extending shelf life, increasing bioavailability and solubility.

Starch hydrolysed by enzymes or acids are inexpensive, has low viscosity in high concentrations are tastelessness this makes them ideal for use as protective wall materials for cores of milk fat, orange oil, fish oil and soy oil. The higher the dextrose equivalent (DE) of the hydrolysed starch, the lower the permeability and the better the protection of the core against oxidation. In addition to higher encapsulation efficiency (Wang et al., 2015; Anandaraman and Reineccius, 1986; Danviriyakul et al., 2002; Sheu and Rosenberg, 1998; Hogan et al., 2001b). Trubiano and Lacourse (1988) reported that the retention of orange terpenes and lemon oil in octenyl succinylated starch was better when compared to gum Arabic. The improved performance of succinylated starch, when compared to native corn and barley starch, was shown regarding flavour retention in the study by (Jeon et al., 2003). They found that the retention of four synthetic meat flavours

of dimethyl trisulphide, 2-mercaptopropionic acid and benzothiazole and benzaldehyde were higher in chemically modified starch as compared to the native starches.

Maltodextrin (MD) derived by hydrolysing corn flour with acid and enzymes, it is used widely for encapsulation purposes in the food industry. The degree of hydrolysis is measured by dextrose equivalent (DE) with the amount of reducing sugar available in the polymer by comparing it with dextrose which is 100 (Madene et al., 2006). Maltodextrin is cheap and does not affect the flavour of the system when it used as a wall material, it has low viscosity and can be produced in different molecular weights it is also able to protect the core from oxidation (Carneiro et al., 2013). Maltodextrin and starch are able to form a helical structure which can be utilised to entrap lipophilic compounds in their hydrophobic pocket (Siswoyo and Morita, 2003).

Moreover, gum Arabic (GA) is considered as an excellent emulsifier and offers high retention of the volatiles, therofre, its used widely in the encapsulation of oils and flavours. Bertolini et al. (2001) used GA to encapsulate monoterpenes and linolenic acid. In other studies by Fang et al. (2006) and Gascon et al. (1999) GA used to encapsulate essential oils used as antioxidants. The availability, cost and impurities in gum Arabic are the main obstacles to the commercial use of GA in encapsulation. However, there is attempts to reduce the cost by combination GA with other wall materials. Many researchers have used blends of MD and GA such as McNamee et al. (2001), who stated that it is possible to replace GA with non-emulsifying carbohydrates to encapsulate soy oil. Replacement of GA with MD was shown to be efficient and able to replace 50% of GA. Glucose and maize starch were evaluated but were unsuccessful as replacements for GA. The blend was also used to encapsulate rice flavour (Apintanapong and Noomhorm, 2003), citrus oil (Thevenet, 1995), volatile compounds such as citral and linalyl acetate (Bhandari et al., 1992), and cardamom oleoresin (Savitha Krishnan,2005). Ethyl n-hexanoate and d-limonene was successfully encapsulated by blends of β-cyclodextrin and GA (Hirokazu Shiga, 2001).

Cyclodextrins are the converted starch produced by enzymes to form rings of oligosaccharide made from sugar molecules. They are used to encapsulate food ingredients and pharmaceuticals because of the hollow cavity formation within the sugar molecules. Bhandari et al. (1998) found that the ratio of 6:94 w/w of lemon oil to β-cyclodextrin ensued high volatile retention. It is also noted that β-cyclodextrin is able to encapsulated d-limonene (Yuliani et al., 2006). The encapsulated core remain stable in

the dried dairy-like emulsion during storage as lactose forms crystals, which resist low Relative Humidities (RH), and the content was only released, when the RH reached 75% and above (Vega and Roos, 2006).

However, carbohydrates by themselves are unable to retain the flavour compounds during spray drying due to lack of emulsifying properties (Bangs, 1985; Bangs and Reineccius, 1982). Therefore, the blend of surface active compounds such as milk proteins (Kagami et al., 2003; Hogan et al., 2003; Bylaitë et al., 2001), gum Arabic (Krishnan et al., 2005; Liu et al., 1999; Thevenet, 1995; Bhandari et al., 1992; Fernandes et al., 2008; Bule et al., 2010) or esterified modified starch produced by adding lipophilic succinic acid ($C4H6O4$) to side chain of the starch (Soottitantawat et al., 2003, Soottitantawat et al., 2005a, Mongenot et al., 2000, Tesch et al., 2002, Bule et al., 2010); with carbohydrates is used to increase the retention of flavours and achieve high encapsulation efficienies when spray dried (Shiga et al., 2001; Soottitantawat et al., 2003; Liu et al., 2000; Kagami et al., 2003; Carneiro et al., 2013).

The different interaction forces between colloidal particles in the delivery system

The active compounds can be delivered by fabricating a colloidal delivery system to form microparticles or nanoparticles by either top-down or bottom-up approaches or the combination of the two methods. For the first type, the particles made up from solid phases such as crystals or bulk liquid such as large oil droplets. While to form a colloidal particle from bottom-up approach small molecules such surfactants, proteins, polysaccharides, lipids, phospholipids, minerals or water are required. The properties of the building blocks determine the possible interactions within colloidal particles and with the surrounding. Particles in a colloidal system interact with each other through different interaction forces such as van der Waals and hydrogen bonding, which are able to attract particles at medium range with a medium magnitude.

Another important force between particles is caused by repulsive steric interactions which occurs when a thick layer is presented on the surface of the colloidal particles which prevent the particles from flocculation or coalescence by repelling the particles. The magnitude of steric forces is strong and occur when the particles are within a short range distance. Electrostatic forces are either attractive or repulsive between the charged particles. The type of charge on the particle surface, pH and ionic strength determines the type and the magnitude of the interaction. On the other hand, depletion interaction

between excluded particles occurs when they are in a medium range of distance. The size and the concentration of the excluded particles determine the magnitude of the attraction forces between the particles. Another important force between the particles is the hydrophobic attraction between the hydrophobic surfaces which may be strong or weak. Finally, the hydrogen bonding as attractive force also controls the interactions between colloidal particles with a medium magnitude and at medium ranges (McClements, 2014).

The biopolymer interactions

When biopolymers are present in a colloidal system, they tend to interact with themselves and/or with other molecules via different interactions by physical and/or chemical forces between particles (Israelachvili, 2011; Jones and McClements, 2010). Various types of interactions between biopolymers are illustrated in (Figure 2.12).

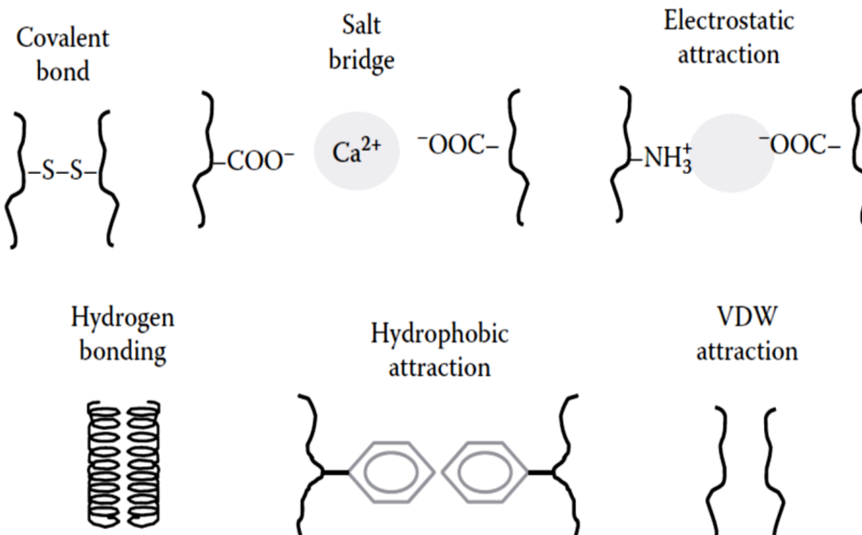


Figure 2.12: The molecular interactions between biopolymers (McClements, 2014).

Biopolymers are able to interact electrostatically through the different charges on their structures. The molecules repel each other if they have the same charge, and attract each other if they have different charges. The degree of the interactions between biopolymers governed by the charge density of the molecules and the dielectric and ionic strength of the solution.

Another type of interactions is ion-bridge as occurring in casein molecules by linking anionic phosphates. Pectin, alginate and carrageenan are linked by Ca^{+2} via carboxylic acid or sulphate groups. Moreover, chitosan has amino groups which can be associated

with calcium ions (McClements, 2014). The interaction, structure and the functionality of biopolymers is also governed by hydrogen bonds occurring between electronegative atoms such as (F, O and N) and electropositive hydrogen atom (Jones and McClements, 2010). These bonds are responsible for the interactions within the biopolymer or with another biopolymer. For example with proteins, it is found that hydrogen bonds are able to stabilise polysaccharides intramolecular structures (sheets and helices). They are also able to form bonds between different biopolymers as observed in cellulose, gelatin and starch. The effect of hydrogen bonds is substantial when a large number of these bonds contribute to the attraction interaction.

Hydrophobic interaction, another type of interaction force plays a great role in determining the functionality and structure of the biopolymers (Jones and McClements, 2010). They are attractive forces occurring between hydrophobic parts of the biopolymers when they are exposed to water (Israelachvili, 2011). This type of interaction appears because the water-water interactions via dipole-dipole bonds dominate the weak van der Waals bonds between water-nonpolar regions of the protein. When proteins are present in the water, the hydrophobic interaction, firstly, changes the proteins to the compact structure by decreasing the unfavourable interaction between hydrophobic parts of proteins and polar parts of water. Secondly, it facilitates the adsorption of the biopolymers on the oil surface or air water interface. Thirdly, they increase the aggregation of polysaccharides and proteins via nonpolar regions. Finally, binding of nonpolar molecules is promoted to the nonpolar areas on the biopolymers such as the hydrophobic pockets in starch helices and some types of proteins (McClements, 2014).

2.2.5 The stability of the delivery system

The stability of the biopolymer delivery systems containing the active ingredients is of great interest in the food industry; the systems should preserve the active ingredients during preparation, storage and after application into food products (McClements, 2010). Emulsions containing biopolymer particles tend to destabilise due to physical and chemical mechanisms such as the separation by gravitational forces leading to sedimentation or creaming and/or the aggregation of the particles such as coalescence and flocculation. Moreover, particles tend to erode or break apart, shrink or swell.

The creaming rate or velocity (U) of the particles depends on many factors such as the particle radius (r) the density differences between the dispersed (ρ_2) and continuous phase (ρ_1) and the viscosity of the continuous phase (η) as shown by Stoke's law:

$$U = \frac{2gr^2(\rho_2 - \rho_1)}{9\eta_1} \tag{2.1}$$

If the result is negative, it means the particles tend to sediment and if the value is positive it tends to cream. If the system contains only biopolymers and water sedimentation may occur because the density of biopolymers is 1500 kg/m^3, the addition of oil into the systems can decrease the difference in density until it reaches the density of water which stabilises the emulsion against gravitational separation.

According to Derjaguin-Landau-Verwey-Overbeck (DLVO) theory the aggregation of the particles occur when the attraction forces such as van der Waals, hydrophobic, hydrogen bonding and depletion dominate the repulsive forces such as steric and electrostatic interactions (McClements, 2005; Israelachvili, 2011) as shown in Equation 2.2. The total interaction energy results from the combination of repulsive and attractive forces between particles in the delivery systems determines the stability of the system. Strong steric and/or electrostatic repulsive forces are required to prevent flocculation and coalescence and increase the stability of the system (McClements, 2005).

$$\boldsymbol{w_{DLVO}(h) = w_{interaction}(h) + w_{repulsive}(h)} \tag{2.2}$$

Where w_{DLVO} is the total energy of the interaction, $w_{interaction}$ are the attractive forces and $w_{repulsive}$ are the repulsive forces between to particles at (h) distance.

When the particles are at a long-distance range, the van der Waals attractive forces dominate the attraction of particles and the potential energy of system reaches the *secondary minimum*. At this point particles flocculate and it is possible to re-disperse them by gentle shaking. Whereas when the particles in the suspension become closer repulsive forces become dominant generating an energy barrier to prevent. Moreover, coalescence may occur when the particles fall into *primary minimum* if the *maximum energy barrier* is not large enough to prevent particles from coagulation (Dickinson et al., 1999) as shown in (Figure 2.13).

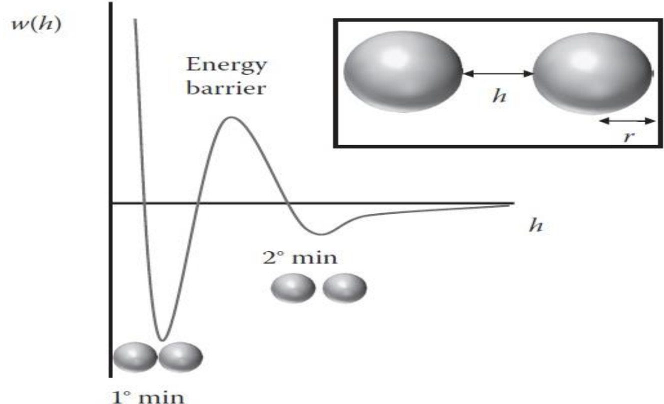


Figure 2.13: The interaction potential between two colloidal particles(McClements, 2014).

2.2.6 The challenges

The selection of an appropriate encapsulation technique is dependent on the type of food ingredients, the application of the active materials, payload, cost, release triggers, storage and handling. For instance, high temperature increases the evaporation of volatiles and denatures proteins which may decrease the encapsulation efficiency or properties of the proteins. Cost is a significant factor; the overall cost can be reduced by applying effective encapsulation techniques which increase the functionality of expensive ingredients such as vitamins, flavours, fatty acids (ω-3 and 6). Choosing materials that are GRAS, keeping the active materials safe and viable during storage, is another challenge in the encapsulation of food applications (Sobel et al., 2014a; Onwulata, 2012; Madene et al., 2006).

2.2.7 The techniques of microencapsulation

There are many methods available for microencapsulation. Physical methods include: spray drying, spray chilling, freeze drying, extrusion, spray cooling, centrifugal extrusion, fluidised bed, spinning or rotating disc and multiple emulsion. Chemical methods include liposomal inclusion, coacervation, interfacial polymerization, phase separation and nanoencapsulation as shown in (Figure 2.14). The selection of technique depends on the physiochemical properties of both the core and coating materials and the properties of the mediums they are being applied to (Sobel et al., 2014a; Oxley, 2014; Onwulata, 2012; Sara, 1995).

Each of the techniques has its procedural steps, for instance in spray drying the core materials should be dispersed in the coating materials and then homogenised, afterwards the atomisation of the emulsion is required to obtain a dry powder of the encapsulated materials (Desai and Park, 2005).

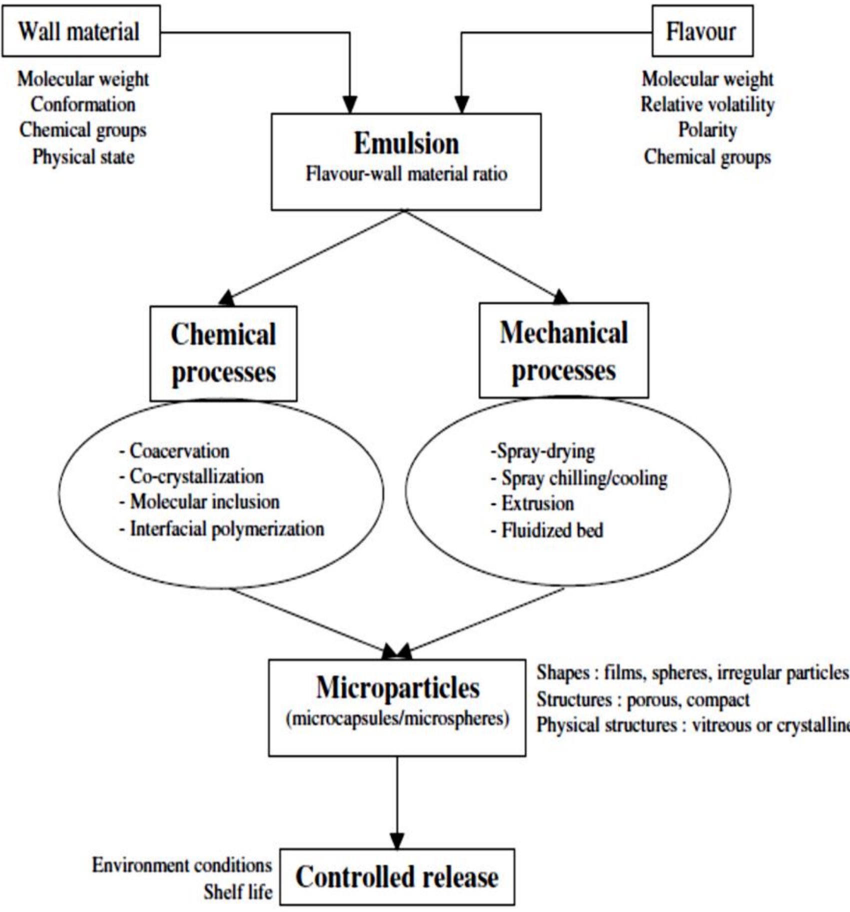


Figure 2.14: Different techniques used in encapsulation of core materials (Madene, et al., 2006).

2.2.7.1 Spray Drying

Spray drying is one of the techniques which is widely employed in the food industry. It was first patented by Samuel Percy for dry milk in 1872 and used to encapsulate flavours in 1930. It is considered a simple and low-cost procedure which is ideal for use in the food industry (Onwulata, 2012; Jafari et al., 2008). It is used to dehydrate liquid food products to preserve foods from undesirable changes by reducing the water activity. Droplets are formed from the liquid phase with solidifying of the capsule shell as a result of water evaporation when the atomised droplets pass through a hot gas stream. There are

many stages in the spray drying process as shown in (Figure 2.15). Different designs of spray dryers are available in the market from laboratory scale which can dry from 3L/ hour to high capacities reaching thousands of litres per hour (Jacobs, 2014b).

There are many examples of solutions, emulsions and suspensions of foods and pharmaceutical products which are converted to a powder by using the spray drying technique such as instant coffee, milk powder, baby food, tomato paste, fruit juices in food, penicillin, ampicillin, and streptomycin in pharmacy (Jain et al., 2011). The technique is also used as encapsulation techniques of flavours and oils due to its ability to convert solutions and emulsions into easy handling powder with low water activity and protecting the core material from undesirable changes during storage (Carneiro et al., 2013). If the solvent is not flammable, such as water, hot air is used as the drying gas. Whereas with flammable solvents such as ethanol and toxic solvents such as methylene chloride, as nitrogen closed loop is used. In the food industry spray drying is the preferred method for drying emulsions containing flavouring oils (Maria et al., 2009; Jacobs, 2014a; Rodea-González et al., 2012).

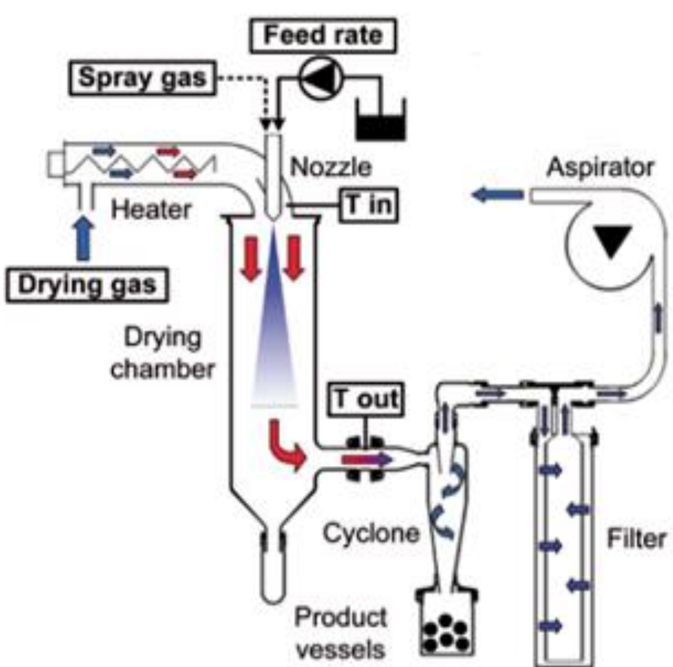


Figure 2.15: Spray drying stages (BÜCHI).

Preparation of the feed

Successful spray drying depends, mostly on the way in which the feed is prepared and on the addition of additives such as stabilisers, antifoaming agents, using emulsifiers and high pressure homogenisers or a high energy mixer to form a homogeneous feed of emulsions, suspensions or solutions (Jafari et al., 2008). In spray dried microencapsules the total solid in the solution determines the amount of the core material that can be encapsulated which is usually between 20-25% of the TS (Soottitantawat et al., 2003).

Feed solutions in the form of homogeneous emulsions can be prepared from maltodextrin or gelatin as hydrophilic polymers along with gum Arabic or different proteins to encapsulate flavour oils. Furthermore, these polymers prevent aggregation of the oil droplets by the hydrophobic attraction during spray drying when they are exposed to each other (Ameri and Maa, 2006). To reduce the risk of fire in the spray drying chamber and to decrease the loss of the volatiles by the surface oil the amount of the volatiles should not exceed 50%. Reducing the load will reduce the loss of the volatiles according to Raoult's law, because the partial vapour pressure (*pi*) decreases and as well as depends on the mole fraction *xi* of component i; and *pi** which is the vapour pressure of the pure component i. as shown in the below equation (Jacobs, 2014a):

$$p_i = p_i^* x_i \qquad (2.3)$$

Moreover, particle size plays a great role in the retention rate. To improve the retention of volatiles during spray drying smaller particle size is preferred. Soottitantawat et al.,(2003) used high shear mixing then passed the mixture through a high- pressure homogeniser to form an emulsion with small size droplets which characterised by high retention rate. Jafari et al. (2008) used different emulsifiers and viscosity modifiers to improve flavour retention. Recently a mixture of biopolymers has been used and was found to enhance encapsulation efficiency (Jimenez et al., 2006; Pérez-Alonso et al., 2008).

Atomization

The first step in spray drying begins by decreasing the size of the droplets by passing the dispersion, solution or emulsion through a nozzle into spray under high pressure, where the particles are atomised, and the surface area to volume ratio is increased to

facilitate the evaporation process. In the next step the particles are exposed to hot air inside the chamber. Atomization can be obtained by other techniques such as spinning disc or an ultrasonic nozzle at the top of the chamber as shown in (Figure 2.16) (Jafari et al., 2008). The size of the droplets are different depending on the orifice size, surface tension and the flow rate of the feed into the nozzle. Moreover, the density of the feed and the pressure mainly control the flow rate of the liquid through the nozzle. If large particles are required low pressure and high density or viscous liquid can be used. The pressure can be controlled by using different orifice sizes (0.4-4mm), that mean for the same feed rate smaller droplets can be formed, by using smaller orifice size which increases the pressure. In the food sector, more than one pressure nozzle is used in large chambers to increase the production (200-1000kg/hour).

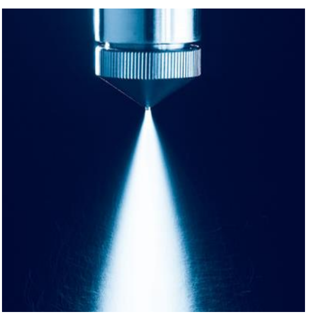


Figure 2.16: Types of atomisers (1) pressure nozzle (2) spinning disc (3) ultrasonic nozzle.

After the feed leaves the nozzle the droplet surface area reaches a high level of heat transferred between the droplet and its surrounding (Gibbs et al., 1999; Gharsallaoui et al., 2007; Stéphane and Frédéric, 2011).

Droplet – hot air contact

After atomization of the liquid, the tiny spherical particles travel by the hot air inside the chamber, where two kinds of current are used in the spray dryers. The first type is the co-current where the droplets and the hot air move in the same direction with the temperature ranging between 150-220 °C. This type of spray dryer is useful for drying heat-sensitive food products because the particles are exposed to moderate temperature around 50-80 °C (Gharsallaoui et al., 2007). As a result of rapid water evaporation from the capsules and formation of a hard crust around the particles, the temperature inside the particles remains lower (<100 ºC) than the chamber temperature (>150 ºC) (Jafari et al.,

2008). As the solvent evaporates from the atomised particles, a semipermeable hard crust forms on the surface of the droplets. This results in an increase in the concentration of large size core material inside the particles and allowing smaller molecules of the solvent to diffuse out of the particle (Zuidam and Heinrich, 2010). Essential oils contain different components with different boiling points. Consequently, the components with high boiling points retain in the core while some of the low boiling point components will be lost during the process of spray drying (Jafari et al., 2008).

The second type of hot air is the counter-current the hot air comes from opposite direction of the droplets flow, this method is not useful for heat-sensitive products as the processing temperature greater in this method which can lead to degradation of the encapsulated core (Gharsallaoui et al., 2007).

Evaporation of water

In this stage, the partial pressure of moisture and balances of temperatures between the gas in the chamber and the droplet governs the drying process. Firstly, the heat transfer into a droplet, due to the differences in the temperature, the droplets heats and reachs constant value called air drying humid thermometer temperature. While the water transfers to boundary layer as a result of the differences between droplets and outside vapour pressure. Secondly, the content reaches a critical value, at which a dry crust film is formed on the droplet surface and the diffusion continuous at lower rates until the temperature of the particles equilibrates with the air temperature (Gharsallaoui et al., 2007; Schuck, 2014).

Many factors affect the encapsulation efficacy by spray drying such as the feed temperature which is crucial because it controls the viscosity and flow of the feed. On the other hand, it must be taken into consideration the effect of high inlet temperature on the heat-sensitive compounds. Also, it leads to rapid water evaporation and cracks on the surface and releasing the content, while low inlet temperature leads to low evaporation rate resulting in dense droplets and agglomeration (Gharsallaoui et al., 2007)

Spray drying is widely used for encapsulation processes because of the flexibility of use, equipment availability, low cost of the processing, retaining of volatiles and prolonging the shelf-life of the dried product. The drawbacks of spray drying are as the following; firstly, the formed powder particles are very fine (<10 µm) which needs further

processing to make them more instantly soluble. Secondly, it may affect the heat sensitive compounds (Nedovic et al., 2011; Stéphane and Frédéric, 2011).

To obtain a semi-permeable film around the droplets, using a high-level solid feed (35-40%) is recommended, which is useful for retaining a high amount of volatile compounds. In contrary, increasing the solid level may result in the poor dissolving of the material and raising viscosity which prevents or decreases the film formation on the surfaces and may lead to the loss of volatile compounds. In order to retain high amounts of volatile compounds inside droplets during spray drying the emulsion of the lipophilic core should be prepared with small sized droplets and a high stable emulsion (Zuidam and Heinrich, 2010). The drying process will change the state of carrier material from liquid to glassy state; introducing low molecular weight carbohydrates at 10-35% will support this transformation. The temperature should be controlled in order to prevent exceeding the boiling point of water inside the droplets, which may lead to movement of water bubbles from inside to outside of the droplets that results in core loss.

Spray drying is useful for encapsulation of volatile compounds as retention of the core material is achieved because the volatile core material remains at lower temperature during drying (Zuidam and Shimoni, 2010). The phenomenon of selective diffusion, described by Thijssen in 1971, explains how water diffuses rapidly from droplet surfaces as it is heated by hot air resulting in drying the surface area, selective diffusion then plays a role and the diffusion coefficient of the core material is reduced to levels lower than that of water. However, with volatile compounds, the rate of core diffusion depends on the molecular diameter rather than molar volume. It has been observed that monoterpenes with the same molecular weight and solubility have different retention rates during spray drying, this is related to the various molecular structures of monoterpene compounds and their boiling points (Bertolini et al., 2001).

The powder formed by spray drying is dusty and contains tiny particles which decrease the flowability. To overcome this problem, new types of spray dryers are being designed to dry the feed in two steps. This approach leads to the formation of agglomerates from the particles which achieved by passing the fine particles through a fluid bed chamber at the bottom of the spray dryer to form agglomerates ranging between 100-300 µm with lower dust content and higher flowability (Jacobs, 2014a).

2.2.7.2 Freeze drying

This technique is used to encapsulate heat sensitive materials. The feed material in the form of an emulsion is exposed to freezing temperatures and then the vacuum is applied to remove water in the form of ice by sublimation. This approach minimises the probability of oxidation. It is not widely used because of high processing cost which is about 50 times than that of spray drying. The drying by this method takes long time. Consequently, this technique is usually used to encapsulate high-valued materials (Stéphane and Frédéric, 2011; Madene et al., 2006).

2.2.8 The principles of choosing applicable encapsulation technology

The process of encapsulation can be selected depending on many aspects such as the influence on food processing and shelf-life, size, payload, cost, morphology and the materials being used. Encapsulation processes can prepare small size particles of a few nanometers to a few millimetres. The final application determines the size requirement of the capsules together with uniformity, stability, mouthfeel and beverage suspension. In addition, the physiochemical properties of the core materials, the process conditions, the storage conditions of the capsules and the food applied to, the mechanisms of release and cost will determine the selection of proper encapsulation technique (Zuidam and Shimoni, 2010).

Different encapsulation processes produce different particle morphologies which also affect the rate and mechanism of the core material release, level of protection and the payload. In microcapsules, the core material which is near the surface follows the first order release mechanism. In contrast, the microspheres accommodate can have multiple cores, with each uniformly protected, the core material is released by a burst release mechanism. The core material is more protected by microspheres compared to microcapsules which have thinner wall material and higher loading.

Emulsion techniques produce submicron droplet whereas physical processes produce particles larger than 1μm (Oxley, 2014; Donsì et al., 2012). The feed of core material and the wall material must be prepared prior to spray drying by high pressure homogenisation (Jafari et al., 2008).

2.2.9 The encapsulation efficiency (EE %)

The success of the encapsulation process is determined by the amount of core material retained within the capsules and the presence of minimal surface oil on the wall materials after drying which is referred as the encapsulation efficiency (Jafari et al., 2008; Carneiro et al., 2013). In the drying chamber water molecules diffuse through a semi-permeable membrane on the surface of wall materials, but the larger molecules of the core material are retained in the capsules. The highest loss of volatile core materials happens at the initial stage of particle formation before the hard crust is formed around the capsules. Many researchers studied the retention of volatiles during spray drying by using the single droplet technique (Hassan and Mumford, 1996; Hassan et al., 1996; Reineccius, 2004). Judson (1995) and Liu et al. (2000) explained the loss of volatiles during droplet drying in different stages. Firstly, when the feed is atomised there is a high chance of volatile loss as the surface area of the emulsion sheets are exposed to the hot air in the drying chamber. Secondly, before the hard shell is formed around the droplets, there is a high water loss rate; that may take some of the volatiles from the droplets because the selective membrane has not had time to form. Finally, if the internal water of the droplets reaches the boiling point, there is a risk of bubble formation leading to rupture the hard membrane causing significant loss of volatiles. This final mechanism usually results in the most catastrophic form of volatile loss.

Jafari et al. (2008) have drawn a useful diagram showing different stages in encapsulation and the factors affecting the encapsulation efficiency as illustrated in (Figure 2.17) and described in the following sections.

The molecular weight (MW), polarity, interaction with wall materials and the degree of volatility of the core material have a great role in the retention rate of the core material (Madene et al., 2006). High molecular weight compounds are less diffusible through the wall material of the atomised droplet and the semi-permeable shell of the capsule; this results in higher retention and encapsulation efficiency. The retention of strawberry flavours in GA was investigated by Voilley (1995), this author found that the encapsulation efficiency of ethyl hexonate with high molecular weight (MW=144) was greater than that of ethyl butyrate (MW=116).

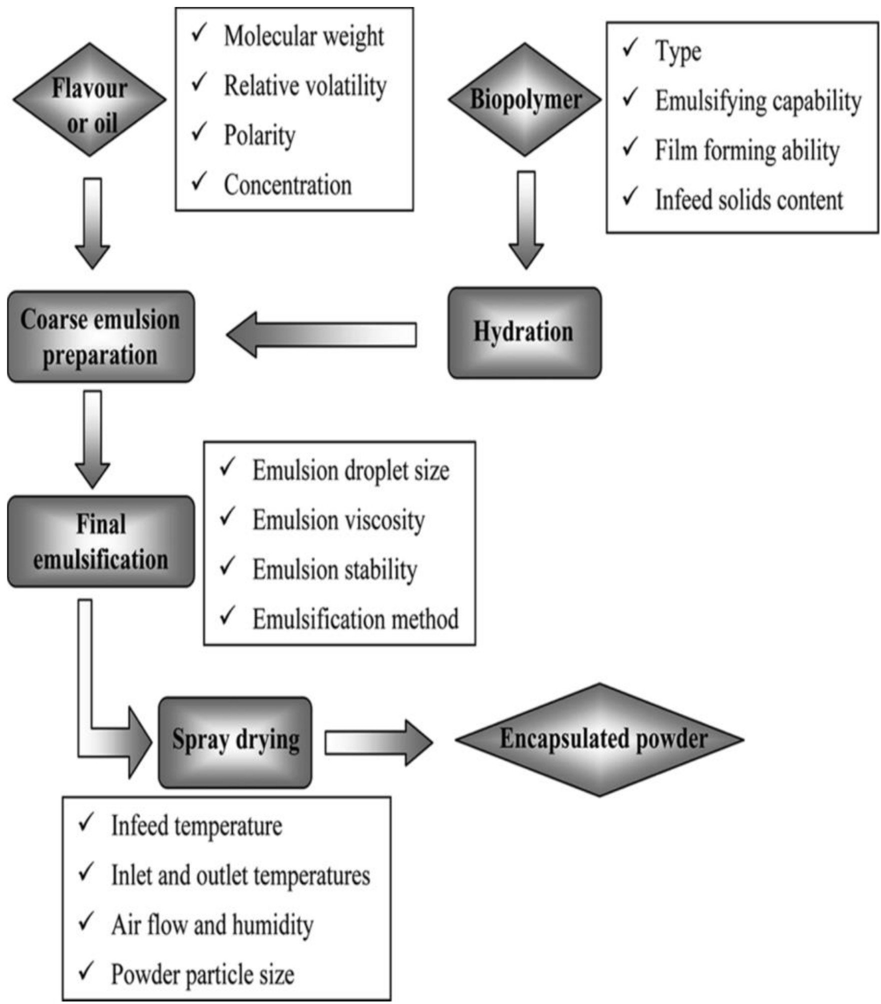


Figure 2.17: Flow diagram showing various stages in encapsulation process and the factors influencing the retention of the volatiles and oils (Jafari et al., 2008).

The effect of the flavour properties

The vapour pressure of the compounds in the core materials, which is known as relative volatility with respect to water, governs the retention of volatiles before the semi-permeable surface has formed (Rulkens and Thijssen, 1972). In a study on the spray dried flavour compounds octanol, octanone and octanal by Bangs and Reineccius (1982) in MD, casein, soy protein and WPI, it was found that lower relative volatility compounds had higher retention in capsules than those with high relative volatility. Coumans et al. (1994) explained the relative volatility α_{iw} as shown below.

$$\alpha_{iw} = \frac{Y_i/X_i}{Y_w/X_w} \qquad (2.4)$$

Where X is the molecular fraction in liquid and Y is the molecular fraction in gas, w is water and i is the volatile compound.

The polarity of the encapsulated compounds plays a great role in their level of retention in the capsules during the drying time as water diffuses through the semi-permeable membrane. Highly polar compounds are more soluble in water than nonpolar compounds. As a result, the loss of these compounds with water is higher than compared to nonpolar compounds (Ré, 1998; Rosenberg et al., 1990).

The concentration or load of the core materials also influences the retention of the encapsulated core material and encapsulation efficiency. It is preferable economically to use less wall material for encapsulation which provides high core retention. However, high core load commonly results in low retention and high surface oil and lower EE% (Hogan et al., 2001b; Rosenberg et al., 1990; Hogan et al., 2001a; Bertolini et al., 2001; Tan et al., 2005). Hogan et al. (2001a) found that the EE dropped from 89% to 18.8% when the ratio between soy oil to SC increased from 0.25 to 3. The ratio of 1:4 (core to wall material) is considered effective in the retention of the core material. For instance, in GA and modified starches, this ratio was found to be an optimal ratio (Madene et al., 2006; Desai and Park, 2005; Soottitantawat et al., 2005a). Moreover, Carmona et al. (2013) found that the retention of orange essential oils encapsulated by WPC and MD was strongly affected by the total solid concentration of the emulsion.

The effect of the feed

The properties of wall materials such as molecular weight, structure, chemical composition, and physical state influence the retention of the core materials and their release rates. High molecular weight wall material able to decrease the diffusion of the core materials as observed with maltodextrins. When amorphous material, in a glassy state are used as wall materials, the movement of the core is limited affecting the rate of diffusion when kept below the glass transition temperature (T_g). The molecular weight of core materials also affects the diffusion rate; low MW compounds have high mobility and diffuse faster than high MW compounds. The carriers which are made from proteins such as sodium caseinate, whey protein and soy protein showed better protection of core materials because of low permeability to oxygen compared to gum Arabic or modified starch (Zuidam and Heinrich, 2010). Orange oil retention was increased when sodium caseinate used as wall material (Edris and Bergnståhl, 2001, Hogan et al., 2001c, Kim et

al., 1996). They also noted that SC with MD are cheap materials which can be used as wall matrix.

The droplet size, stability, total solid and viscosity of the feed emulsion plays a significant role in determining the retention of volatiles. Increasing the dissolved total solid content improves the retention of volatiles by promoting the formation of the semi-permeable membrane, similarly increasing the viscosity of the wall material helps the formation of the hard crust in the spray dried particles in shorter time (Ré, 1998; McNamee et al., 2001; Rulkens and Thijssen, 1972; Coumans et al., 1994; Soottitantawat et al., 2005b; Minemoto et al., 2002). However, excess total solid may result in high loss and low retention for the following reasons: firstly adding more solid beyond the solubility limit reduces the encapsulated core and secondly it increases the viscosity of the emulsion. Total solid content should be optimised to allow effective encapsulation of the required core material (Rosenberg et al., 1990; Sankarikutty et al., 1988). In contrast, the retention of core material in some systems depends on the physiochemical properties of the core materials more than the on the amount of the total solid. For example in a study by Liu et al. (2001), they found that the dissolved total solid did not affect the retention of d-limonene (95%) while the type of flavour and the emulsion stability played a major role in flavour retention during spray drying. In contrast, the retention of ethyl propionate and ethyl butyrate improved with an increase of total solid concentration. The total solids of feed also govern the powder particle size; the higher the total solid, the larger the particles are, since these tend to dry very rapidly when compared with those of lower total solid feed (Reineccius, 2004).

The viscosity of the emulsion has a pronounced effect on the retention of volatiles as discussed above. The viscosity of the emulsion can be increased by adding thickeners such as alginate, gums, carboxyl methyl or cellulose. For example, when sodium alginate with MD was used by Silva and Re (1996) to encapsulate allyl guaiacol and by Rosenberg et al. (1990) to encapsulate ethyl caproate. They found that the retention of the flavour compounds is proportional to the viscosity increment, and the optimum viscosity was 105mPa in the first study and in the second study it was 125 to 250 mPa. Gelatine with GA optimised the retention of ethyl butyrate, by an effect which is explained by the improvement in crust formation (Liu et al., 2001). In contrast to previous works when xanthan gum is used no significant improvements were observed in flavour retention (Reineccius and Coulter, 1969). It is not practical to increase the viscosity of the initial

emulsion beyond the optimum rate because it affects the droplet formation and the loss of volatiles during atomisation; larger particles with irregular shapes get formed (Ré, 1998; Bhandari et al., 1992; Silva and Re, 1996).

The stability of the initial emulsion plays a role in retaining the flavour during spray drying; stable emulsions have higher encapsulation efficiency (Rosenberg and Sheu, 1996; Danviriyakul et al., 2002; Minemoto et al., 2002). Hogan et al. (2001a) and Hogan et al. (2001b) stated that the retention of soy oil encapsulated in SC decreased when the emulsion droplet size increased as a result of using low pressure during the homogenisation process. Liu et al. (2000) and Liu et al. (2001) also found a correlation between emulsion stability and retention of volatiles when using blends of GA and MD to encapsulate ethyl butyrate, ethyl propionate and d-limonene.

The size of emulsion droplets found to influence the retention of volatiles and encapsulation efficiency (Sheu and Rosenberg, 1995; Shiga et al., 2001; Soottitantawat et al., 2003; Re and Liu, 1996; Soottitantawat et al., 2005a; Liu et al., 2001; Minemoto et al., 2002). The stability provided by smaller droplet emulsions (< 1 µm) further improves the retention of the volatiles during spray drying and the final powder characteristics. The stability of linoleic acid against oxidation is increased by decreasing droplet size; this may be due to the reduction of surface oil on the powder particles (Minemoto et al., 2002). Same results also confirmed by Soottitantawat et al.(2001) and Liu et al. (1999) when they demonstrated that the retention of d-limonene was improved with finer emulsion droplets (< 2 µm) due to the stability of the smaller droplets during both atomisation and spray drying. They also found that the emulsion droplet size does not affect the size of the spray dried particles. There is a difference in retention rate depending on the solubility of the core materials, hydrophilic compounds such as ethyl butyrate and ethyl propionate showed lower retention even with small droplet size comparing to d-limonene which is hydrophobic. This phenomenon can be explained as smaller droplets have a larger surface area which may facilitate the diffusion of hydrophilic compounds through the wall material (Soottitantawat et al., 2003). The amount of surface oil is correlated with the emulsion droplet size, the larger the droplets, the greater the amount of surface oil on the powder particles (Sara and Gary, 1988; Danviriyakul et al., 2002; Soottitantawat et al., 2003). They stated that larger droplets loss more volatiles during atomisation because of shear effects. Larger particles loss volatiles as a result of droplet disruption during atomisation and evaporation of the volatiles during drying and/or insufficient

encapsulation of large oil droplets after breaking the large emulsion droplets. Thus, the surface oil is more prone to oxidation and off-flavour formation which shortens the shelf-life of the powder.

The spray dryer condition and encapsulation efficiency

The spray dryer conditions influence the retention of core materials. The variable factors include feed flow rate, inlet and outlet air temperature, the speed of the centrifugal atomiser or the size of the orifice and pressure in the nozzle, the air flow and the size of the formed powder particles.

The size of the powder particle is determined by the physical properties of the feed for instance the viscosity, the total solid content and by the condition of the spray dryer such as flow rate, temperature and the atomisation (Masters, 2002; Finney et al., 2002; Reineccius, 2004; Fang et al., 2006; Jafari et al., 2007). The size of the particles depend on the type of the atomiser as reported by Finney et al. (2002); they noted that spinning atomisers produce smaller particles than nozzle atomisers, and the effect of atomisers type is greater than that of temperature during spray drying on the size of the particles. However, the operational temperature influences the particle size, small particles can be produced with low inlet temperatures and when there are large differences between inlet and outlet temperatures. However, this led to slow drying and shrinkage of the particles as the particles take a longer time to form and to stabilise their structure. The influence of the atomised particle size on encapsulation efficiency is clearly understood. Some researchers such as Blackebrough and Morgan (1973) and Fang et al. (2006) have stated that the lower surface oil and higher encapsulation efficiency are achieved when large particles are produced. In contrast, (Reineccius and Coulter, 1969) and Finney et al. (2002) reported that the encapsulation efficiency cannot be affected by the size of the atomised particles but rather by the solid content of the feed. Additionally, (Soottitantawat et al., 2005a) stated that the encapsulation efficiency is more affected by the emulsion size and they also noted that the particle size does not have a major influence on retention of flavour. The retention was low for the large atomised particles compared to the intermediate particles, as shown by (Chang et al., 1988) when encapsulating orange oil in modified starch.

Table 2.3 shows that different particle sizes were obtained by changing the rotational speed of the atomiser. The respective core retention levels were decreased, and

corresponding surface oil levels increased. The increase in surface oil in the medium and large size particles was in contrast to the finding of (Finney et al., 2002), as the surface area of large particles in relation to the volume are smaller than with small particles which results in surface oil reduction. Soottitantawat and co-workers explained that the rise of surface oil in medium and large particles were due to the longer time required by large particles to form a film and dry. As a result, there was a greater loss of the core materials during spray drying.

Table 2.3: The relation between particle size, retention rate and surface oil.

Atomised particle size (µm)	Retention rate %	Surface oil %
42.5	15.2	0.67
53.2	15.9	1.68
66.6	12.8	7.10

Another explanation may be that behaviour is related to the particle morphology, as the large particles require a longer time to dry, the particles may damage (shrink or defect) which increase the particle's surface area and increases the loss of the flavours and surface oil. Although large particles are at a disadvantage, regarding flavour retention, they are often preferred as they are easier to disperse in water; small particles tend to form lumps which are non-dispersible in cold water (Buffo et al., 2002; Turchiuli et al., 2005). Large particles are produced to enhance the dispersity, wettability, flowability, and solubility of the powder alternatively fluidised bed agglomeration techniques are used to attach small particles together.

Atomisation is the first stage in spray drying, the loss of volatiles occurs directly when a thin sheet of the emulsion is form with high surface area prior to breaking into droplets which are then sprayed into the drying chamber together with turbulent air. The retention of volatiles is related to the pressure in the nozzle and the speed of the centrifugal atomiser. High pressure leads to a breakup of the emulsion sheets rapidly to droplets, the

longer this process takes, the greater the loss of the volatiles occurs due to the evaporation of solvent from the atomised sheets.

In a study by (Judson. K., 1995) when the pressure in nozzle increased from 1.83 to 3.55 and 7 MPa the retention of propyl acetate increased respectively by 31, 35, and 45 % by producing smaller droplets which tend to form thin films very quickly due to their high surface area. He also stated that the droplets dried faster when their contact with hot air increased, this can be achieved by using a wide spray angle. Volatile retention can be improved when a centrifugal unit wheel is used by increasing the speed of the rotation. In contrast, Finney et al. (2002) reported that the retention of orange oil did not change with a change in temperature and atomiser types.

Feed temperature is another factor affecting the encapsulation efficiency of the volatiles. The investigation of feed temperature is found in several studies, for example, Rulkens and Thijssen (1972) used different spray drying condition to examine their effects on volatile retention; they used methanol, n-pentanol and n-propanol in maltodextrin. They stated that volatile losses occur principally from the point when the emulsion leaves the nozzle until the droplet is formed. High feed temperature decreases the viscosity of the feed. As a result, the time duration of the liquid in the liquid cone is reduced, reducing volatile losses and encouraging the rapid formation of droplets. High feed temperature also increases the solubility of solids allowing higher loading. (Sivetz and Foote, 1963) commented in their book on coffee processing, they identified that low feed temperature resulted in high retention of aroma compounds; this was attributed to an increase in the viscosity of the feed. High viscous feed increases the droplet size and limits circulation inside the droplets while improving the retention of the volatiles.

Retention of volatiles depends on the contact between hot air and atomised droplets. The mass transfer of the liquid inside the droplets can be improved by increasing the droplet contact with hot air and rate of heat transfer from the droplets (Coumans et al., 1994). The humidity of the inlet air also affects the rate of drying, rapid drying of the droplets can be achieved when dryer inlet air is used (Reineccius, 2004).

The inlet air temperature also governs the encapsulation efficiency; several researchers studied this relation (Shiga et al., 2004; Lee et al., 2005; Bangs, 1985; Rosenberg et al., 1990; Bhandari et al., 1992; Liu et al., 2000). They found that the optimal retention of flavours could be obtained with temperatures between 160-220 ^{o}C,

due to the rapid formation of a crust membrane on the droplet surfaces. On the other hand, the particles may be damaged if higher temperatures are used due to internal liquid boiling and bubble formation which can lead to a breakdown of the droplet membrane causing rapid evaporation of volatiles. This phenomenon is referred to as ballooning it is affected by both the design of spray dryer and types of wall materials (Drusch et al., 2006). In contrast, Bhandari et al. (1992) did not observe any ballooning when they used high temperature of 400 ^{o}C in Leaflash spray dryer to encapsulate a mixture of linalyl acetate and citral in maltodextrin and gum Arabic; they were able to retain 84% of the flavour compounds in the powder. Similarly, Shiga et al. (2004) also stated that the retention of shiitake flavour is increased with increasing air inlet temperature and solid content. In contrast to the previous findings, Anker and Reineccius (1988) and Finney et al. (2002) reported that the air temperature had no effect on the retention of orange oil and diacetyl but that higher temperature decreased the surface oil level. Finney et al. (2002) stated that the surface oil level is increased when higher inlet temperatures are used. Nevertheless, Bhandari et al. (1992) indicated that the surface oil decreased when higher temperatures were used due to the rapid formation of the hard crust around the droplets preventing volatiles from draining out of the surface. The conclusion must be that the effect of high inlet temperature on volatile retention is different according to the types of the volatiles. The retention of ethyl caproate increased to some extent when the temperature increased from 40 to 100 ^{o}C while the d-limonene retention did not change with temperature (Liu et al., 2000). It was also related it to the high stability of the emulsions of d-limonene and ethyl caproate during spray drying comparing to the emulsions of ethyl butyrate or ethyl propionate. When the temperature is increased to 115 ^{o}C, the droplets tend to break and the loss of these compounds increases. Rosenberg et al. (1990) evidenced that ethyl caproate retention increased at high inlet temperature and high concentration of solids. However, when high inlet temperature used the crust around the droplets forms rapidly which prevents the volatiles from evaporating increasing encapsulation efficiency.

2.2.10 Controlled release of active compounds

The encapsulated ingredients remain inside the capsules until a trigger is applied to release the active materials at the required time and location. Understanding the release mechanism of food ingredients from the capsules is crucial when establishing any release

model (Sobel et al., 2014b; Garti and McClements, 2012; Pothakamury and Barbosa-Cánovas, 1995).

In some applications, it is advantageous for the encapsulated components to be discharged rapidly and within a short time, this is referred to as “Burst release”. In other applications, the encapsulated materials needed to release at controlled and more controlled rate, this is referred to as “Sustained release”. While when the components are released as a result of changes in the surrounding medium it is called “Triggered release” for example dissolution of powder by water, saliva or solvent, enzymes, changing pH, heating or cooling, breaking the capsules by shears or pressure by chewing in the mouth. Starch is often considered to be an ideal wall material as the amylase in the saliva able to break the wall easily. When components are released at a specific location, this is called “targeted release” for example by releasing in a specific part of the gastrointestinal tract by using specific enzymes and pH. Proteins and carbohydrates are used as encapsulation materials when the release trigger is water as they are water soluble materials. In the case of coffee, chocolate drinks, tea, soup, cocoa drinks, heat or thermal trigger is used to release the active ingredients from capsules conatining wax, lipid or fats as wall materials. (Jan, 2009; Gibbs et al., 1999; Sobel et al., 2014b; Reineccius, 1995).

Before performing encapsulation of active ingredients, the developer should take into account the interactions of the active materials with other materials in the system, cooling and heating, aeration, moisture addition, shear forces which might be used during food preparation process. It is also important to consider stability during storage and handling as it is important that when used by the consumer the active ingredients are delivered as required (Sobel et al., 2014b).

The mechanisms of controlled release

There are many mechanisms which can be utilised to release the active components within the food or beverage, diffusion, dissolution, erosion, fragmentation and swelling are examples of these mechanisms. In the diffusion, the core materials diffuse through the wall material. The chemical potential difference between the encapsulated materials inside and outside the capsules is the major driving force behind the diffusion; an example is differences in vapour pressure particularly for volatile compounds (Gibbs et al., 1999; Sara, 1995; Stéphane and Frédéric, 2011; Reineccius, 1995). In addition, the polarity,

molecular weight of the core material and wall material properties, the characteristics of the particles, and the differences in concentration gradient (McClements, 2014).

The second mechanism is the dissolution of the particle when added to aqueous food or particular solvent. When the capsules dissolve, the dispersed active material is released into the medium. The dissolution rate directly depends on the degree of matrix dissolution. Thirdly, the erosion of the wall material by strong acid or base (chemical) or high temperature (physical) or using enzymes such as amylase, lipases or protease to degrade the wall material can be exploited to release the encapsulated active ingredients. Another mechanism of release is the disruption of the capsules by chemical, physical and enzymes and fragmenting of the capsules. The release from fractures occurs by all mechanisms mentioned earlier but at a higher rate due to the small size of the fractures. Finally, in some delivery systems such as hydrogel particles the release of the active ingredient depends on the swelling by absorbing solvent from the surrounding and increase the pore size which let the small molecules release which passes to the outside of the particles (McClements, 2014). There are many steps in the release of the active ingredients including wetting of the surface, hydration followed by swelling of the wall material, fragmentation, erosion of the wall and moving the active ingredients through the wall towards the lower concentration gradient outside of the capsules.

The release rate depends on the steps presented above and the rate of release which may change over time. The release kinetics is defined by dissolution of the matrix and solubilisation of the active ingredients. The system can be designed to determine the time and location of release. For instance, the active ingredients remain for a longer time in the system when the matrix is eroded slowly. This approach is beneficial when the slow release is required, for example releasing enzymes in cheese during the ripening process. Whereas, when a rapid release is required the wall materials must be very erodible, for example when releasing yeasts in dough. The active ingredients physiochemical properties, capsules morphology, and type of wall materials are all important in determining the rate of release.

Chapter 3 Chemical and Physical Characterisation of Thyme Essential Oils

3.1 Abstract

Thyme essential oils has been used for many years in folk medicine and as a food flavouring agent. They contain different compounds mainly comprised of terpenes. The composition of the extracted oil depends on many factors such as extraction method, plant origin, drying method, season and type. Therefore, it is difficult to compare the physiochemical and bioactivity of thyme essential oils published in the literature. The presence and fraction of each terpene determine the general properties of the oil regarding solubility, surface tension, partitioning and stability of the delivery system. The objective of this chapter was to identify the chemical composition and determine the physical characterisation of thyme EOs used in this study. The chemical analysis carried out using gas chromatography-mass spectrometry (GC-MS) to identify and quantify different compounds. The physical properties such as solubility of the monoterpenoids, octanol/water partitioning, surface activity and the self-assembly were also measured. Results from the chemical analysis showed that there were 29 compounds in thyme EOs, 11 of which are oxygenated, and the rest are non-oxygenated. The results of this study revealed that thymol was the main compound, constituting about 37 % of the total compounds in thyme EOs. Moreover, the results of physical experiments revealed that thyme essential oils were able to produce a short term stable emulsion when homogenised with water without adding any surfactants probably due to the presence of many amphiphilic compounds in the thyme EOs. It was also shown that monoterpenoids such as thymol, carvacrol, terpinene-4-ol, camphor, α-terpineol and thyme EOs had a solubility of 0.96g/L, 1.27, 2.6, 1.5, 1.80 and 0.95 ml/L, respectively in water. And the octanol/water partitioning coefficient results showed that camphor was the most hydrophilic and γ- terpinene the least hydrophilic compound among eight terpenes used in the experiment. Further, the monoterpenoids were able to reduce the surface tension of deionised water and reached the critical micelle concentration below their solubility limit.

3.2 Introduction to thyme essential oils

There are different species of *Thymus* such as common or garden Thyme *T. Vulgaris* L., Spanish thyme *T. zygis* L., wild thyme *T. serpyllum* large wild thyme *T. pulegioides* L., which are all belongs to the Lamiaceae family. The name comes from the Greek word thymos which means strength or courage. They are grown in the Mediterranean area, North Africa, Asia and South Europe. In the past, thyme was used as a treatment of many

diseases and in food as a seasoning agent. Moreover, thyme gets a mention in sixteenth century books on herbal remedies such as Pear Matthiolo (1505-1577) and Leonhart Fuchs (1501-1566), which described the medicinal curative effect of thyme herbs on cough (Stahl-Biskup, 2004). Nowadays thyme is used in foods for flavour enhancement and preservation, in cosmetics, and in the aroma industry.

The EOs in thyme contains two groups of secondary products, volatiles and non-volatiles components. They are liquid, aromatic and have a pleasant smell (Grigore et al., 2010; Chang et al., 2012). EOs are usually stored in glandular trichomes on thyme leaves. The typical spicy aroma of thyme emits from the EOs constituents. The Essential oils content in dry thyme plant ranges between 1-2.5%. A variety of chemical constituents are present in the EOs of thyme as shown in (Figure 3.1).

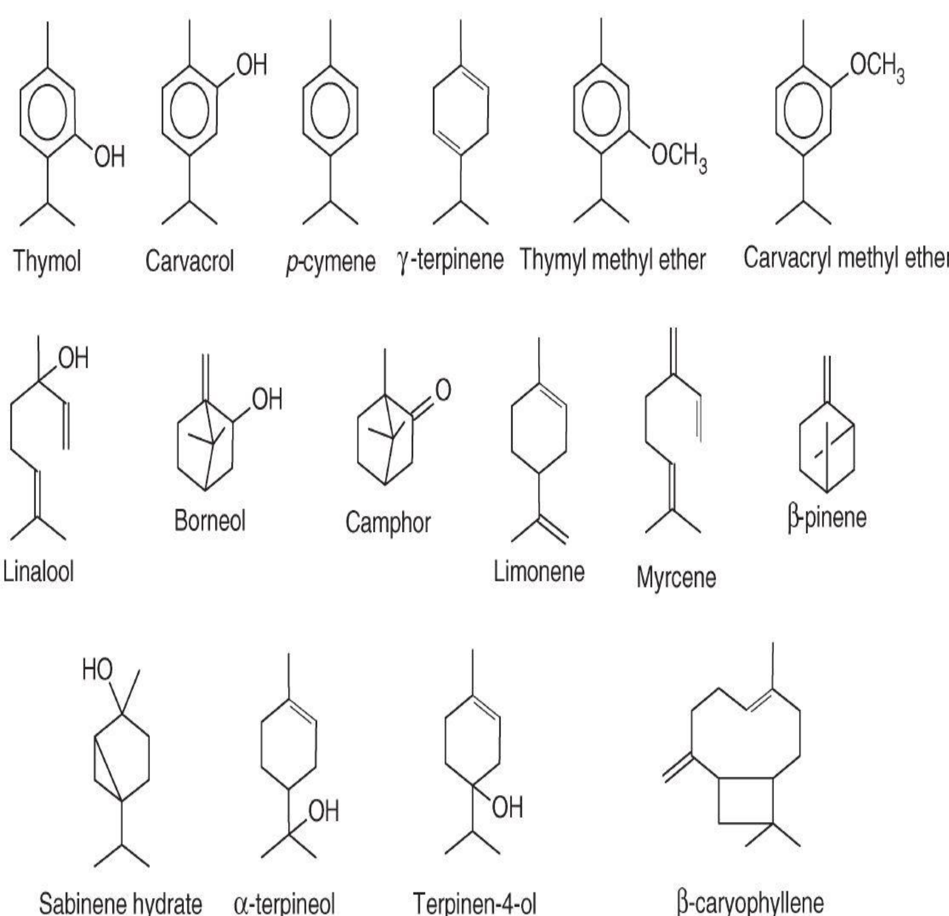


Figure 3.1: The chemical composition of thyme essential oils (Stahl-Biskup, 2004).

The chemical profile of thyme essential oils vary, which influences the physiochemical properties of the oil. Regarding the composition of thyme essential oils there is limitation of obtaining the same compounds in all batches due to the geographical origin, species, growth stage, stress and watering. Therefore, it is crucial to analyse the oil to identify the compounds present in it before investigating properties such as encapsulation or antimicrobial effects. The profile composition of thyme EOs constituents determines their interactions and activity. For instance, the phenolic compounds thymol and carvacrol in thyme EOs are the main components responsible for the antibacterial activity.

Different physical properties of the individual components in thyme EOs such as solubility, surface activity, self-association and partitioning in octanol/water solution should be measured as they are the main factors determining the stability of the delivery system containing them. For instance, the partitioning of these compounds in bacteria cell wall is important when used as an antibacterial agent. The amphiphilic compounds such as thymol, camphor, carvacrol and terpinene-4-ol may extend the stability of the colloidal particles by reducing the surface tension of the system resulting in the decrease in the free energy of the emulsion system which is thermodynamically favourable.

To the best of our knowledge, there is little information on the physical characterisation of thyme essential oils and its components. Therefore, the objectives of this chapter were twofold: (1) to identify and quantify various components present in thyme EOs using GC-MS. (2) To investigate the physical properties of thyme EOs and main compounds in EOs to understand their interactions and roles in the stability of delivery system containing these compounds.

3.3 Materials and Methods

3.3.1 Materials

White thyme essential oils and standards including borneol 97%, p-cymene 99%, camphor 98%, linalool 97%, γ-terpinene 97%, α-terpinene 85%, and carvacrol 98% were obtained from Sigma-Aldrich Co Ltd. Other standards such as terpinen-4-ol 97%, α-terpineol 96%, geraniol 97% and thymol 98+% were obtained from Fisher Scientific Ltd. (Acros Organics). SPME fibre (PDMS/DVB) obtained from (Supelco, Bellefonte, USA).

3.3.2 Methods

3.3.2.1 The chemical analysis of volatile compounds of thyme essential oils by head space (HS)-solid phase micro extraction (SPME)-gas chromatography mass spectrometry (GC-MS).

HS-SPME-GC-MS is an excellent method to analyse essential oils because it only determines the volatiles that are extracted from the head space of the sample vial by SPME. Therefore, the sample preparation is simple and the identification of the volatiles is easier. Moreover, it can be used to extract analytes from both liquid and solid samples.

Thyme essential oil and all standards were analysed by Gas Chromatography-Mass Spectrometry (GC-MS) to qualify and quantify the different components in the EOs. Physical characterisation of the EOs and some standards also carried by measuring the solubility of the monoterpenoids, surface activity measurements, partitioning in octanol-water and modelling the self-association of thyme EOs amphiphilic compounds in water.

Analysing of EOs by GC-MS requires a capillary column. The properties of the stationary phase, length of the column, internal diameter and thickness of the stationary phase are very important when choosing these types of columns. The ideal parameters for columns to be used in GC analysis of EOs are 25-50m length, 0.20-0.32 mm internal diameter of column and 0.25 μm the thickness of the stationary film. The separation of the EOs components occurs according to their boiling point in nonpolar stationary phase. While in polar materials the polarity of the components plays a key role in resolving the components in the stationary phase. However, the components in EOs have a narrow range of boiling points this may result in elution of different compounds in narrow retention time when a nonpolar column is used. Using slower temperature rate may

overcome this problem and led to wider elution and retention time. Using a polar column is another option as oxygenated compounds elute after hydrocarbon compounds elution (Barbarad et al., 2015).

Solid phase microextraction (SPME)

The solid-phase microextraction (SPME) is one of the most used techniques for extracting aromatic compounds in herbs, plants, fragrance and spices. The SPME is considered as a more useful technique for volatile analysis than other techniques due to the ease of sample preparation, low cost, rapid detection, in addition, to its ability to identify aromatic compounds in either solid or liquid samples that are not possible to inject them directly into a gas chromatography (da Rochaa et al., 2014). . Thus it is preferable to separate the volatile compounds into headspace prior to analysis by gas chromatography (Karl-Heinz, 2015). In this technique SPME syringe was used with a coated fibre on the fused silica mounted onto GC syringe as shown in Figure 3.2.

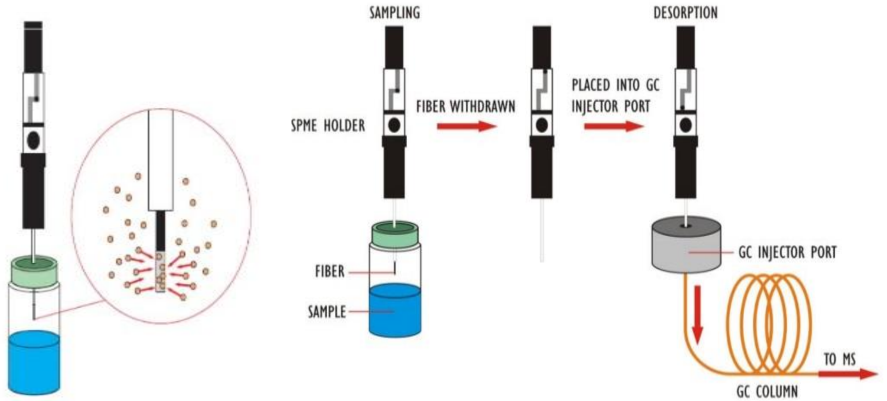


Figure 3.2: Diagram shown principles and different steps in SPME (Schmidt and Podmore, 2015).

The principle of volatile extraction in SPME depends on the partition coefficient of the analytes in the fibre and the volume of stationary phase. The adsorption of the analytes onto the coated fibre occurs depending on many factors as shown in Equation 3.5.

$$n = \frac{K_{fs}V_fV_sC_0}{K_{fs}V_f+V_s} \qquad (3.1)$$

Where n is the amount of the analytes extracted by the coating material, K_{fs} is the partitioning coefficient between sample and coating materials, V_f is the volume of coating material on the fibre, V_s is the volume of the sample and the C_0 is the initial concentration of the analytes in the sample. The equation clearly shows a direct relationship between the amount of extracted analytes and their concentration in the sample.

The polymers are used as a coating material, such as liquid polydimethylsiloxane (PDMS) and Carboxen-CAR/PDMS which are made from a mixture of solid and liquid polymers. The properties of the compounds to be analysed are important in choosing an appropriate fibre. The fibres are coated with certain polymer and thickness depending on the different properties of the analysed compounds. Commercially, PDMS (7, 30 and 100µm), polyacrylate (85 µm) and a variety of mixed polymers are available (Karl-Heinz, 2015).

The conditioning of SPME fibre

Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) (65 µm) fibre which is recommended by Supelco and (Rochaa et al, 2014) for volatiles and nitro-aromatic and amines of different molecular weights (50-300 Da). Moreover, da Rochaa et al. (2014) found that among different fibres PDMS-DVB (65 µm) was the most efficient fibre in extracting the thyme EOs constitutes therefore, it was selected to be used to analyse the thyme EOs and its components in this study. The fibre was first conditioned by injecting the fibre into the GC-MS injector at 200 ºC for 30 min according to the instructions provided by the supplier.

Gas Chromatography – Mass Spectrometry (GC-MS)

GS-MS was used to analyse thyme essential oils, all the standards and the recovered EOs from encapsulated samples, surface oil, available thymol, release rate, and log K_{ow} experiments were analysed using the same GC-MS condition. A headspace technique was used to analyse thyme EOs and the standard components. The method optimised by assessing different times of extraction, agitation temperature and using different solvents, temperature programming and analysis time.

Sample extraction and adsorption

An aliquot sample containing 1 mL of sample and 14 mL of methanol: water (50:50) solution were added into magnetic crimp vial with geraniol, (0.025 mg/mL) as internal

standard. The COMBI PAL autosampler was used to deliver the glass vials with samples into agitator at 250 rpm and heated to 50 ºC for 5 min to facilitate the equilibration and the extraction of the EOs components. Then the SPME fibre on the auto-sampler exposed to the headspace of the vial in the agitator to extract and adsorb the compounds onto the fibre for further 10 min.

***Sample* injection**

After sample had been extracted the fibre was withdrawn from the head space into the housing and transferred to the injector valve of the GC-MS system which was connected to the capillary column. The complete desorption of the volatiles from the fibre was occurred at 250 ºC for 15 min in the GC-MS injector.

GS-MS analysis of thyme essential oils

The analysis of essential oil composition of different samples was performed using VARIAN (CP-3800 Gas chromatography) and (Saturn 2200 Mass Spectrometry) equipped with FID detector. The GC-MS technique has numbers of benefits including sensitivity, low cost, a small sample required, rapid and ease of use. Low-polar Phenomenex (Zebron) capillary column (30 m length X 0.25 mm internal diameter X 0.25 µm film thickness) coated with the stationary phase of 5% Phenyl 95% dimethylpolysiloxane was used to separate the different compounds of the EOs. Helium was used as carrier gas at the flow of 1 mL/min and the pressure maintained at 0.1 psi during the analysis. The injector temperatures was 250 ºC. The oven temperature gradients was set from 40 ºC to 250 ºC. The temperature was held for 4 min at 40 ºC then raised to 150 ºC by increasing rate of 10 ºC / min. After holding for 4 min at 150 ºC the temperature then increased by another 10 ºC / min increment and held for another 4 min at 250 ºC. The identification of different components of the EOs was based on the comparison of retention times of the unknowns with the retention times of the reference standards, and those available from the mass spectrum reference library of National Institute of Standards and Technology (NIST 2008) and from literature. Five concentrations of (0.0625, 0.125, 0.25, 0.5 and 1 mg/mL) thyme EOs were used, the average and standard deviations were calculated for each component. The percentage of each compound in the thyme essential oils was measured based on the total peak areas under the chromatogram of thyme EOs.

Quantification of the thyme EOs components

In order to quantify various components of thyme EOs in different samples, standard curves were generated for all available standards. The concertation used to generate the standard curves ranged between (0-1 mg/mL). The standard solution were run through the GC-MS, the peak areas of each standards diveded by the internal standard peak area and plotted against their concentration to generate the calibration curve and straight line equation . The R^2 for all standards were above than 0.99. The quantification of the unknown concentration was carried out using the linear equation obtained for each standard curve as shown in (Figure 3.3) for thymol as an example.

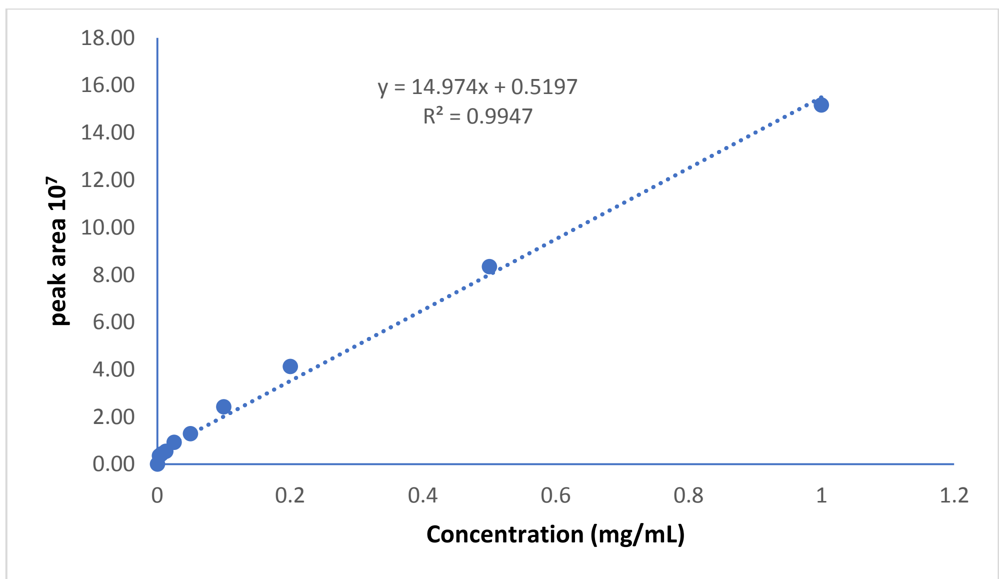


Figure 3.3: Thymol calibration curve.

3.3.2.2 Physical characterization of thyme essential oils

Studying the physical properties of thyme essential oils is important to understand the different interactions with surrounding when used as core materials in oil-in-water emulsions. Solubility, surface activity, partition coefficient, density, and diffusivity can affect the encapsulation efficiency, release rate and activity of the EOs.

Density

The density of essential oils is important as it is the key factor in gravitational separation in emulsions. An Anton Paar digital density meter (Graz, Austria) was used to

measure the density of thyme EOs and different emulsions. The instrument contains a hollow oscillating U-tube which can be filled with any liquids to determine the density. The oscillation of the tube by the filler materials generates different frequencies which can be measured electronically. The principle of this method is the "Mass-Spring" model, where the frequency of the container is dependent on the mass of the samples. To determine a sample density the U-tube was cleaned with deionised water for several times then the density of pure water determined at 25 ºC. Subsequntly, the densities of each sample was determined 3-4 times at the same temperature.

Solubility of thyme oil and its components in water

Thyme essential oil and the oxygenated compounds were tested for their volume fractions in deionised water. For thyme EOs 10 mL of oil mixed with 990 mL of deionised water in a 1L standard flask. The content was then transferred into a separating funnel, and the mixture was shaken vigorously for 30 min to guarantee mixing of the two phases. The mixture was left overnight in separation funnel to settle. On the following day, 100 mL of water phase transferred into Clevenger as described in Section 4.3.2.4.6 and the recovered thyme EOs volume measured in the graduated tube and recorded as fractionated oil in water (Rao, 2005).

The terpenoids are slightly soluble in the water phase (0.05-1%) due to their polarity (Fleisher and Fleisher, 1991). Some of the terpenoids such as (thymol, carvacrol, α-terpineol, terpinen-4-ol, geraniol, and p-cymene) were also were tested for their fractioning in deionised water. A set of the experiments were conducted to measure the solubility of oxygenated compounds in water. Hydrophobic Nile red dye was mixed with individual terpenes in order to follow the oil droplets when they were dispersed in water using a micropipette. Beakers containing 100 ml deionised water were placed in a water bath at 20 ºC with continuous stirring, after temperature equilibration individual terpenoids standards were added to the water dropwise using a micropipette. The mixtures were continuously stirred to allow the droplets to distribute and disappear inside the water phase. Adding components continued until the first undissolved droplets observed which indicated the end of solubility of the components is reached. The concentrations of dissolved chemicals were recorded as the highest amount to be dissolved in water.

Octanol-water partitioning of the EOs monoterpenoids

The partitioning of the EOs components in biological membranes is the key point when they are used as an antibacterial agent. This is determined by the partition coefficient ($K_{o/w}$) of these compounds between the octanol and water phases. The partition coefficient is widely used to evaluate the partitioning of medicine, pesticides and other chemicals.

Ten milligrams of solid standards and 10 µL of the liquid standards of thyme EOs (p-cymene, carvacrol, thymol, camphor, borneol, terpinene-4-ol, α-terpinene and γ-terpinene) were added to 5 mL octanol. The solution was then mixed with 5 mL water the mixture stirred on a magnetic stirrer for 30 min and left overnight for equilibration at 25 ºC. On the following day, samples were centrifuged at 5000 rpm for 10 min to separate the phases. The upper layer of octanol was withdrawn from all tubes using syringe with removable needle, and 1 mL from each layer was added separately into two different GC-MS vials containing 14 mL methanol: water (50:50) solution and for each vial geraniol was added as internal standard. The $log\ K_{o/w}$ of the various standards in the both phases was calculated depending on the peak area of the compounds in each phase obtained using the GC-MS (OECD, 1995).

$$K_{ow} = A_o/A_w \qquad (3.2)$$

Where A_o and A_w are the peak areas of the chemicals in octanol and water respectively.

The determination of the surface activity of thyme EOs and some monoterpenoids

Surface tension is produced by attracting the molecules of liquid material to each other that generate cohesive force at the interface which can be determined by different methods of surface tension. The stronger cohesion forces between the molecules at surface forms a film on the surface due to an imbalance force of the neighbouring atoms on all directions (Figure 3.4).

The overall properties of food emulsions are dependant on the interfacial tension, the presence of surface active materials at the interface strongly affects the emulsion properties such as free energy, droplet size, structure, and rheology.

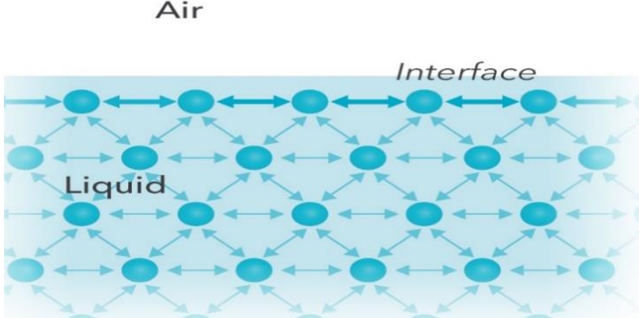


Figure 3.4: Surface tension in liquids (Biolin, 2015).

These properties can be varied according to the type and concentrations of the surface active materials in the system. On the other hand, the quantity and the type of the colloidal forces between the droplets in an emulsion are affect by the structure and thickness of the interfacial layer. Some of the EOs components poses surface activity to some extent. In this study, the oxygenated compounds were tested for their surface activity as this could affect their diffusion during encapsulation and release, when they were added to aqueous solutions. Wilhelmy plate method (Figure 3.5) was used for measuring the surface tension as described in Equation 3.3:

$$\sigma = \frac{F}{L.cos\theta} \qquad (3.3)$$

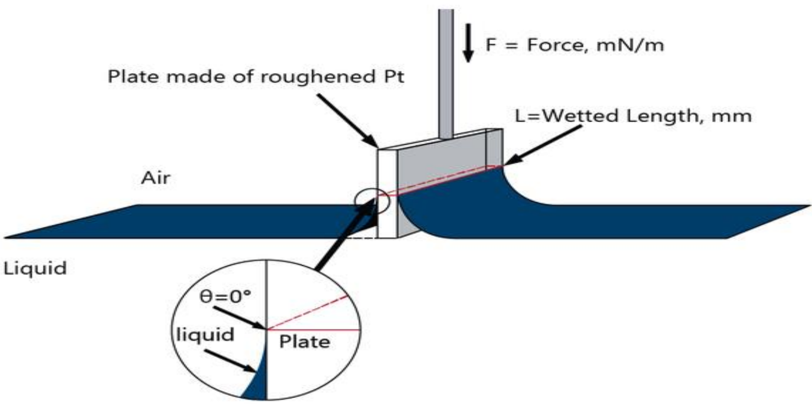


Figure 3.5: Wilhelmy plate method (Krüss, 2016).

In this method, a platinum plate is used to measure the surface tension of different solutions. The platinum plate is easily cleaned and inert chemically. The contact angle θ

is zero (cos θ=1) which means optimally wetted by the liquid. The unit used in surface or interfacial tension measurements is the force in Newton (N) per the length of the interface (m^{-1}), or it can be expressed as energy in Joule (J) per interfacial area(m^{-2}) (McClements, 2004a).

A Krüss (K 10 ST) tensiometer was used for surface and interfacial tension measurements. The determination of surface tension or interfacial tension of every sample was started by cleaning the plate by heating up it above bunsen burner for 10-15 seconds, continuously turning until it glowed. Stock solutions of the standard terpenoids and thyme oil were prepared by dissolving the upper solubility limit of each component according to the results obtained from solubility experiment and series of dilutions were prepared which used individually to measure the surface tension. The glass vessel was also thoroughly cleaned and rinsed with deionised water. The tensiometer was adjusted to 0.00 mN/m after dangling the plate, using the adjustment knob. The sample vessel was raised until the plate dipped into the surface of the sample. Then the sample vessel was lowered slowly, and the servomotors was automatically started and pulled the plate out from the liquid until the lower edge of the plate tangent to the liquid surface. Later, the motor stopped at the end of the measurement, and the surface tension values were recorded from the digital display on the tensiometer and also in the computer.

For interfacial tension measurement, the ring was used. The vessel was filled with the essential oil phase and the ring was dipped into it. Then the tensiometer was adjusted to zero, then the ring and the vessel were removed. The cleaned vessel was filled with water (heavy phase) and a cleaned ring was dipped into it. The thyme essential oil was gently overlaid onto it using a glass spatula. The sample vessel was lowered until the servomotors started and the interfacial tension value was recorded from the digital display on the tensiometer. The provided table for ring measurement and equation (3.4) of correction factors was used for obtaining the correct interfacial value.

$$F = 0.725 + \sqrt{0.4036x10^{-3}x\frac{\sigma}{(D-d)} + 0.0128} \qquad (3.4)$$

Where F is the correction factor, σ is the measured interfacial tension in mN/m, and D, d are the densities of both phases in g/cm$^{3.}$ The measured interfacial tension then was divided by the correction factor to get the actual value.

The self-assembly of thyme EOs in water

To evaluate the self-emulsifying properties of thyme EOs, an emulsion of thyme oil 10 % w/w in water was prepared without adding surfactants. Thyme oil was added to water dropwise with stirring, the mixture was passed three times through a high pressure jet homogeniser. The samples were scanned in Acoustiscan instrument cells at 25 ± 0.4 ºC for 60 days. The speed of sound is measured while it passes through different parts of the materials, the sound is attenuated because of the particles are present inside the medium, and then the velocity is expressed as follow:

$$v = \frac{d}{t} = \lambda f \tag{3.5}$$

Where d is the distance or the thickness of the medium, t is the time of the travelled wave through specific distance, λ is the wavelength and f is the frequency.

The first transducer produces acoustic wave and the second transducer detects the wave after passing through the emulsion (Figure 3.6). The received signals then converted in to electrical signals by the oscilloscopes. These signals are then transferred to the computer software for the data analysis.

Low power of ultrasonic is used to pass across materials (food, emulsions), that has no destructive effect on the food composition. While high power ultrasonic is used in cleaning, homogenization and welding processes (McClements and John, 2001).

The concentration of dispersed materials can be measured by using ultrasonic techniques. As the velocity of the sound decreases and the attenuation increases, due to the influence of diffused particles in the continuous phase. Ultrasound has been used to measure the volume fraction of vegetable oil by McClements and Povey (1989). Crystallisation, particle size, flocculation and creaming were measured by using ultrasonic techniques by many researchers. Flocculation can be detected accurately by ultrasonic, as the droplets change the attenuation values significantly, according to a number of flocculated droplets in a particular place (McClements and John, 2001).

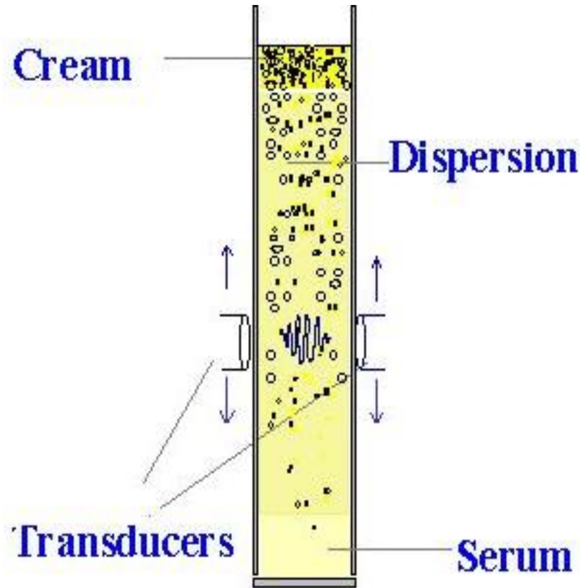


Figure 3.6: Diagram showing the Acoustiscan cell with sample and transducers used to scan emulsions (Povey, 1997b).

A procedure has been desinged and developed by Povey (1997b) to study the stability of emulsions. The system is computerised and called AUP, which refers to Acoustiscan Ultrasonic Profiler; it measures the speed of sound from the top to bottom of the cells that contain the emulsions. According to change in the velocity at different heights, a graph was obtained containing both velocity and height. The temperature is controlled because any change in temperature will affect the reading (1 ºC changes the velocity by 3 ms^{-1}) (Povey, 1997b)

The volume fraction Φ of oil in water measured using the following equation along the cell's height.

$$\Phi = \frac{\frac{1}{v^2} - \frac{1}{v_2^2}}{\frac{1}{v_1^2} - \frac{1}{v_2^2}} \qquad (3.6)$$

Where v is the measured velocity in the sample, v_1 and v_2 are respectively the velocities in thyme oil and water. (The speed of sound in water at 25 ºC was 1496.701 ms^{-1} and it was 1285.738 ms^{-1} in thyme oil).

Statistical analysis

The results were analysed using (IBM SPSS 24), the data were presented for all values as the means ± standard deviation in chapters 3, 4 and 5. To identify the significant differences between means at ($p < 0.05$), one way ANOVA and univariate analysis was used followed by Tukey's test. Moreover, when significant differences were presented between means, different letters were given to the values.

3.4 Results and discussions

This section contains different results obtained from chemical analysis and physical characterisation of thyme essential oils and the standards. Including gas chromatography-mass spectrometry (GC-MS) analysis, solubility, partitioning in octanol / water, critical micelle concentration (CMC), and possible self-assembly of thyme EOs.

The physiochemical properties of thyme essential oils and its components

The essential oils of thyme contains various compounds, each of them with specific physiochemical properties. Studying their interactions with the surrounding matrices, as well as other different compounds found in the delivery systems are important when designing an efficient delivery system.

3.4.1 The chemical analysis of thyme EOs and standards

The chromatogram from GC-MS revealed the existence of 16 mono and 13 sesquiterpenes which are classified as 11 oxygenated compounds and 18 non-oxygenated terpenes. These represent approximately 90% of the total identified components in the thyme EOs. Only compounds which presented at concentrations of greater than 0.25% were identified in thyme oil and listed as the chemical profile of the thyme EOs in this study. The percentage fraction, the molecular weight of the identified compounds are shown in (Figure 3.7) and (Table 3.1).

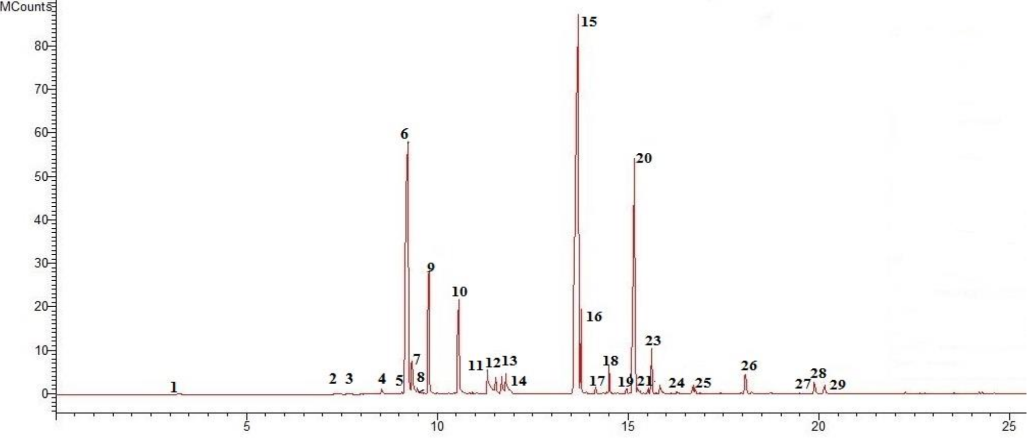


Figure 3.7: The chromatogram of thyme essential oils obtained from GC-MS analysis (each peak number represents a compound in thyme EOs as shown in Table 3.1).

Using GC-MS with SPME to analyse the samples in the current study was advantageous regarding the simplicity of sample preparation, speed of tests and reliability of the technique. Thymol as phenolic monoterpene was the most abundant compound comprising (37%) of the total oil. According to the variation in the compound's fraction in different types of *T. Vulgaris,* there are seven different chemotype of thyme. Therefore the thyme oil chemotype in the current study is thymol. The presence of a high fraction of thymol in thyme EOs affects the physicochemical properties and biological activity when applied to food systems. The composition of thyme oil used in this study differed in composition from that used in other studies such as (Jouki et al., 2014) who found that thymol comprised 46.42%, carvacrol 12.42% and p-cymene 22%. In another study, thymol was lower than carvacrol comprising only 3.63% while carvacrol was 21.89% (Chamanara et al., 2012). Albanian thyme was found to contain different quantities of chemical compounds depending on cultivation regions (Asllani and Toska, 2003).

P-cymene as a monoterpene and thymol precursor was found to be the second most common compound in thyme oil with a fraction of (16.24%). The third most common compound was γ-himachalene (10.38%) which is not identified in other studies on thyme EOs. Originally this compound belongs to the EOs of anise seed, its presence in thyme EOs in the current study at high concentration is not fully understood. However, it may added to thyme EOs by mistake during preparetion, or these particular thyme plants may produce it. Alternatively the detection was not accurate as the standard for it was not available in the current study and for its identification only NIST library was used.

Table 3.1: The retention time, percentage content, chemical formula and molecular weight of the identified compounds in thyme EOs.

No.	Components	Retention time (min)	Relative content %	Chemical formula	Molecular weight (g/mol)
1	α-Thujene	3.20	0.032	$C_{10}H_{16}$	136.24
2	α -pinene	7.32	0.041	$C_{10}H_{16}$	136.24
3	Camphene	7.65	0.026	$C_{10}H_{16}$	136.24
4	β-pinene	8.54	0.891	$C_{10}H_{16}$	136.24

5	α-terpinene	9.07	0.057	$C_{10}H_{16}$	136.24
6	P-Cymene	9.20	16.24	$C_{10}H_{14}$	134.21
7	Eucalyptol	9.33	1.84	$C_{10}H_{18}O$	154.24
8	γ-Terpinene	9.47	0.310	$C_{10}H_{16}$	136.24
9	Carene	9.77	3.53	$C_{10}H_{16}$	136.24
10	Linalool	10.55	5.48	$C_{10}H_{18}O$	154.25
11	Camphor	11.17	1.97	$C_{10}H_{16}O$	152.24
12	Endo-Borneol	11.53	0.80	$C_{10}H_{18}O$	154.25
13	Borneol	11.68	1.68	$C_{10}H_{18}O$	154.25
14	Terpinen-4-ol	11.79	1.39	$C_{10}H_{18}O$	154.25
-	Geraniol	12.90	-	$C_{10}H_{18}O$	154.25
15	Thymol	13.58	37.24	$C_{10}H_{14}O$	150.22
16	Carvacrol	13.72	2.05	$C_{10}H_{14}O$	150.22
17	α- Cubebene	14.14	0.129	$C_{15}H_{24}$	204.35
18	Copaene	14.50	0.54	$C_{15}H_{24}$	204.35
19	α-Caryophyllene	15.03	0.054	$C_{15}H_{24}$	204.35
20	γ-Himachalene	15.16	10.38	$C_{15}H_{24}$	204.35
21	β-Caryophyllene	15.24	0.099	$C_{15}H_{24}$	204.35
22	Aromadendrene	15.52	0.114	$C_{15}H_{24}$	128.17
23	α-Humulene	15.61	1.54	$C_{15}H_{24}$	204.35
24	Allo-aromanderene	16.27	0.328	$C_{15}H_{24}$	204.35
25	Isoledene	16.70	0.25	$C_{15}H_{24}$	204.35
26	Caryophyllene oxide	18.07	0.98	$C_{15}H_{24}O$	220.35
27	Isoaromadendrene	19.43	0.396	$C_{14}H_{22}$	190.32
28	Isoaromadendrene epoxide	19.81	0.67	$C_{15}H_{24}O$	220.35
29	Alloaromadendrene oxide	20.14	0.58	$C_{15}H_{24}O$	220.35

This finding is in agreement with studies in which *Thymus* species were found to be rich in phenolic compounds such as thymol or carvacrol (Stahl-Biskup, 2004). Carvacrol is an important compound and the fraction (2.05 %) found in this study is still within the

range reported in other studies (da Rochaa et al., 2014; Nickavar et al., 2005). The presence of other terpenes at high levels decreased the carvacrol and thymol fraction in the oil. While phenolic compounds comprised 45.5% of thyme EOs when analysed by (Fachini-Queiroz et al., 2012).

The specific chemical composition of the EOs depends on many different factors. For instance, the production of the main EOs components (carvacrol and thymol) relies on the pathways from the precursor p-cymene and γ-terpinene which are dependent on location and harvest period. Thyme species contained 35.83%, and less of phenolic monoterpenes are considered poor (Chorianopoulos et al., 2004). Moreover, Marino et al. (1999) observed a variation in chemical composition of *Thymus vulgaris* depending on plant development. They found that the precursor p-cymene and γ-terpinene were at their highest level when the plant was at a premature stage, these levels then tend to decrease as the plant approaches its flowering period. During the flowering period, carvacrol was found to be the major component of the EOs, this then decreased sharply at the end of the flowering stage to be replaced by thymol as the major constitute. It is also reported that the antimicrobial activity of essential oils is found to be at its highest level during or directly after the flowering stage (Chorianopoulos et al., 2004).

In this study, the plant from which the thyme oil extracted was harvested is likley to be at a premature stage as indicated by p-cymene content. However, according to thymol and carvacrol content arguably the plant seems to be at the end of its flowering period. Therefore, it can be concluded that the thyme essential oils in this sample came from blends obtained from different plants which were at different stages of their development and/or from different origins. It would have been more appropriate to use essential oils extracted from plants at the same stage of development and from same area and harvesting time.

In many studies, the identification of compounds of thyme EOs carried out until eluting carvacrol fraction on the chromatogram. While as it is shown in the (Table 3.1), another 13 compounds which comprise 16.06% of the total oil eluted after carvacrol were identified in this study. The presence of these compounds decreases the fraction of other compounds when establishing the thyme EOs profile. Therefore, it is considered to be necessary to report and list all eluted compounds from GC-MS analysis. No detailed data are available on the importance of these compounds regarding their activity against microorganisms or their antioxidant activity. Therefore further investigation suggested

from the current study to evaluate the action of these compounds and their roles in thyme EOs. These compounds may work synergistically, antagonistically or they do not pose any action when present with other compounds. However, not all species contain the same compounds and at the same quantities. The level of the phenolic compounds such as thymol and carvacrol is directly related to their activity as an antibacterial in the food systems. Thus, using thyme essential oils containing high levels of these compounds, is highly recommended.

3.4.2 Physical characterisation of thyme EOs and standards

The physical properties of the individual components in thyme EOs determines the overall properties of the oil. Their solubility, surface activity, hydrophobicity, their interactions with and their affinity for different phases are important in designing efficient delivery system.

3.4.2.1 The solubility of thyme EOs and some terpenoids

The solubility of thyme EOs and some constitute standards were measured by dissolving different amounts of the individual standards in deionised water. The solubility limits of the various compounds are presented in (Figure 3.8), terpenin-4-ol was the most soluble compound among the oxygenated monoterpenes and reached a concentration 2.6 g/L. The second most soluble compound is α-terpineol 1.80 mL/L, followed by camphor 1.5 mL/L, carvacrol 1.27 mL/L, thymol 0.96 g/L, thyme EOs 0.95 mL/L and geraniol 0.55 mL/L.

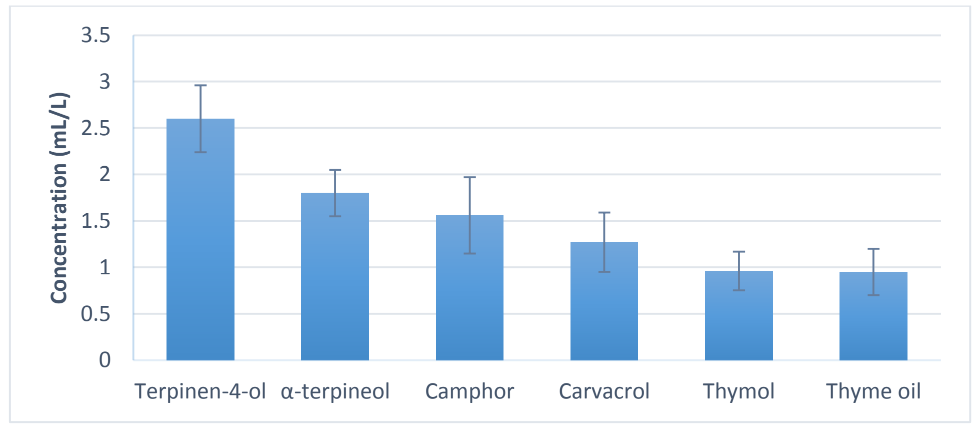


Figure 3.8: Solubility of thyme EOs and some monoterpenoids in deionized water at 20 °C.

Regardless of the presence of one polar group attached to all of the monoterpenes, there was a variation in their solubility. This may be due to the location of the polar groups on the molecules

3.4.2.2 The partition coefficient of the thyme oil components

The partitioning coefficient experiment is carried out by dispersing the individual standards in octanol and water solutions and analysing different phases by GC-MS for a fraction of the standards components in each phase. The value of log $K_{o/w}$ with standard errors is shown for the six standards in (Table 3.2) over the entire range of the partitioning coefficient values the standard errors were between 0.01-0.06 except for thymol which it was 0.20. These values showed the precision in results when GC-MS used to determine the log $K_{o/w}$ for essential oil components. From the values obtained it is clear that the most hydrophobic compound was γ- Terpinene with log $K_{o/w} = 4.34$, conversely, camphor with log $K_{o/w} = 2.78$ was the most hydrophilic compound. The log $K_{o/w}$ of the hydrocarbon compounds are roughly the same with no significant difference between them. As expected compounds with attached hydroxyl or oxygen groups showed lower lipophilicity compared to the hydrocarbon compounds. The values of the partitioning coefficient of the oxygenated compounds were significantly different despite containing only one polar group on all of them. Moreover, terpinen-4-ol, carvacrol and thymol were between the other two groups of hydrocarbons γ- terpinene, α-terpinene and p-cymene and oxygenated compounds of borneol and camphor.

From the results of the compounds partitioning in of these compounds between octanol and water, these compounds can be organised according to their hydrophobicity as follows: γ- Terpinene > α-Terpinene > P-cymene > Carvacrol >Thymol > Terpinen-4-ol > Borneol > Camphor.

The solubility of the hydrophobic bioactive components in various matrices of food such as water, fats, oils and carbohydrates, is crucial when designing a delivery system. Additionally, it is also important to take into account the fractioning of the active compounds within the delivery system. It is evident that the degree of solubility depends on the different interactions between the hydrophobic bioactive compounds with other materials in the system (Israelachvili, 2011). The thermodynamic affinity of the hydrophobic bioactive components determines its partitioning behaviour in other phases (McClements, 2004b). Despite the presence of one polar group on each of the terpenoids

standards, there was a variation in both their solubility and partitioning coefficient. This might be due to the fact that location of the polar group on the monoterpenoid compounds may govern their interactions with polar compounds in the system

The partitioning of thyme EOs compounds in octanol and water depends on the presence of the attached hydroxyl group on their molecules. The absence of –OH group on hydrocarbon compounds such as γ- terpinene, p-cymene and α-terpinene and the hydrophobic affinity raised their tendency for dissolution in the octanol phase rather than water phase. Thus, they are more lipophilic. A detailed knowledge of partitioning coefficient of the individual constitutes of the EOs is essential when designing a delivery system for bioactive compounds to target a cell wall of microorganisms. Because bacteria cell walls are formed mostly from phospholipids bilayers.

Table 3.2: The partitioning coefficient of thyme EOs standards.

Standards	The average peak of the standard area in the water phase	The average peak of the standard area in the octanol phase	$K_{o/w}$	Log $K_{o/w}$ ± SD
γ- Terpinene	2.29E+06	5.12E+10	2.24E+04	4.34 [a*] ± 0.03
α-Terpinene	2.24E+06	4.28E+10	1.92E+04	4.28 [a] ± 0.01
P-cymene	7.63E+06	1.06E+11	1.39E+04	4.14 [a] ± 0.04
Carvacrol	1.00E+06	3.08E+09	3.08E+03	3.48 [b] ± 0.06
Thymol	1.70E+06	5.39E+09	3.18E+03	3.46 [b] ± 0.20
Terpinen-4-ol	6.10E+06	1.21E+10	1.98E+03	3.29 [b] ± 0.02
Borneol	8.30E+05	6.41E+08	7.72E+02	2.88 [c] ± 0.02
Camphor	7.36E+05	4.48E+08	6.08E+02	2.78[c] ± 0.02

*Log $K_{o/w}$ with different letters are significantly different ($p < 0.05$)

A higher log Ko/w of the compounds means that they have an affinity for and are able to partition into the bilayer membranes of the microorganisms which result in membrane swelling. Consequently, the partitioning of other compounds such as terpenoids become easier in distorted membranes when compared with un-distorted membranes.

The variation in log $K_{o/w}$ between hydrocarbon and terpenoids compounds is directly related to the presence of hydroxyl group. Compounds containing -OH are less lipophilic, and there is a possibility to show solubility when introduced into the aqueous phase. Moreover, the insignificant differences of log $K_{o/w}$ between hydrocarbon compounds (p-cymene, γ- terpinene and α-terpinene) is due to their similar chemical structure and molecular weights. A similar pattern is observed within terpenoids that have the same chemical conformation and molecular weights. The reason for significant differences between thymol, carvacrol and terpinene-4-ol with borneol and camphor which have roughly the same molecular weight may be due to their geometry and location of the polar group on the molecules. The polar group is more intact and closer to the molecule's skeleton which has more hydrophobic groups such as methyl group $-CH_3$ in the first group of the compounds, this may cancel or reduce the polarity of the molecules. However, the -O and-OH groups are far from the molecular skeleton in camphor and borneol making them more polar; this effect is also seen in NH_3, where the polar head (N) located far from the hydrogen atoms.

The presence of -OH also helps to decrease unfavourable interaction between water and oil by reducing the surface tension that is to say a reducing in the free energy (ΔG) of the system. As a result, thermodynamically stable emulsions can be produced when EOs is present in the system. The hydroxyl group is also necessary to increase the availability of the active compounds and bringing them closer to the targets of interest in the food systems, such as bacteria cell wall, as they have the ability to diffuse in the aqueous phases.

Determination of partitioning coefficient of compounds which are used in food and pharmaceutical products is imperative to understanding and predicting their binding behaviour to the different compounds in the system. Their physicochemical properties govern the distribution and adsorption of the EOs constitute in food systems. Moreover, the diffusion of the bioactive materials across the cell membrane of bacteria, for instance, occurs by passive transport which depends on the affinity of the compounds to the

lipophilic cell membrane and the permeability of the cell wall. Additionally, in the cosmetic industry, terpenes are found to improve the permeability of skin for other compounds as they are able to disrupt the stratum corneum (Cal, 2006). The partitioning coefficient of the active chemicals in cell membranes plays a significant role in their activity. Weber and de Bont (1996) stated that the compounds with partitioning coefficient of more than 3 could interact more efficiently with membranes. The determination of the partitioning coefficient of carvacrol in both liposome-buffer and octanol-water were determined by (Ultee et al., 2002) and were 3.26 and 3.64 respectively. The log $K_{o/w}$ of the standards found in the present study was in agreement with the results reported by (Griffin et al., 1999a).

3.4.2.3 The surface activity of thyme EOS

The surface activity of thyme EOs was investigated by preparing different concentrations of thyme EOs and different monoterpenoids below their solubility limit. The results from the surface tension reduction of deionised water using different concentrations of thyme EOs and monoterpenoids standards are shown in (Figure 3.9).

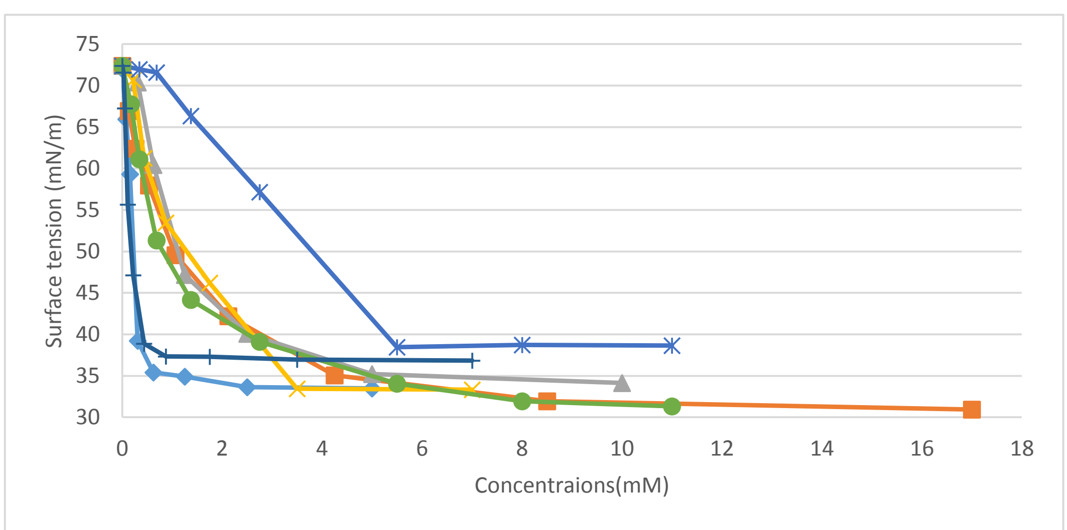


Figure 3.9: The critical micelle concentration of thyme essential oils and some terpenoids at 20 °C —+— thyme oil, —▲—carvacrol, —✕— thymol, —— terpinen-4-ol, —●— camphor, —◆— geraniol and —✳— a-terpineol.

It is clearly shown that the surface tension of deionised water decreased gradually with increasing thyme EOs concentration. The surface tension decreased from72.01 mN/m to 38.86 mN/m when the concentration of thyme EOs rose from 0 to 0.43 mM.

However, using higher concentrations from 0.43 mM to 7 mM of thyme oil solutions did not change the surface tension dramatically and it remained relatively constant. The reason of surface tension reaching a relatively constant value for each standard at higher concentrations, may be due to the aggregation of some amphiphilic compounds inside thyme EOs through hydrophobic interactions which in fact may even form micellar structures. The critical micelle concentration (CMC) is the concentration of the solubilised compounds at which the slope of surface tension changes abruptly. Thus, thyme EOs reached the CMC when its concentration was 0.43mM.

In other experiments, the highest soluble amount of oxygenated terpenes in deionised water was determined. The results for the oxygenated monoterpenes concentrations with surface tension are shown in the (Figure 3.9). The effect of thyme oil components on the surface tension of the water revealed that some terpenoids could reduce both the surface tension (i.e. the free energy of the system). These compounds tend to self-assemble and aggregate when their concentrations are increased. The monoterpenoid standards reduced the surface tension and reached CMC at different levels. The first compound to reach CMC was geraniol at 0.625mM, followed by thymol 3.5 mM, terpinene-4-ol 4.25 mM, carvacrol 5 mM and finally camphor and α-terpineol at 5.5 mM.

The interfacial tension of thyme essential oils and deionised water was also determined, as shown in (Figure 3.10). A reduction of the interfacial tension occurred from ~72 mN/m down to ~ 43 mN/m, when tested between water and thyme EOs.

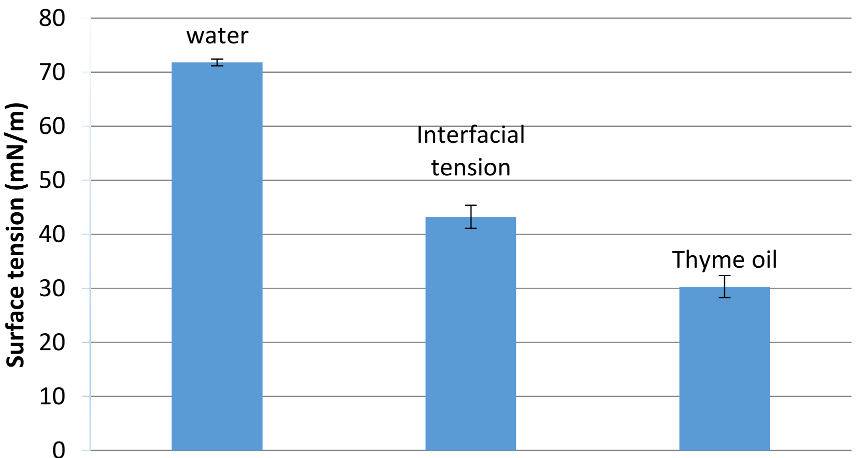


Figure 3.10: The surface tension of deionised water and thyme EOs and the interfacial tension between them at 25 ℃.

The self-aggregation of the terpenoids occurred due to the coexistence of both lipophilic and hydrophilic regions on these molecules. When the concentration of terpenoids in the aqueous solution increase above the CMC, they tend to self-aggregate to form micellar–like structures. There is a lack of explanation of the self-association structure of these molecules but it is believed that it is due to a rigid structure with a short chain of the hydrophobic segments in EOs components.

It is clear that the CMC for thyme EOs is between the CMC's of the monoterpenoids examined in this study. However, the oxygenated and hydrocarbon compounds together in thyme EOs determine the solubility and surface activity of thyme oil. The terpenoids as weak amphiphilic molecules may emulsify the hydrophobic molecules and separate them from polar heads of the water by hydrophobic interactions. The result is, a decrease in the surface tension of water and thereafter, free energy of the system. The CMC results for some oxygenated compounds in the current study were in general agreement with the results reported by Turina et al. (2006), they found that the CMC of some oxygenated compounds such as thymol, camphor, menthol, geraniol and cineole ranged between 3 to 8 mM. They also noted that all monoterpenes partitioned and penetrated themselves into the model membrane of dipalmitoyl-phosphatildylcholine used in their study. Additionally, they found that the monoterpenes located themselves in the polar region of the membrane. All standards and thyme oil reached the CMC below their limit of solubility, indicating that Kraft point is below room temperature for these compounds.

In many systems, the surface or interface between two immiscible phases is critical because it governs the behaviour of the individual bulk phase characteristics separately and the whole system as a result. Very small changes in solubility of one phase in another influence the bulk phase characteristic through the interactions between the solute and the solvent. Only one boundary surface can be present between two immiscible phases. To understand the role of a surfactant in controlling and modifying the interface between different phases it is important to understand both the chemical and physical properties of the molecules at the surface. Adsorption of surface active materials at the interface will change the nature of the mixed phase significantly even if adsorbed layer is only one molecule thick (Dickinson, 1998). The physical property of the interface is different from that of the bulk phases. For instance, the various force fields of the atoms and molecules which are exposed at the surface are distinct from that of the molecules in the bulk phases.

Chemical composition, pressure and temperature are the main factors affecting the electrical properties and interfacial energy of the different systems. Changing the pH, by the addition of neutral electrolytes or changing the chemical composition of the system will change the dielectric constant and as a result, alter the charge characteristic of the system. Therefore, small change in bulk phase will alter the surface tension and surface energy of the systems especially if the system is liquid or high energy solid (Myers, 2006).

Thyme oil contains different types of chemical structures, some of which may induce emulsifying effect when they are introduced to food mediums. This behaviour is due to hydroxyl groups and oxygen on some of the thyme oil compounds which are considered as hydrophilic groups and tend to interact with water molecules by different interaction forces. The ability of these groups to bind with water molecules or an oil phase depends on the solubility of the amphiphilic compound in either water or the oil phase. This balance is referred to as Hydrophilic-lipophilic balance (HLB). The stability of an emulsion depends on the HLB value which is different depending on the type of the emulsion. High HLB surfactants such as Tweens tend to form oil—in-water emulsions and low HLB surfactants tend to form water-in-oil emulsions such as Spans.

3.4.2.4 The self-association of thyme EOs components

From the results of the previous expirements Sections 3.4.2.1 to 3.4.2.3, it was considered if worthwhile to examine the ability of thyme EOs to self-emulsify. An emulsion of water and thyme EOs prepared by mixing water and thyme EOs without adding surfactants and passing the mixture through high pressure homogeniser for 3 times at 300 bar to obtain 10% w/w O/W emulsion. The formed emulsion then monitored for eight weeks at 25 ^{o}C and scanned in the Acoustiscan instrument to track the emulsion stability by measuring the volume fraction of the EOs in water at different heights of the cell.

At the beginning of the experiment, the emulsion was formed as shown in (Figure 3.11) , and no layer of the separated oil could be detected, and it is confirmed by the data obtained from volume fraction. After 4 days of storage, a thin layer was observed to have separated from the mixture. Then the oil layer increased with storage time until all of the oil separated from the water and the developed serum was observed after 60 days of storage, at the bottom of the cell. However, a white interface remained for 60 days as

which may be due to the presence of amphiphilic compounds in EOs organising themselves at the boundary between the two different phases.

In agreement with the visual observations, the measured volume fractions showed the same pattern. The creaming profile of the mixture was obtained by scanning the cells and calculating volume fraction this increased gradually during storage time, as shown in (Figure 3.12). The value of the volume fraction at the beginning of storage was around 0.4 ± 0.05 and then increased to reach 0.95 ± 0.07 at the end of storage.

The Acoustiscan as ultrasound instrument was used to track the fractioning of the EOs in water during storage time. The instrument had to benefit of being simple, very fast and reliable and allowed for measurements is not possible to measure by visual observation or other light scattering techniques. There is a possibility of self-emulsification of thyme oil components, because it contains some molecules that have both hydrophobic and hydrophilic groups.

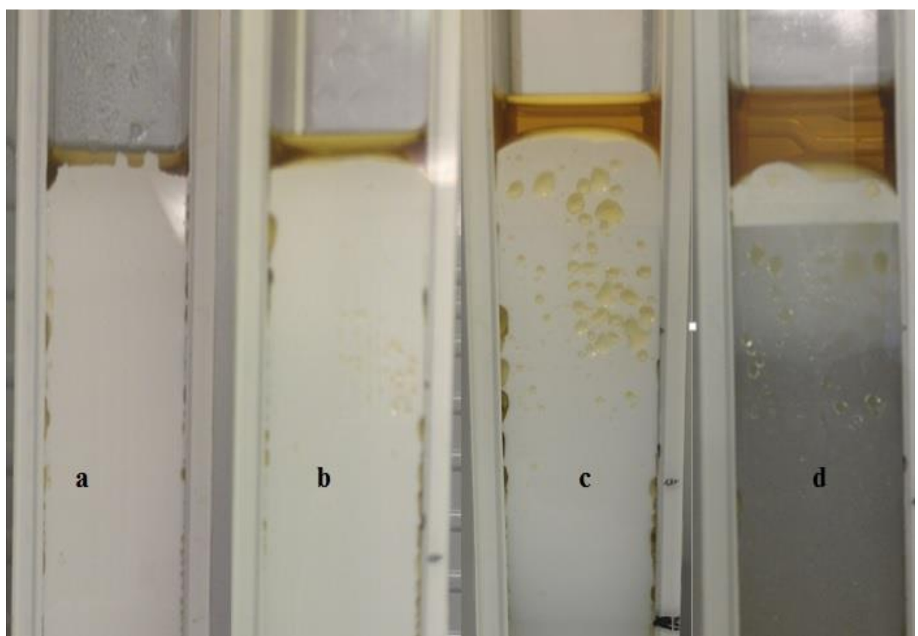


Figure 3.11: Thyme oil emulsion without surfactant (a) after 4 days (b) after 14 days, (c) after 30 days and (d) after 60 days at 25 ºC.

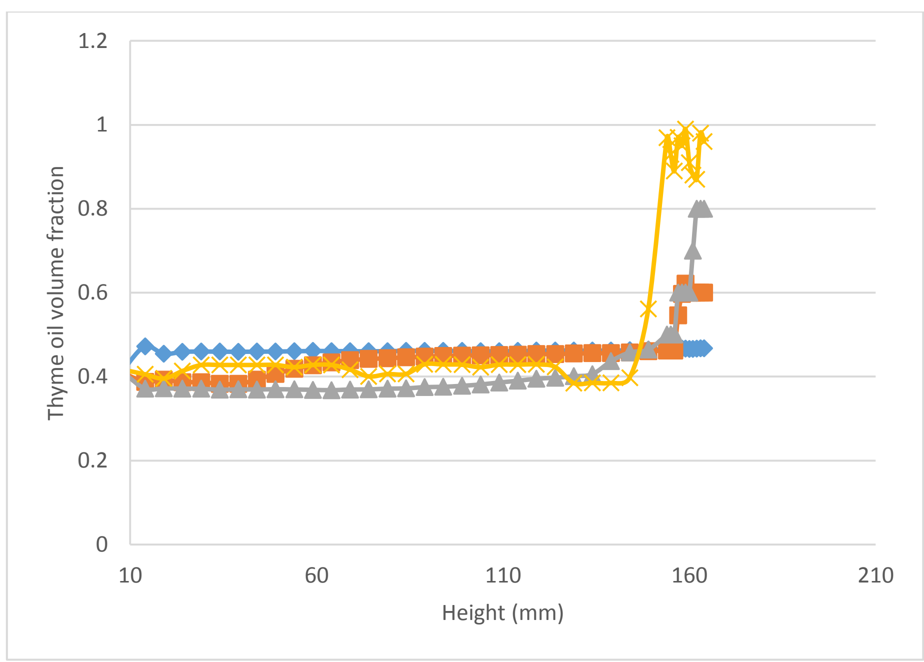


Figure 3.12: The volume fraction of thyme EOs in water and thyme oil systems without addition of surfactant during 60 days at 25 °C —◆—first day, —— after 15 days, —▲— after 30 days and —×— after 60 days.

The emulsion was formed and then separated; this may be due to the structure of the hydrophobic groups of thyme oil components preventing full stabilisation. Thymol, carvacrol and p-cymene contain only 10 carbon atoms as a phenolic ring which represents its hydrophobic group, and it is probable that this is insufficient to form a strong interactions. Subsequently, they cannot remain together in the aqueous phase, because the self-associating tendency depends on many factors; such as actual structure, distribution and number of hydrophobic groups, optimal surface area, geometry and their relative sizes as discussed by (Livney, 2012; Israelachvili, 1992).

There is a lack of fast and accurate procedure to predict packing arrangements of these molecules containing aromatic ring in contrary to surfactants which have long hydrophobic chain which can be estimated calculating critical packing parameter as suggested by Israelachvili (2011). Therefore, empirical observations and computer-aided modelling was used to understand the way the amphiphilic compounds aggregate. The 3D alignment of some thyme oil components was used to draw and display the structures as shown in (Figure 3.13). It contains one molecule of thymol, carvacrol, p-cymene, gamma-terpinene, borneol, geraniol, linalool, alpha-terpineol and beta-caryophyllene. This model was developed using Marvin Sketch 6.0.1, 2013 by Chemaxon. As it is noted

in this figure, the molecules interact with each other, although the hydrophobic groups (grey colour) are more associated and higher in number than the hydrophilic groups (-O and –OH, red colour) on the molecules are in contact with aqueous phase .

The 3D model suggests that the packing of these compounds is more like plate or sandwich sheets than that expected of cylindrical packing. It is more probable that when two or more of the aromatic rings are available in the medium one on the top of another, they tend to interact and overlap face-to-face with each other through π-π intermolecular interaction this is called π stacking which occur between aromatic rings as observed between nucleobases in DNA, RNA and protein molecules (Cockroft et al., 2005; Luo et al., 2001). Alternatively, they may interact by T interactions (right angle 90°) when they overlap face-to- edge which is weaker than the first type of interaction. Moreover, other major interactions via hydrogen bonds, van der Waals, dipole-dipole and hydrophobic interactions collectively support the self-association of the compounds in EOs.

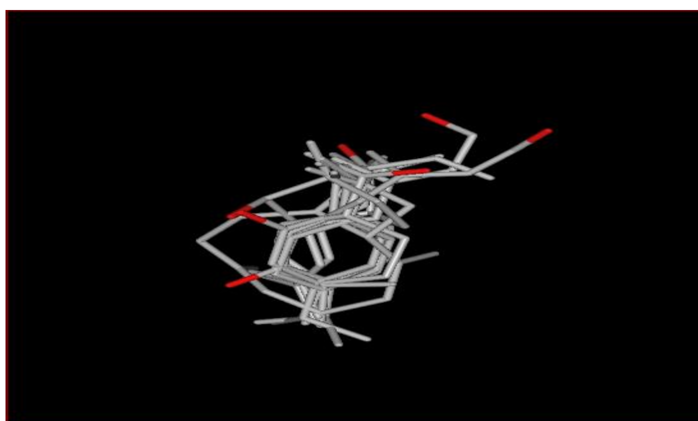


Figure 3.13: A 3D structure model, of some dominant thyme oil components, the polar groups shown in red colour on the outside and the hydrocarbon groups in grey (Chemaxon, 2013).

3.5 Conclusion

HS-SPME-GC-MS as the analysis method employed to identify and quantify thyme essential oils in different samples was precise and straightforward. There is a significant advantage of using SPME to analyse thyme essential oils as it only detects the volatiles, it can be used to extract volatiles from liquid samples and solid samples such powders which contain volatiles. The optimised method resulted in detection of large numbers of compounds in thyme EOs in a short time. The method was precise to detect different compounds with narrow peak area and generated separate peaks for compounds which have similar spectra such as thymol and carvacrol. The results showed that thymol was the dominant compound followed by p-cymene, there was also γ-himachalene which tentatively identified as it is not usually identified in thyme EOs at relatively by high level which affected the proportion of other compounds such as thymol in the oil.

Results from physical experiments showed that oxygenated compounds in thyme EOs were able to solubilise in water to some degree. Moreover, partitioning of these compounds in octanol-water and their surface activity was investigated to understand how these compounds behave when they present in the colloidal system. Thyme EOs was able to form an emulsion when homogenised with water without using emulsifier. The amphiphilic compounds such as thymol, carvacrol, terpenen-4-ol, camphor and linalool were able to emulsify other terpenes such as *p*-cymene, γ-terpinene in water and the emulsion was stable for a short term.

Chapter 4 Emulsification and Encapsulation of Thyme Essential Oils in Skimmed Milk

4.1 Abstract

The delivery of phytochemicals to the desired location and at the preferred time is of interest in food, cosmetic and pharmaceutical industry. Encapsulation as an innovative approach is able to protect phytochemicals from undesirable changes during preparation, processing and digestion. The encapsulation enhances the solubility and availability of flavours, colourants, bioactive chemicals, which are oil based when they introduced to aqueous systems. However, the cost, availability and safety of some wall materials used in encapsulation are the limitation of their use in food industry. The objective of this chapter was to develop a new delivery system based on choosing natural food product which can be used in many industries at low cost and less processing steps. Skimmed milk was chosen as a source of proteins in combination with maltodextrin to be utilised as wall material for thyme essential oils. The results revealed that skimmed milk is an excellent material to emulsify thyme EOs compared to sodium caseinate. The prepared emulsions characterised by monomodal and small particle size distribution (< 240 nm) with a zeta potential above -30mV and remained stable without significant changes in these parameters over 60 days of storage. The addition of MD improved the powder particle properties. Small powder microspheres produced by spray drying with a size distribution of ~ 9 μm with small embedded oil droplets similar to the initial emulsion. The increase of MD reduced the surface oil and increased the retention of thyme EOs when dried in a spray dryer. Higher encapsulation efficiency of 97% was observed when 3% w/w MD combined with skimmed milk to emulsify 5% w/w thyme EOs. The thyme EOs powders characterised by rapid dissolution and higher release rate of the bioactive compounds reaching 80 % in thymol and lower rate for other compounds depending on their solubility and concentrations in the capsules. These properties are important when designing delivery system as they affect the bioavailability and activity of the encapsulated bioactive materials.

4.2 Introduction to encapsulation of thyme essential oils

The bioactivity of the EOs is insufficient in food systems due to limited solubility, high volatility, weak interaction with microorganisms and low dispersity in aqueous food systems (Liang et al., 2012; Ghosh et al., 2014). This problem can be overcome by means of encapsulation where the hydrophobic compounds can be dispersed more efficiently in aqueous systems which result in bioactivity enhancement (Cheong et al., 2016). A free-

flowing dry powder containing essential oils can be produced by spray drying an emulsion containing EOs which can be introduced into other products with prolonged biological activity (Jan, 2015). Recently in a study by Chen et al. (2015) thymol and eugenol were encapsulated in casein and zein proteins and then spray dried. The hydrated powder showed stable dispersion with small particle distribution (200nm) with the improvement of bactericidal and bacteriostatic effects when the powder was added to milk. In another study, thymol has been encapsulated in sodium caseinate and showed high stability during 30 days of storage. The encapsulated thymol showed higher activity against milk microorganisms compared to non-encapsulated thymol (Pan et al., 2014). These studies are suggesting the possibility of using encapsulated EOs in food products. Similar results with encapsulated rosemary also observed when used against *L.monocytogenes* in sausage of pork liver compared to bulk rosemary EOs (Pandit and Shelef, 1994).

The versatility of encapsulation makes it a successful technique to be used in different industries such as pharmaceutical, food, agriculture and chemicals. The main reason for applying encapsulation procedure is to protect and extend the shelf-life and delivery of the active compounds (Vasisht, 2014; Madene et al., 2006). In the food industries, the active compounds in different physical states can be isolated by a thin film which separates them from other food components and adverse environment factors (light, moisture, oxygen and elevated temperatures). Encapsulation is used to prevent undesirable changes to the core material that may affect the quality of the active compounds until "controlled release" (Sobel et al., 2014b; Davidov-Pardo et al., 2015; Jafari et al., 2008; Brownlie, 2007). In addition, encapsulation can decrease evaporation of the core materials or mask the undesirable flavours of the active compounds. The delivery of the functional compounds for instance antioxidant, antimicrobial, vitamins, minerals, fatty acids or probiotics are other reasons behind encapsulation (Chen et al., 2006; Onwulata, 2012; Vasisht, 2014; Wang et al., 2015). Encapsulation also allows liquid flavours to be handled as a powder with desirable characteristics enabling them to be added to food (Brownlie, 2007).

Furthermore, encapsulation increases the activity and bioavailability of the active ingredients if compared with non-encapsulated ingredients as a result of enhancing the dispersity in aqueous foods particularly hydrophobic compounds (Davidov-Pardo et al., 2015). Encapsulation is also employed in food fortifying compounds such as flavours,

essential oils, spices, fatty acids, vitamins, minerals, peptides, fish oils, artificial sweeteners, colourants and preservatives (McClements, 2014).

The size of microcapsules vary between submicron and several millimetres and have great diversity in their shape (Desai and Park, 2005, Gharsallaoui et al., 2007, Nedovic et al., 2011, Gibbs et al., 1999). Encapsulation has been used to introduce many types of ingredients into food such as oil, flavours, antimicrobials, peptides, vitamins, and minerals which are difficult to incorporate them directly to aqueous mediums, because of immiscibility (Garti and McClements, 2012). Essential oils are usually volatile; their use can be significantly extended using encapsulation. This approach can be applied to food, pharmaceuticals, textile and the cosmetic industry. This procedure will also extend the shelf-life and bioavailability of the essential oil components (Jan, 2009). To encapsulate essential oils, they are often dispersed as an emulsion using suitable surfactants and the droplets surrounded by a hydrophilic coating forming capsules. The emulsion can then be spray dried to produce microspheres or microcapsules.

Because of the different variety of chemical compound and immiscibility of essential oils, it is hard to incorporate it into aqueous delivery systems without emulsifying and encapsulation. Encapsulation of essential oils is an effective way to incorporate its active compounds into food systems. This technique also protects the active compounds from chemical, physical and biological changes and prolongs shelf-life by lowering the EOs volatility and by protecting it from surrounded environment. Encapsulation can also protect against undesirable reactions such as oxidation, between the essential oils components with food ingredients such as proteins (Garti and McClements, 2012, Burt, 2004).

However, some of the encapsulation techniques require high cost and long time for preparation or they made from non-food grade materials. Therefore, searching for alternatives is important to be used in industry at a lower cost. The objective of this chapter was to use skimmed milk and maltodextrin as a natural food material in a liquid state as a novel delivery system to emulsify thyme essential oil then drying it using spray dryer.

4.3 Materials and methods

In this section the methods of assessing the composition of skimmed milk used as developed delivery system for thyme EOs, the preparation approaches of thyme oil emulsion and their charecterisation are illustrated. Moreover, different the powder preparation and characterisation also explained.

4.3.1 Materials

Thyme essential oils obtained from Sigma-Aldrich. Skimmed milk was obtained from Tesco Stores Ltd., sodium caseinate was purchased from (Fisher Scientific Ltd. Acros Organic) and maltodextrin (DE 19) obtained from (ROQUTTE-France).

4.3.2 Methods

4.3.2.1 Characterization of skimmed milk

The characterisation of the skimmed milk which was used to emulsify thyme EOs was essential to determine its composition, such as proteins and carbohydrates as they are crucial when designing a delivery system. It was also beneficial in measuring the exact content of the formed emulsion when thyme EOs and different concentrations of maltodextrin were added to it. Skimmed milk was obtained from Tesco Stores Ltd., and according to the label its composition is presented in (Table 4.1).

Table 4.1: Skimmed milk composition as described on the label.

Typical Values	100 ml contains	Typical Values	100 ml contains
Energy	147kJ (35kcal)	Fibre	0 g
Fat	0.1g	Protein	3.4 g
Saturates	< 0.1g	Salt	0.1 g
Carbohydrate	5 g	Vitamin B12	0.4 µg
Sugars	5 g	Calcium	124 mg

Determination of Protein content

In order to determine the protein content of skimmed milk, a quantitative method of Kjeldahl (AOAC official method, 2012) was used which is considered as a standard method for protein content determination in food products.

Determination of Fat content

In order to determine the fat content of skimmed milk the Rose Gottlieb method was used (AOAC official method, 2012).

Ash determination

The ash content of the skimmed milk was determined using (AOAC official method, 2012).

Moisture content determination

The moisture content of milk sample was determined by using an oven method. Four clean stainless steel dishes with lids were labelled and weighed. In each dish 5 g of skimmed milk was added and weighed again then placed in an oven at 105 ºC for 24 hours. The samples were cooled down at room temperature in desiccator for 30 min and the moisture content of the milk was calculated as shown below:

$$\text{Moisture content \%} \left({}^{\mathrm{W}}/_{w}\right) = \frac{(w_1 - w_2)}{weight\ of\ milk\ sample}\, x\ 100 \qquad (4.1)$$

Where W_1 and W_2 are the weights of the dishes before and after drying in the oven.

Carbohydrates determination

The carbohydrate content of the milk samples were calculated by difference as shown in the Equation 4.5:

Total carbohydrates = 100 - (Protein % + Fat% + Moisture %+ Ash %) (4.2)

4.3.2.2 The preparation of thyme oil emulsion

In this study, the core material was thyme essential oils obtained from Sigma-Aldrich. The aqueous solution was a mixture of different concentrations of maltodextrin (DE 19) obtained from (ROQUTTE-France) and Skimmed milk (Tesco Stores Ltd.).

The loading capacity of the skimmed milk

A preliminary experiment was carried out to investigate the loading capacity of skimmed milk. Different concentrations of thyme EOs (1%, 2%, 3%, 4% and 5% w/w) were homogenised in skimmed milk. The results were reported as the amount of EOs that had been emulsified by the skimmed milk components.

Thyme oil emulsion stabilised by skimmed milk and maltodextrin

The aqueous solution prepared by dispersing different amounts of maltodextrin (DE 19) in skimmed milk to obtain (0%, 1%, 2%, 2.5% and 3% w/w MD using high shear laboratory mixer (SILVERSON, Model-L5 series, from Silverson Machines Ltd. England) at 10,000 rpm for 3 min to ensuer a fully hydration of the maltodextrin. The mixture was then placed in a water bath at 37 ºC and the solution stirred at 300 rpm using lab stirrer (LTE Scientific LTD, UK). The total solid concentrations of different mixtures were varied depending on the amount of maltodextrin added as shown in (Table 4.4). Subsequently, thyme oil was added dropwise to each solution to form 5% w/w thyme oil-in-skimmed milk and MD solutions with continuous stirring. The mixture was stirred by a high shear laboratory mixer for 3 min at 10,000 rpm prior to homogenisation. Different codes were given to the different types of emulsions which contained 5% w/w thyme EOs. SM referred to skimmed milk, and the number is the percentage %w/w of maltodextrin in the dispersion phase. For example SM0 is 5% w/w thyme oil in skimmed milk, SM1 is 5% w/w thyme oil in in skimmed milk containing 1% w/w maltodextrin, SM2 is 5% w/w thyme oil in skimmed milk containing 2% w/w maltodextrin, SM 2.5 for 5% w/w thyme oil in skimmed milk containing 2.5% w/w maltodextrin, SM3 for 5% w/w thyme oil in skimmed milk containing 3% w/w maltodextrin and SC for 5% w/w thyme oil in 3% w/w sodium caseinate. The premix was then passed twice through the top table laboratory homogeniser (GEA Niro Soavi's PandaPlus 2000), at two different stages of homogenisation, first valve was at 300 bars and the second valve was adjusted to 50 bars, to prepare an oil-in-water (o/w) emulsion. One litre of each emulsion was prepared, 750 mL was used for spray drying, and the remaining emulsions was kept for further characterisation of the emulsions over the storage period. Duplicate samples were prepared for each emulsion.

Thyme oil emulsion stabilised by sodium caseinate

Other emulsions were prepared using sodium caseinate (Fisher Scientific Ltd. Acros Organic) as wall materials. The continuous phase solutions prepared by dispersing 3% w/w of sodium caseinate in deionised water stirred for 24 hours to allow fully hydration. Thyme EOs was added dropwise with continuous stirring and was then mixed by a high shear laboratory mixer and passed through high-pressure homogeniser twice as described in section 4.3.2.2. About 500 mL of the emulsion was used for spray drying and the remaining was stored at room temperature for further characterisation.

Emulsion characterisation

The stability of the emulsion depends on the properties of formed droplets. The properties of the droplets or particles can be determined by using various physical analytical methods such as particle size distributions, zeta potential, and microscopic observations.

Emulsions droplet size measurements during storage time

Dynamic light scattering (DLS) was used to measure droplet size distributions (DSD) by using a Zetasizer (Nano Series, Model: ZEN3600-Malvern LTD, UK). In this technique, the laser is used as a light source when the light hits the particles in a suspension the light scatter, and the scattered light can be captured from another side by the single detector from one angle (Figure 4.1). As a result of the particle motion in the suspension, the scattered light fluctuate frequently, and an average value of the fluctuation is obtained during a specific time. The movement of the particles in the suspension associated with diffusion properties of the particles which affect the frequency of the scattered light on the detector. The diffusion of the particles in the dispersion medium is called Brownian motion which is caused by the random movement and collision of the particles or molecules with each other. “Stokes-Einstein equation” describes the relation between the size of the spherical particles and the diffusion of the particles in a suspension (Amanda et al., 2005).

$$D = kT/6\pi\eta r \qquad (4.3)$$

Where D is the coefficient of diffusion, k is Boltzmann's constant (1.380×10^{-23} J/K), T is the temperature in Kelvins, η is the viscosity of the suspension and r is the particle radius.

From the equation the particle size and diffusional coefficient is inversely related, the small particles move faster in the suspension than the larger particles. Smaller particles have a high frequency of fluctuation while large particles have lower fluctuation frequency (Figure 4.2). From the relation between detected intensity and time of the diffusion the DSD is calculated (Amanda et al., 2005).

Dynamic light scattering is able to measure the small droplets of less than 100nm diameter. The DLS can be used only with liquid samples because it depends on the Brownian motion of the droplets.

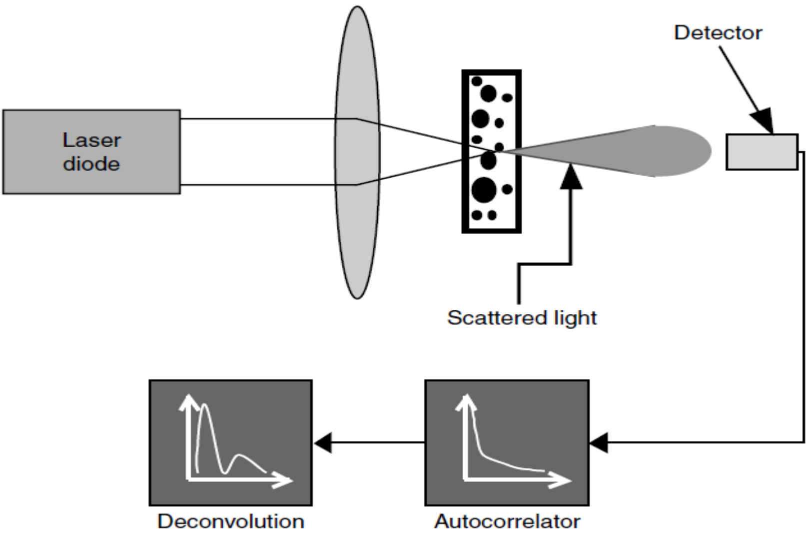


Figure 4.1: Schematic of dynamic light scattering technique (Amanda et al., 2005).

In order to investigate the stability of thyme oil emulsions, the size distributions of the emulsion droplets was considered as a quality parameter. Triplicate samples of emulsions were stored at room temperature for 60 days. The particle size distributions of freshly prepared emulsions were measured at 25 °C and subsequent measurements were made every 10 days intervals during storage period. For each measurement, the sample was diluted with deionised water to about 25 times to ensure the count rate (kcps) was above 300 during the measurement. Each sample was measured in triplicate, and each measurement had 60 running cycles. The average particle size with standard deviation were plotted against the time of storage to evaluate the emulsion stability.

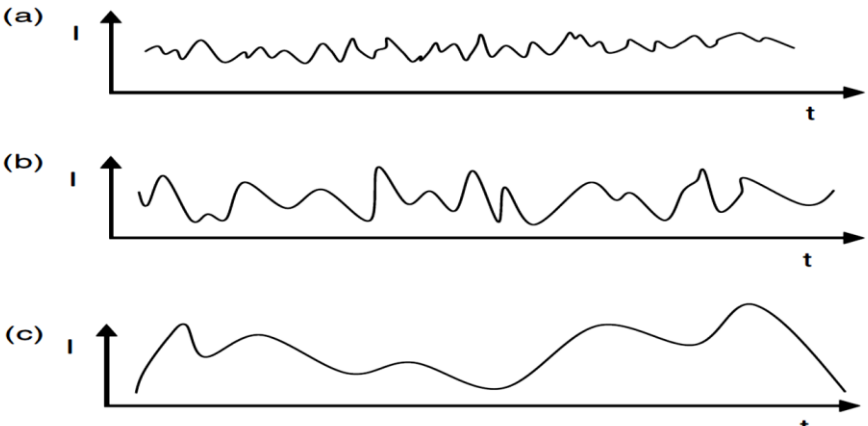


Figure 4.2: The differences in scattered light intensity between different size particles: 1-100 nm particles, (b) 100-500nm particles, (c) 500-1000 nm particles (Amanda et al., 2005).

Droplet charge

The molecules of the biopolymers used in stabilising emulsions have an electrical charge which can be measured by tracking the motion of the particles in the suspension by Laser Doppler Micro-electrophoresis (LDM). In this technique, an electrical field was applied in the dispersion. As a result, the particles move towards the oppositely charged electrode. The velocity of the particles depends on the magnitude of the ζ-potential. The electrophoretic mobility of the particles in the suspension can be calculated by measuring the velocity using phase analysis light scattering (PALS). Henry's equation is used to determine the Zeta potential (ζ-potential) as shown in Equation 4.7.

$$\boldsymbol{z} = \frac{\boldsymbol{U_E 3\eta}}{\boldsymbol{2\varepsilon f(Ka)}} \qquad (4.4)$$

Where z is zeta potential, U_E is electrophoretic mobility, η is the viscosity of the suspension, ε is dielectric constant and $f(Ka)$ is Henry's function (1.0-1.5).

The electrostatic interactions between droplets or particles are important in particle aggregation and stability of the emulsion. The degree of the ζ-potential is an indication of the strength of repulsion forces between particles in the suspension. ζ- potential depends on the pH and ionic strength of the emulsion (Jahanshahi and Babaei, 2008).

ζ-potential of different emulsions was measured by diluting the samples with deionised water in 1:25 ratio. The diluted samples were then added to plastic folded cuvette equipped with gold electrodes, three measurements for each sample were carried out in the Zetasizer for 60 cycles. Each sample was measured at the first day and every 10 days intervals for 60 days. The data obtained statistically analysed and plotted in graphical forms.

Droplet morphology and micrograph of the emulsion

Conventional microscopy (Leica, Wetzlar, Germany) equipped with a camera (Leica DC100) was used to visualise the emulsion droplets and powder particle microstructure. For optical microscopy, samples were diluted and placed on a microscopic slide using a dropper and covered with a coverslip. The images were labelled according to the degree of magnification used. The scale used for each magnification was drawn on the images to visualise the particle size distributions which were compared to the size distributions obtained by light scattering equipment (Zetasizer).

Viscosity measurements of the emulsions

The dynamic viscosity was measured for different samples using glass capillary viscometer (u-tube) which is called Ostwald viscometer. 25 mL samplewas added into the viscometer which was held vertically and submerged in a water bath at 25 °C for 15 min prior to measurements to control the temperature of the samples. Then the emulsion was raised through the narrow bore capillary tube using a suction pump to fill the upper bulb up to the mark above the capillary tube. The liquid then allowed to flow to the lower bulb again. Dynamic viscosity was measured by the time required for the liquid to pass through two marks above and below the upper bulb multiplied by a viscometer factor of 0.01404.

Recovery of thyme essential oil using Clevenger apparatus

To recover essential oils from different samples, Clevenger which is a circulatory apparatus was used, in which known amount of sample and water were placed in the round-bottom flask. The round-bottom flask with its content placed on a heated mantle and connected to a condenser with a graduated tube to measure the volume of the separated oil. The water steam takes volatiles into condenser then falls into the tube, the oil layer float on top of the water layer and the excess water recirculate back into the

round-bottom flask from the bottom of the graduated tube (Figure 4.3). The volume of the trapped EOs was recorded and withdrawn from the bottom tap of the graduated tube. The collected essential oil was transferred into Eppendorf tubes and centrifuged at 4000 rpm for 10 min. Subsequently, the oil phase was transferred into another Eppendorf tube which was stored in a freezer for further investigations. The Clevenger apparatus recovers most of the essential oils from the sample due to re-distillation of the recirculated water into the flask over 3 hours of the process (Rao et al., 2005).

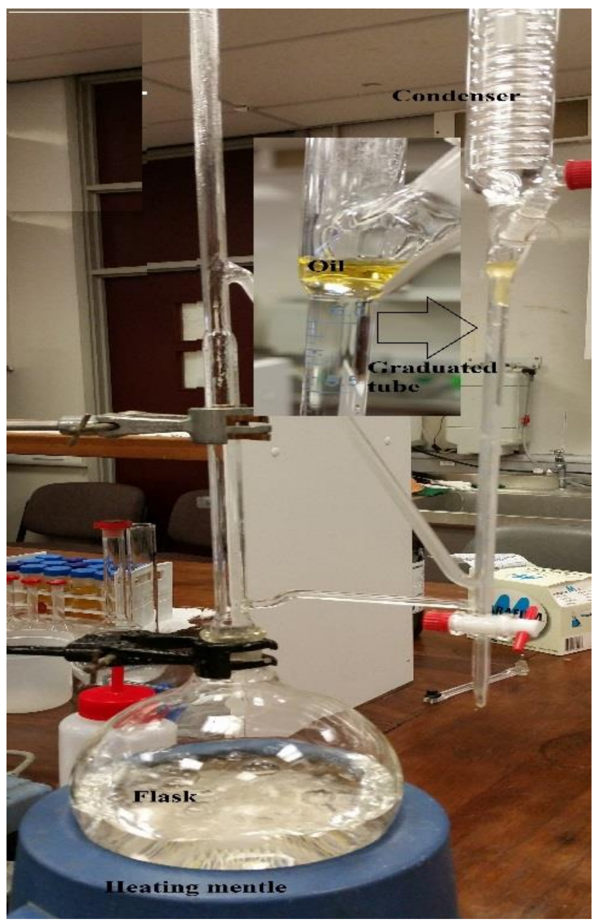


Figure 4.3: The Clevenger apparatus

4.3.2.3 Spray drying of the thyme oil emulsions

Thyme EOs emulsions were prepared as described in Section 4.3.2.2 and 4.3.2.3 and spray drying technique was used to prepare dry powder containing thyme essential oils. BüCHI mini spray dryer (B-290-Switzerland) was used to perform the drying of the different thyme EOs emulsions. The co-current hot air was used with air pressure nozzle

to atomise the emulsion into the drying chamber. The operation conditions of the drying were as the following: the inlet temperature was 120 °C ± 2, outlet temperature 70 ºC ± 5, the aspirator speed was 100% (35 m^3/h), pump speed was 20% (6 mL/min), 0.4mm nozzle orifice was used and the air flow was adjusted at 50 on the flow meter (0.75 bar). Each emulsion was spray dried in duplicate and the samples were collected and weighed, vacuum packed and stored at room temperature. The powder production yield was calculated by using the following equation:

$$P_Y = \frac{W_{PP}}{W_{TS}} X\ 100 \qquad (4.5)$$

Where P_Y is the production yield, W_{PP} is the weight of the produced powder and W_{TS} is the weight of the total solid of the initial emulsion.

The characterisation of the encapsulated thyme EOs

Spray dried powder was characterised in terms of encapsulation efficiency, particle size and morphology, moisture content, surface oil and dissolution in water and release rate were evaluated. The chemical composition of the released oil during the dissolution of the powder also analysed using various techniques as described in the following sections

Cryo-scanning electron microscopy (CSEM)

A cryo-scanning electron microscopy (FEI Quanta 200F FEG ESEM -USA) was used to study the micro structure and morphology of spray dried powders. Samples were prepared by mounting sample stub on the CSEM transfer holder then a small portion of the specimen was added and fixed by cryogenic glue then mounted to another device called vacuum transfer. Liquid nitrogen was added to the slushing station and vacuumed to slush the nitrogen and the samples frozen. After freezing the slushing station was vented and the sample was transferred into cryo preparation chamber. Once the sample was transferred into the sample chamber, a high voltage of 10kv electron beam was applied to the specimen and the interested areas of the samples were magnified and images were taken.

The moisture content of spray dried thyme EOs emulsions

The moisture content of produced powders was measured directly after the powder was formed and every 30 days for 6 months by Karl Fischer (MKS-500- Japan) which depends on volumetric titration and it is more accurate, rapid and selective than other methods. The instrument consists of different parts as shown in (Figure 4.4). The moisture content of the sample is determined according to the volume of the reagent reacted with water. The Karl Fisher vessel was cleaned and dried using methanol. Then 50 mL methanol was added to the titration vessel to cover the Pt electrode then pre-tittered until reached the dehydration of the cell. The reagent used was (HYDRANAL-Composite 5, Fluka). Reagent factor (mgH_2O/mL reagent) measured by injecting a known amount of pure water into the vessel and the result appeared when end point (EP) had detected then the result stored in the instrument. Subsequently, triplicate measurements of the samples were carried out by adding known amount of the sample into the cell and the measurement carried out as described above. The moisture content of the samples was automatically calculated depending on the reagent factor, and volume used to dehydrate the sample. The measurement of water content from the Karl Fischer titration is based on the reaction of water with iodine and sulphur dioxide in the presence of alcohol and base as shown below.

$$H_2O + I_2 + SO_2 + CH_3OH + 3RN \longrightarrow [RNH]SO_4CH_3 + 2[RNH]I$$

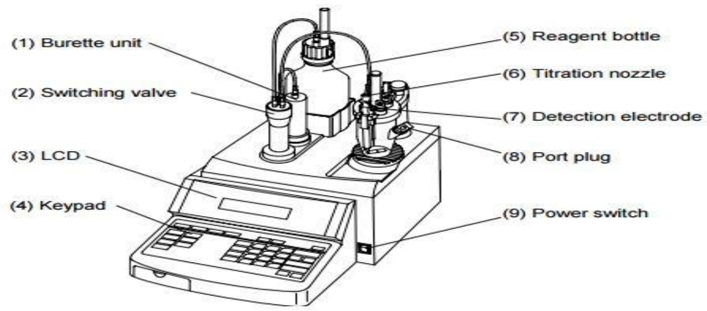


Figure 4.4: Karl Fischer instrument.

The surface oil measurements of the encapsulated thyme EOs

In order to quantify of the amount of EOs migrated onto the surface of the particles during spray drying was determined by extracting the surface EOs with solvent. One gram of spray dried powder was added to 9 mL of methanol in 10 mL plastic test tubes. The

tubes were shaken gently for 1min, the suspended solution was centrifuged at 3000 rpm for 5 min. One millilitre of the supernatant which contains the EOs was mixed with 14 mL methanol: water (50:50) solution in a glass vial and was analysed by GC-MS.

Encapsulation efficiency of thyme EOs in different powder formulation

To quantify the encapsulated thyme essential oils in the powder, a Clevenger apparatus was used. For each sample about 5g of powder was added into round-bottom flask mixed with 300 mL deionised water and stirred using a magnetic stirrer to ensure complete dissolution of the powder in the solution. After heating on setting 1 on the heated mantle for 3hours, the volume of the collected EOs in the graduated tube was recorded and multiplied by the specific density of the EOs (0.917 g/mL) to obtain the weight of the recovered thyme EOs. The encapsulation efficiency was calculated using the equation below:

$$Encapsulation\ efficiency\ \% = \frac{Recovered\ oil\ \ (g) - Surface\ oil(g)}{Theoretical\ oil\ content(g)}\ x\ 100 \qquad (4.6)$$

As the EOs are prone to evaporation and loss during spray drying, therefore, the recovered and theoretical oil content was calculated. The retained EOs in all types of powder were measured on the first day of the powder production and followed every 30 days during a storage time of 6 months.

The evaluation of thyme EOs powder dissolution in water using UVM

The dispersion of the powders, when introduced to the aqueous phase, is one of the important properties of the powder. In the food industry, the term “Instant” is usually used with powdered milk, soups, cocoa, coffee and sugar to describe the dissolution and dispersion of these food products in water in a short time. Dissolution of powder occurs in four steps including wetting, sinking, dispersing and solubilising.

To determine the dissolution rate of the samples non-destructive technique of ultrasound velocity meter (UVM-Cygnus) instrument which is designed by Povey (1997a) was used (Figure 4.5). The device is sensitive to detect the aggregated structures in the dispersion medium. The instrument consists of central processing unit, ultrasonic transducer (transmit/ receive), temperature sensor and magnetic stirrer. The dissolution was tracked by transmitting and travelling of the ultrasound waves through the dispersion and receiving the signal by the ultrasound receiver probe (Povey, 1997a).

The UVM cell placed in a water bath to control the temperature at 25 °C and filled with water (70 mL) to cover the ultrasound transmitter-receiver probe. The measurement was carried out in deionised water with continuous stirring prior to sample addition until a constant value of the ultrasound velocity in the cell is reached. Subsequently, 1g of spray dried powder sample was added on the top of the water in the cell. The presence of agglomerated particles in the suspension medium resulted in the loss of ultrasound signal for some time depending on the rate of dissolution of the powder.

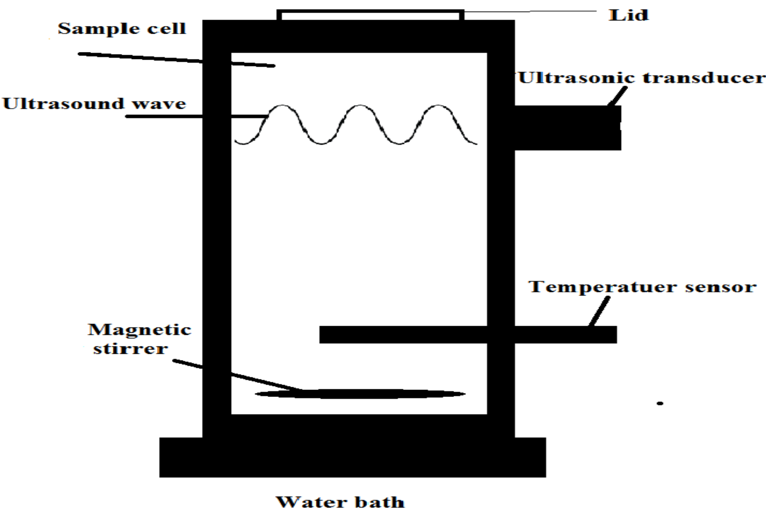


Figure 4.5: Cygnus ultrasound velocity meter (Povey, 1997)

After complete dissolution of the powder and forming homogeneous suspension, the signal appeared again which was proportional to the time required for the powder to disintegrate. The differences between the times when the signal disappeared and reappeared was determined and recorded as the dissolution rate time. For each test, at least three samples were used, the dissolution time presented as the mean time with the standard deviation for each sample.

The release rate of the essential oils from spray dried powders

The release of the EOs components for all types of spray dried powders was determined for 60 min of powder dissolution in the UVM cell. Samples were taken from the reconstituted powder for 4 times (every 15min) by syringe. The samples were then filtered into Eppendorf tubes using 0.45 syringe filters. Samples were stored in a freezer for GC-MS analysis. On the day of the analysis, the frozen samples were allowed to thaw at temperature and 0.5 mL of the sample was added to 14.5 mL of methanol: water solution (50:50) in GC-MS vial and analysed. The release rate was measured depending

on the release of three principal components (thymol, carvacrol, and p-cymene) in thyme EOs. The concentration of these components was calculated according to their peak areas in the GC-MS vial. The percentage of the released components was then calculated according to the encapsulation efficiency of the powder and the percentage of the components in the encapsulated EOs. The release rates were plotted against the release time.

4.4 Results and discussion

This section contains different results starts with the composition of skimmed milk which was used as the continuous phase in encapsulation of thyme EOs. The composition of different emulsions and their characterisation are reported. Moreover, the results from composition and characterisation of the powders produced from thyme oil emulsions is demonstrated. These involve particle morphology, surface oil, encapsulation efficiency, dissolution, and the release rate of various powders.

4.4.1 Skimmed milk

Commercially available skimmed milk was analysed in order to confirm its content as it would be used as developed delivery system for thyme essential oils. To reduce variability, three samples were used for each measurement. The results from the skimmed milk analysis indicated slight differences in content between measured values and the values claimed on the label. The protein content was lower by 0.12 % which was considered normal. The fat content was higher than the label by 0.03% and the carbohydrate by 0.15%. The ash and moisture content showed lower levels by 0.2 % and 0.66% respectively. The label also claimed that 124 mg calcium is present as shown in (Table 4.2).

Table 4.2: The comparison between the measured and labelled composition of skimmed milk.

Composition	Measured %	Labelled %
Proteins	3.28 ± 0.03	3.4
Fat	0.13± 0.02	0.1
Carbohydrates	5.15± 0.03	5.0
Ash	0.8± 0.008	0.1
Moisture	90.64± 0.01	91.3
Total	100	100

Table 4.3: The milk protein fractions (Swaisgood, 2008).

Protein fraction		%	Protein fraction		%
Casein	α- s1 casein	~44	Whey	β-lactoglobulin	~55
	α- s2 casein	~11		α-lactalbumin	~24
	β -casein	~32		Serum albumin	~5
	κ-casein	~11		Immunoglobulins	~15

The amount of the protein in skimmed milk is sufficient to allow it to be utilised for emulsifying 4 to 5% of thyme essential oils. The proteins are originally available to emulsify between 3-4% of fat in whole milk. Milk proteins comprise of 80% casein and 20% whey protein (Swaisgood, 2008). Caseins are divided into four different fractions Table 4.3, they are flexible and disordered proteins in the solution. On the other hand, whey proteins are comprised of a number of different proteins.

The properties of the emulsions prepared with caseins are governed by the presence of polar and nonpolar groups on the caseins. The emulsification properties of whey proteins are directly linked to the presence of β-lactoglobulin. Emulsions prepared with a mixture of these proteins may improve the stability. However, Dickinson (2011) stated that the presences of these types of proteins affect the interfacial composition of the coating film as they tend to compete to adsorb at the interface which can result in decreasing emulsion stability. To the best of our knowledge, there is a lack of reported research using liquid skimmed milk as the original solution to emulsify oils. Successful use of milk to deliver essential oils is potentially important for use in food systems, pharmaceuticals and/or cosmetic industries.

4.4.2 The characterisation of thyme emulsions in skimmed milk

Different parameters are measured to characterise emulsions produced in this work. The stability of the emulsions as an important parameter was monitored by visual observations and through instrument-based approaches using Zetasizer, viscometer and light microscope. The particle size distribution and zeta potential as established procedure

for thyme oil in skimmed milk emulsions were measured during the storage of the emulsions.

4.4.2.1 The emulsion capacity of skimmed milk

The optimum concentration of thyme EOs in skimmed milk, required to form a stable emulsion, was found by adding different concentrations of EOs and inspection of the emulsion stability. It was found that the emulsions contained 3, 4 and 5% w/w thyme EOs were the most stable emulsions among those produced. However, lower concentrations of the essential oils in the emulsions resulted in instability with phase separation, these were discarded and were not used in further experiments (Figure 4.6). The highest ratio of EOs 5% w/w was nominated for further experiments as it gave around 30% w/w essential oil after spray drying in the final product. Therefore, this fraction was considered the optimum ratio of core material to wall material which it was found to have increased stability during drying and storage. As shown in the later section the emulsions were characterised by relatively small particle diameter < 225 nm and a monomodal particle size distribution.

From the (Figure 4.6), it is clearly illustrated that the emulsion remained stable more than one week when the amount of EOs was greater than 3% w/w. However, in general, stability monitoring of these emulsions was undertaken for only 1week. And more measurements were carried out on other emulsions as illustrated in the later sections.

The emulsions at lower concentrations of the EOs formed a gel-like structure without separation of the EOs. This may have occurred because the emulsion system contains different particles with different sizes such as large oil droplets and small protein of casein micelle molecules and higher quantity of proteins in emulsions with lower oil content. Therefore a possible reason for the instability of the emulsions containing lower amounts of thyme oil may be due to depletion flocculation due the excess proteins (nonadsorbed) in the emulsion (Dickinson and Golding, 1997) in addition to excess carbohydrates in the systems. The osmotic pressure and depletion interactions encourage the steric exclusion of the smaller size polymers or casein micelles, expelling them from the regions between the large oil droplets, when the width of that region is equal to the radius of the polymers or radius of casein micelles.

Figure 4.6: The ability of the wall material to hold different concentrations of thyme EOs, (1) 1%, (2) 2%, (3) 3%, (4) 4% and (5) 5% w/w thyme EOs in skimmed milk after 1 week of storage.

As the concentration of the protein decreased in the area between two large oil droplets, it increases in the remaining part of continuous phase. Therefore, to reduce the concentration of protein in these continuous phase regions to these parts of solution with the solvent tends to move from excluded region to these parts of solution without particles. Thus, the distance between two droplets decreases which results in aggregation of oil droplets. Another explanation is that when lower concentrations of oil is used, different proteins compete and displace each other to adsorb on the surface of the oil droplets. The remaining displaced proteins in the solution tend to cause flocculation for the same reasons mentioned above (Dickinson, 2011). Therefore, higher concentrations of oil resulted in more surface area for all proteins in the emulsion to be absorbed, leaving little or no free polymer in the continuous phase they leading to more stable emulsions.

4.4.2.2 The composition of delivery system of thyme essential oils in skimmed milk and different concentrations of maltodextrin

All oil-in-water emulsions were prepared using high shear laboratory mixer followed by two passes through GEA homogeniser. The compositions of the emulsions were varied determined by the desired content within the matrices. All emulsions contained the same EOs concentration 5% w/w with different maltodextrin (MD) concentrations. Maltodextrin was used because it added many features to the emulsion and the subsequent powders; these relating to total solid and EOs retention during spray drying, as mentioned separately in the literature review and as discussed in the later sections. The emulsions

denoted SM followed by different numbers which represents the MD percentage in different systems Table 4.4. Thyme oil emulsion prepared in a sodium caseinate (SC) solution was found to contain lower total solids at 8% w/w. However, the total solids of the other emulsions with skimmed milk (SM) were comparable to the amount of the added maltodextrin, with this increasing from 13.90 % w/w in SM0 to 16.61% w/w in SM3. The oil content of all emulsion remained constant at 5% w/w, the variation in other emulsion components resulted from the addition of different amounts of MD. As the MD content increased from 0 to 3% w/w the protein content also decreased to somewhat from 3.11% w/w to 3.01% w/w. Similarly other components such carbohydrate, ash, fat content also all decreased with increase of MD.

Table 4.4: The expected composition of 5% thyme oil emulsion in, 3% w/w (SC), SM0 without MD, SM1 with 1% w/w MD, SM2 with 2% w/w MD, SM2.5 with 2.5% w/w MD and SM3 with 3% w/w MD.

Emulsion composition	SC	SM0	SM1	SM2	SM2.5	SM3
Thyme oil %	5	5	5	5	5	5
Maltodextrin %	0	0	1	2	2.5	3
Protein %	3	3.11	3.08	3.05	3.03	3.01
Carbohydrate (Lactose) %	0	4.89	4.84	4.78	4.76	4.73
Ash%	0	0.76	0.75	0.74	0.74	0.73
Fat %	0	0.12	0.12	0.12	0.12	0.11
Total solid %	8	13.89	14.79	15.70	16.15	16.61
Moisture %	92	86.10	85.20	84.29	83.84	83.38
Total %	100	100	100	100	100	100

In this experiment, the visual stability of thyme EOs emulsions in skimmed milk were monitored for creaming or phase separation during storage period of 60 days. Other stability parameters such as particle size distribution and zeta potential are shown Sections 4.4.2.4 and 4.4.2.5.

4.4.2.3 The stability of thyme EOs in skimmed milk and different MD concentrations in comparison with thyme EOs in sodium caseinate

The emulsions with different concentrations of MD, showed stability over this storage time. No creaming or separation of oil layer was observed during this period. All samples showed a similar pattern regarding the stability as shown in (Figure 4.7).

0 day

After 60 days

Figure 4.7: The stability of the 5% w/w thyme oil emulsion during 60 days of storage at room temperature in skimmed milk (SM0), SM with 1% w/w MD (SM1), SM with 2% w/w MD (SM2), SM with 2.5% w/w MD (SM1) and in SM with 3% w/w MD (SM1).

After 60 days samples formed light gel-like clumps without any distinct layer of cream or oil thus the tracking of size and zeta measurements were terminated at this point. However, no phase separation was detected for the emulsions containing MD even after 10 months of storage and the flocculated emulsions reproduced when shaken.

On the other hand, to compare the differences in stability between thyme oil emulsions in skimmed milk another thyme oil 5% w/w emulsion prepared using sodium caseinate 3% w/w. The prepared emulsion rapidly formed an extensive creamed layer, within 24 hours of storage. As shown in (Figure 4.8) after 7 days a thick layer of creaming appeared. Further storing over weeks resulted in EOs separation, this usually called “oiling off” due to flocculation and coalescence. When samples were investigated by conventional microscope after a week, it was clearly observed that flocculation had taken place as shown in (Figure 4.9). The flocculation and particle coalescence also confirmed by particle size distribution measurements in section 4.4.2.4.

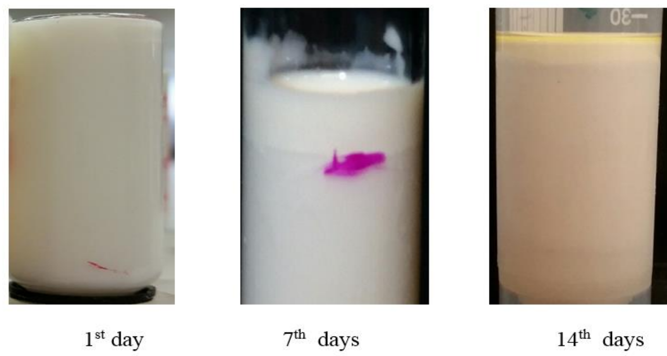


Figure 4.8: Thyme oil 5% w/w emulsified in sodium caseinate solution 3% w/w.

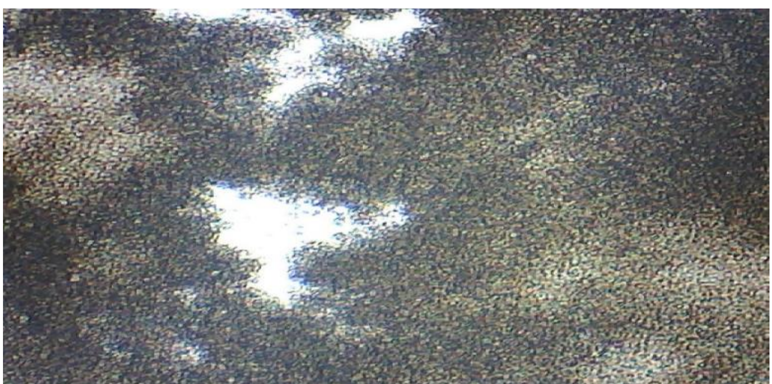


Figure 4.9: Flocculated thyme oil droplets emulsified in sodium caseinate solution after 7 days of storage at room temperature.

Using skimmed milk to emulsify EOs was successful regarding the stability, cost, simplicity, time of preparation, availability and the size of the droplets. The stability of emulsion was estimated by measuring droplet size and zeta potential using Zetasizer. The mean diameter of the droplets (nm) of the emulsions remained below 250 nm during 60 days of the storage. There are several different components in formulation all of which can influence and alter colloidal interactions between oil droplets. These include milk proteins, added polysaccharide and calcium. Interactions between molecules such as electrostatic, van der Waals and steric may affect the colloidal particles interactions as well. The particle interactions govern the aggregation, the association, size of the colloidal particles. The droplet interaction determines the stability, sensory, texture, appearance and mouthfeel of the food emulsion (Dickinson 2006, 2010, 2013).

The emulsions in skimmed milk and MD showed high stability during 60 days of storage. The improved stability may be due to many factors including the amphiphilic behaviour of the milk protein at the interface where the lipophilic parts tend to adsorb on the oil droplets, and the hydrophilic parts remain in aqueous phase this allowing protein to mediate steric repulsion, hence preventing the droplets from aggregating (Xue et al., 2013). The smaller diameter of the emulsion droplets is another factor in preserving the emulsion from creaming. Creaming behaviour accelerated with the size of the droplets. The presence of high density MD around the small droplets of EOs decreases the density differences between dispersed and continuous and increases the viscosity of the system thus slows down the gravitational separation. Moreover, a stable emulsion may be due to the presence of polar groups (-OH or -O) on some of the compounds in essential oils such as carvacrol, thymol, terpinene-4-ol, borneol and camphor which may interact with molecules on the interface. They may able to interact with other molecules in the surrounding such as proteins and water via van der Waals, dipole-dipole and hydrogen bonding. Whereas other oils such as vegetable (triacylglycerol) are non-polar and only interact with water molecules at the oil-water interface via relatively weak van der Waal interactions this is a result of the lack of polar groups which considered relatively weak compared to the hydrogen bonds (McClements, 2015). In the first case, a thicker and a more flexible interfacial region is formed which is promotes the stability of the emulsion. The absence of creaming or sedimentation due to flocculation and coalescence, over the storage time, is a strong evident that the repulsive forces such as electrostatic and steric are dominant upon the attractive van der Waals forces in the current system.

To visualise the interface, which is hard to see in small emulsion droplets, a coarse emulsion of thyme essential oil in skimmed milk was prepared to visualise the interfacial region between thyme oil droplets and water. The image confirmed the distribution and adsorption of different biopolymers molecules on the oil droplets which were used as wall materials (Figure 4.10). The presence of calcium and salt in the skimmed milk used for forming the emulsions may contribute to the binding and cross-linking of different proteins and amino acids within the same protein at the surface of the oil droplets. From the figure, it is evident that a thick interfacial layer was formed which is favourable regarding the steric stability of the emulsion.

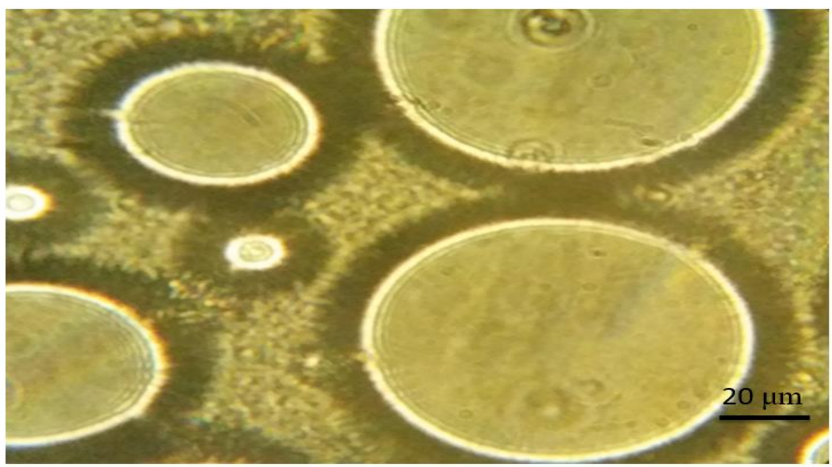


Figure 4.10: Light microscopy images showing the thick interfacial layer at the interface between thyme oil droplets and water.

To investigate the difference between essential oils which contains amphiphilic compounds and sunflower oil which contains only hydrocarbons an experiment was carried out by preparing 5% w/w sunflower oil in skimmed milk and prepared in the same way to thyme EOs. Results clearly showed that there were a differences in particle size distribution and stability when skimmed milk was used as the dispersion phase for sunflower oil to form oil-in-water emulsion. The emulsion showed relatively large particle size distribution 336.46 nm when measured directly after preparation and increased. Whereas thyme oil emulsion 5% w/w in skimmed milk was around 225 nm and remained relatively small over storage time for 2 months. In contrast, the particle size of the sunflower oil emulsion dramatically increased and reached 425.85 nm after 21 days of storage. It is more likely that a thin interface layer was formed which may induce

flocculation and coalescence, increasing droplet size but increasing the instability of the emulsion.

From the results, it can be suggested that proteins can be easily utilised to deliver hydrophobic EOs into aqueous food products. Caseins as flexible protein contain more hydrophobic groups which able to reduce the interfacial tension between water and oil (Dickinson, 2011). The presence of polar groups on some EOs compounds formed smaller particles sizes, thick interfacial layers with resulting increased stability.

4.4.2.4 Particle size distribution of the thyme EOs emulsions in skimmed milk and different maltodextrin concentration and in sodium caseinate

The same thyme EOs emulsions which prepared as illustrated in the previous section were used to monitor both particle size and zeta potential behaviour during storage time. The effect of different concentrations of MD on the emulsion droplet mean diameter over 60 days of storage is shown in (Figure 4.12). The mean diameter of skimmed milk was 174.29 nm and for emulsions were between 225 and 237nm for all samples with a narrow distribution of particle size when 0 to 3% MD was used. Figure 4.11 shows an example of the data obtained from SM3 emulsion when analysed by Zetasizer instrument.

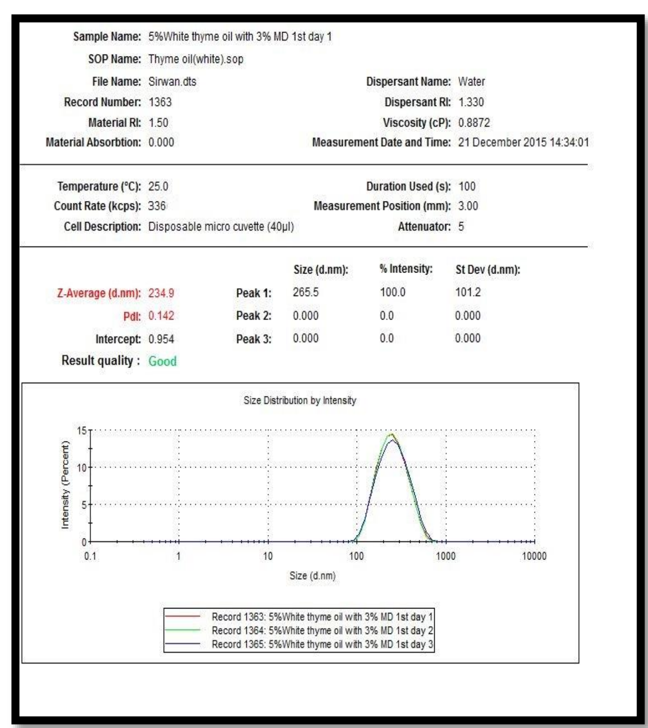


Figure 4.11: An example of particle size distribution in sample SM3 directly after preparation.

As shown in the example above the emulsion had a monomodal distribution with an average particle size of 234.9 nm and a relatively broad standard deviation σ value 101 nm. No large particles can be observed as there was absence of other peaks of large particles. The absence of large particles sizes was confirmed by microscopic images of the prepared emulsions as showing in (Figure 4.13).

Similarly, thyme EOs emulsion prepared with sodium caseinate showed small particle size at the beginning of the storage but then increased rapidly after 14 days reaching 286 nm. The phase separation was clearly observed during and after 14 days of storage (Figure 4.8). Similar instability of thyme EOs emulsion in SC was observed by Xue and Zhong (2014), they reported that the creaming of the emulsion appeared after 2-3 days of storage. They also stated that the emulsions became unstable when stored for longer times. They suggested possible causes for creaming of the thyme oil emulsion to be depletion flocculation (resulting from the excess SC in the continuous phase) which led to increased particle size and coalescence. The current study suggests that sodium caseinate is not a suitable as the stabilising dispersant in EOs encapsulation, if used without any chemical treatments.

Adding MD into skimmed milk altered the size of the droplets. There is a significant difference in the droplet mean diameter when different amounts (1-3% w/w) of MD were added into the dispersing phase. The average size distribution of emulsions containing 1% MD (SM1) was 226 nm, which is larger by around 5 nm than the emulsion without MD (SM0) and were statistically significantly different. The different value of particle size distribution then increased and reached 13 nm when MD in the system was increased to 3% w/w. The statistical comparison of the means showed significant differences ($P < 0.05$) between SM0 and SM1 and other emulsions. Statistically insignificant differences were observed between SM2, SM2.5 and SM3 whereas SM1 was significantly different to all the other emulsions. No significant ($p < 0.05$) changes in mean diameter were observed throughout the 60 days of the storage for all groups of the emulsions. All emulsions were characterised by a monomodal and narrow mean diameter distribution. Moreover, there is consistency between the mean diameter results and the image of the emulsion shown in (Figure 4.13).

Using skimmed milk to emulsify thyme EOs has been shown to be a practical and reliable alternative for other emulsifying techniques to produce a small size particles with extended stability. The main reason for emulsion instability arises from the interactions

between two colloidal droplets via attractive van der Waals forces, the density differences between dispersed phase and a continuous phase, coalescence and Ostwald ripening. The small particle size of the nanodroplets and thick interfacial layer adsorbed on their surfaces in this work are the main factors controlling stability. The small droplets in the emulsions remained apart because attractive forces between the droplets fall off with separation distance more rapidly than the repulsive forces perhaps due to a stable interfacial layer. Moreover, the lack of creaming or sedimentation observed in the emulsions is also attributed to the small size of the droplets as they have a higher Brownian motion which counters gravitational separation. Additionally, the interfacial layer on the oil phase somewhat decreases the differences in density with the water phase which in turn improves stability against creaming (McClements, 2015).

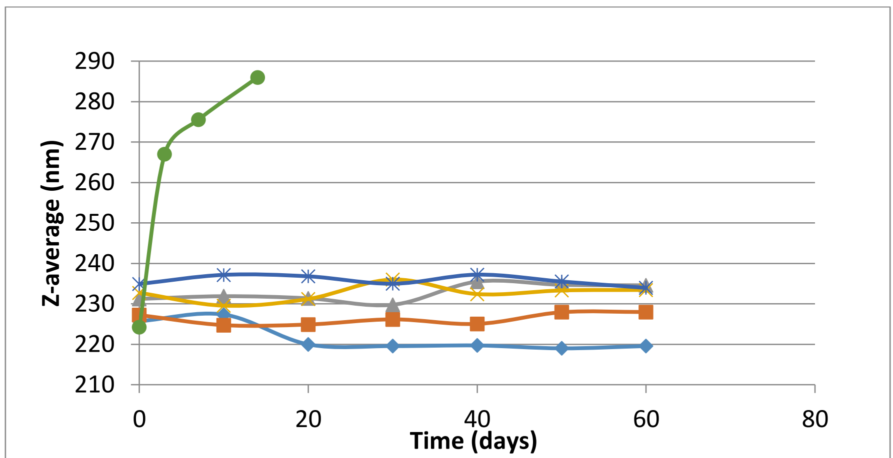


Figure 4.12: The mean diameter of different emulsions over 60 days of storage at room temperature, SM0, SM1, SM2, , SM2.5, SM3 and SC.

The caseins, and whey proteins in milk as a globular proteins are able to adsorb and form a thick layer (10nm) on the oil droplets during the process of homogenisation (Dickinson et al., 1998). The stability against flocculation and coalescence of the emulsion is due to repulsion forces from steric, and electrostatic forces between droplets arising from the adsorbed layer at the interface (Hu et al., 2003). The emulsions containing nanodroplets are usually more stable than conventional emulsions due to a small particle size which improves resistance to destabilisation factors (McClements and Rao, 2011, Li and Lu, 2016). The coadsorption of the amphiphilic protein whey and casein at the interface reduces the interfacial tension of the system which is favourable thermodynamically. The whey proteins on the interface are able to produce a viscoelastic

film on the droplets which makes them more resistant to rupture and heat stability during process (McClements, 2004c).

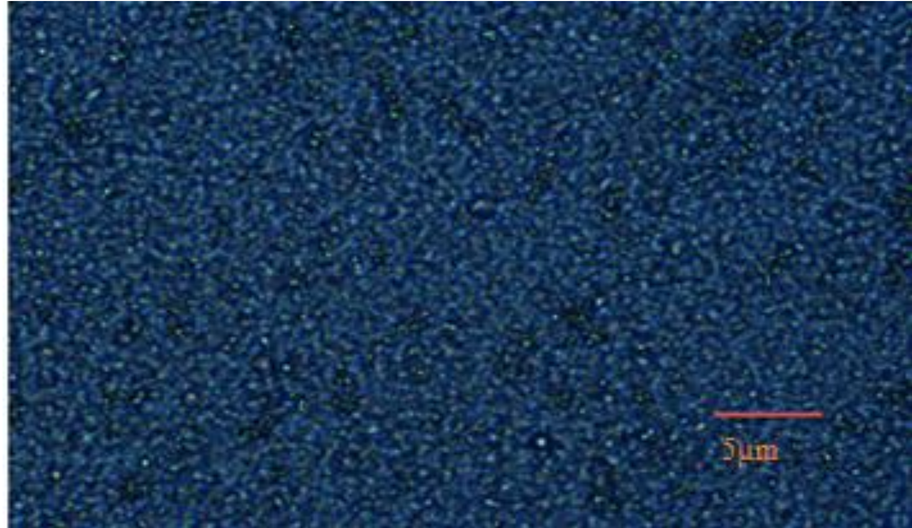


Figure 4.13: Microscopic image of 5% thyme EOs with 3% MD in Skimmed milk.

Moreover, the skimmed milk in this study contained calcium as indicated on the label (12.4 mg/100 ml). Therefore the adsorption of the casein increased as the calcium was able to induce aggregation of casein at the interface and increase surface load (Dickinson, 2011). However, the explanation of the behaviour of systems containing whey protein, casein, MD and minerals in addition to essential oil remains complex to be fully understood at present. Some researchers reported that systems which contain only casein and whey protein are complex to designate and interpret the adsorbed layer (Zhang et al., 2004). Some of the essential oils components with polar groups are able to interact by different means at the interface, as described earlier these are able to make a flexible interface though are likely to be displaced by casien from the surfaces when later is present.

Liquid skimmed milk shown to have have a high encapsulation efficiency, produces stable emulsions, its ease of use, and of showing better compatibility with EOs than ordinary vegetable oil with regards to the stability of the emulsions. Moreover, the emulsion was prepared in a single step procedure within a short time and without requiring complex preparation of the continuous phase solution in advance. This avoids complex procedures such as hydration of the protein for 24 hours and adjusting pH by using acids and bases as required steps in preparing other emulsion systems, such as whey or casein protein stabilised emulsions . The milk components did not experience high

drying temperature treatment which it is required when casein or whey protein isolates are prepared from milk. Casein developed and isolated by many steps may affect its functionality and requires long time preparation for precipitation by lowering pH to 4.6, then resuspension and addition of NaOH to neutralise the solution, followed by spray drying to finally obtain sodium caseinate (Liu et al., 2012). Finally, one can suggest the blend of MD and proteins in skimmed milk as an excellent emulsifying agent for EOs due to the high stable emulsion, small droplets and narrower size distribution produced. The combination is advantageous over using each biopolymer separately because proteins are able to produce small emulsion droplets on the other hand emulsions containing polysaccharides are more stable when the emulsion environment changes such as pH, temperature, ionic strength (McClements, 2004b; McClements, 2015). The improvement of emulsion stability by MD was also reported by Turchiuli et al. (2014) who stated that MD supported acacia gum and resulted in narrower particle size distribution and stable emulsions. More likely that high amount of MD reduces the Brownian motion and slows down creaming.

The small size of particles obtained in the current study is beneficial when delivering active materials due to their high surface area, better stability and rapid diffusion rates (Lopez-Rubio et al., 2006; Davidov-Pardo et al., 2015; Jafari et al., 2008; Wang et al., 2015).

4.4.2.5 The Zeta potential of thyme EOs emulsions in skimmed milk and different MD concentrations

The Zeta-potential of the emulsions were measured for different emulsions prepared with skimmed milk and various concentrations of MD. The measured zeta potential of skimmed milk was -24.46 ±1.09 mV at pH 6.75. Directly after emulsion preparation, the pH value of the emulsions ranged between 6.7-6.9. All emulsions had a negative charge. The SM0 emulsions had the highest zeta potential value -32.12 ± 4.18 mV directly after preparation, and there was a slight reduction with the value reaching -29.68 ± 2.99 mV after 60 days of storage. The pH of the SM0 emulsions remained high at 6.72 ± 0.06 reduced slightly from 6.81± 0.05 at the beginning of the storage time.

The zeta potential of the thyme oil emulsions containing maltodextrin decreased insignificantly compared to the zeta potential of thyme oil without added maltodextrin. The Zeta potential for SM1, SM2, SM2.5 and SM3 emulsion were -31.49 ± 3.3, -31.13

± 3.11, -30.21 ± 2.18 and -30.30 ± 1.48 at (0 day) of the storage and changed slightly to -26.51 ± 2.17, -27.82 ± 4.09, -28.23 ± 1.7 and -28.57 ± 5.73 mV respectively after 60 days of storage. Generally, there was a variation in zeta potential within emulsion over the storage period. However, these variations remained too small and insignificant to have any major consequences and stability. As shown in (Figure 4.14), the magnitude of zeta potentials of all the emulsions were high during the storage time.

The surface charge of the particles is necessary to determine the stability of the emulsions for long term storage. The amphiphilic molecules which are adsorbed on the interface determine the nature of the interactions between droplets in the emulsion. The charged molecules induce repulsive forces between droplets through electrostatic interactions which prevent films from rupturing and coalescence (Ivanov, 2000). The negative charge of the emulsion is associated with the presence of protein on the surface above its isoelectric point (McClements, 2015).

There are no observed significant differences ($P < 0.05$) in zeta potential within the different emulsions as a result of the storage time. The values of the zeta-potential at the beginning of storage were well below -30 mV which is considered ideal for particles stability in the emulsion. The nanoparticles which have a zeta-potential below -25or greater than +25 mV are predicted to be highly stable against flocculation and coalescence due to the repulsive forces between droplets having the same charge (Heurtault et al., 2003, Israelachvili, 2011).

The zeta-potential values for all samples exhibited relatively high values over the storage time due to relatively constant pH (6.5-7). The reason for this is the ability of thyme oil to inhibit microbial growth such as lactic acid bacteria which converts lactose to lactic acid. Therefore, the milk proteins remain far from their isoelectric point, with high-charged molecules and relatively high zeta potential. As a result, the particles in the emulsions repel each other, and there was a reduced chance of particle collision and subsequent aggregation.

The results indicate that no significant impact was found for maltodextrin in the emulsions on the zeta potential. Thus, the origin of the negative charge may come from the casein and whey protein adsorbed at the surface of droplets and because of the pH of the systems remaining high and above the isoelectric point of the proteins. It is worth mentioning that essential oils also contributed to increasing the value from -24 mV of

skimmed milk to -32mV in skimmed milk containing 5% thyme EOs. However, the zeta potential of pure thyme EOs was not reproducible changed in magnitude and change continuously when tested. Thus, it is probable that when the EOs is added the negative heads of EOs compounds associate themselves at the interface to modify the magnitude of the zeta potential of the emulsion.

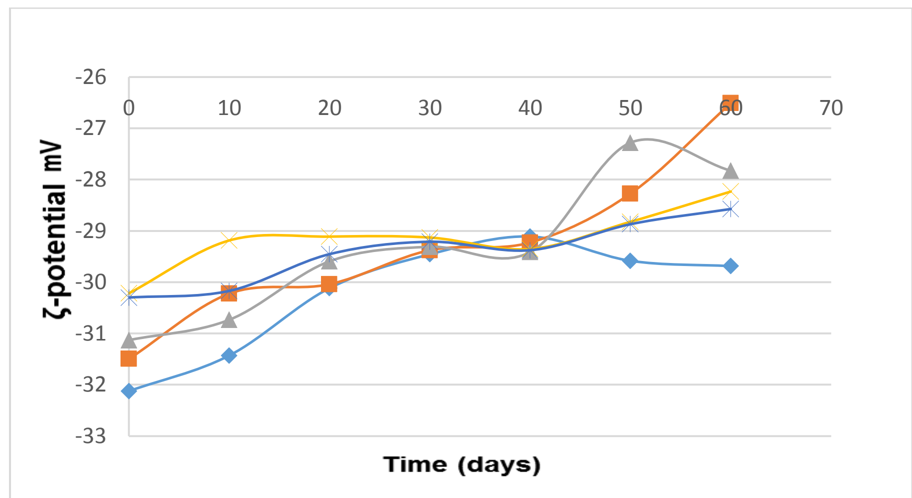


Figure 4.14: The zeta potential of the various emulsions during 60 days of storage at room temperature showing insignificant variations of only 3-4 mV during this time, SM0, SM1, SM2, , SM2.5 and SM3.

The results of the zeta potential of the emulsions in the present study were greater in magnititude than that reported by (Bonilla et al., 2012), who found that the zeta potential of thyme emulsion in distilled water was of the order of -13.4 and -10 mV dependent on the use of different homogenisation techniques. They also found that the emulsion charge was positive when they used chitosan as a wall material at pH 3.2 at which the amino group is positively charged.

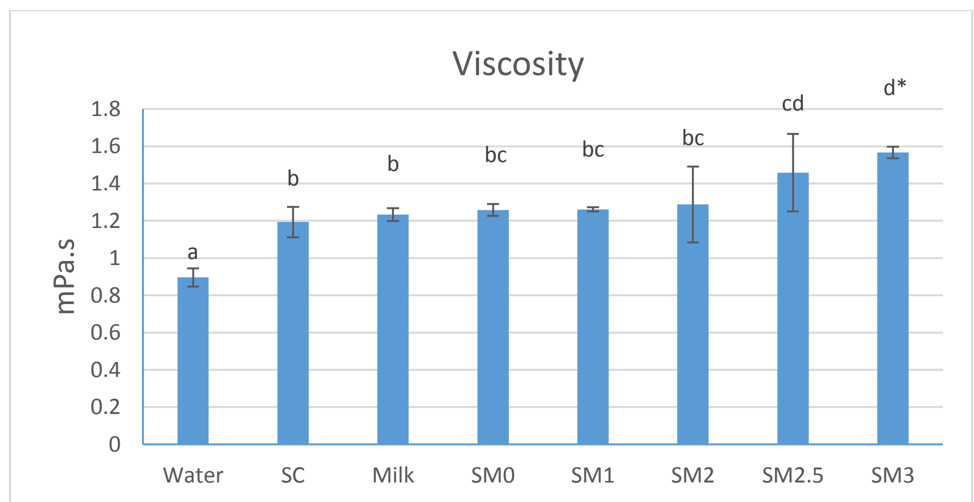
The main repulsive forces which stabilised the emulsions are steric and the electrostatic which may increase the energy barrier between the stable and unstable state of the emulsion in addition to the optimum size of the emulsion droplets which may help the emulsion to be stable (McClements, 2012). The small size of the particles also stabilised the emulsions against gravitational separation as according to Stoke's law the velocity of creaming decreases with small particles in addition to higher Brownian motion

and less density difference between dispersed phase and the oil by the interfacial layer (McClements, 2014).

4.4.2.6 The viscosity of different Thyme oil emulsions prepared by skimmed milk and MD and in SC

The viscosity of the solutions directly related to the concentration of maltodextrin in the systems. The lower viscosity value was for water (0.89 mPa.s) followed by sodium caseinate solution 3% w/w which were significantly different with each other. No differences were observed between SC, Skimmed milk, SM0, SM1 and SM2. For these solutions as the concentrations of maltodextrin increased to 2.5 and 3% w/w the differences became significant. However, the addition of higher concentrations of maltodextrin (3% w/w) raised the viscosity of the SM3 by 1.5 times that of water viscosity.

The addition of maltodextrin increases the viscosity and the densities of the dispersions. In contrast, no dramatic changes in viscosity occurred by increasing the proportion of MD. The bulk viscosity of the dispersion phase, and even more significantly the interfacial viscosity interface between water and oil is important in preventing coalescence between emulsion droplets.



* Bars with different letters indicate significant differences at ($p < 0.05$).

Figure 4.15: The dynamic viscosity of emulsions, as well as water and skimmed milk, at 25 ºC.

This is because when the viscoelastic layers are formed around the oil droplets, it helps to reduce surface distortion and prevents the formation of corrugations. In contrast,

droplets with low interfacial viscosity are more susceptible to rupture by fluctuating wave's mechanism which then leads to cause coalescence (Ivanov, 2000). In addition it is preferable to use low viscous feeds when spray drying the suspension. High viscosity suspension produces larger droplet when atomised in the spray drier and become harder to dry completely and increases the moisture content of the particles after drying (Turchiuli et al., 2014).

4.4.3 The characterisation of the spray dried powders of thyme oil emulsions

The microcapsules were formed by spray drying of thyme EOs emulsions and then characterised according to different wall materials and the concentrations of maltodextrin. Maltodextrin was selected based on the results of previous studies. In addition, fresh skimmed milk in the liquid state was used as a wall material and this has not been previously reported to be used for encapsulating essential oils of thyme. This section describes the spray drying of the emulsions, the composition, the morphology, the encapsulation efficiency, the dissolution rate, particle size, surface oil and the release rate of differently spray dried powders.

4.4.3.1 The composition of the formed powders from different emulsions

After the drying, the emulsions which had different concentrations of maltodextrin with skimmed milk were found to contain different amounts of EOs (Table 4.5) D letter is added to the name of all emulsions here when spray dried to identify spray dried thyme EOs powder from thyme EOs emulsions. The powder yields were high for all samples containing maltodextrin and increase with MD content; the yield increased from 74% w/w in DSM0 to 80 % w/w when 3% w/w maltodextrin added to the initial emulsion. Consequently, fewer particles were observed on the walls of the drying chamber and the cyclone of the spray dryer. No agglomeration of the powders was observed when collected from the product vessel which reflected the rapid drying of the wall material during drying for samples containing higher MD concentrations. The calculated EOs content of different powders according to the EOs content of the initial emulsions should theoretically contain from 60.37% w/w in powders prepared with sodium caseinate to 28.93 % w/w in powder produced from skimmed milk and 3% w/w maltodextrin. The actual EOs content was determined after recovering the oil from the samples. The total solid content of the initial emulsion determined the oil content of the powders, therefore,

when higher total solids were used the EOs content decreased. For example, the EOs of DSM0 was 34.61% w/w and decreased to 28.39 % w/w when the MD concentration in the initial emulsion was increased to 3%. Powders prepared with skimmed milk and maltodextrin contained lower amounts of EOs compared with powders formed with SC due to the presence of other milk components such as proteins, carbohydrates, salt and ash in the first emulsion system.

Table 4.5: The composition of the different powders formed from various emulsion formulations.

Emulsion composition	DSC	DSM0	DSM1	DSM2	DSM2.5	DSM3
Thyme oil %	60.37	34.61	31.28	29.48	29.30	28.39
Maltodextrin %	-	0	6.26	11.79	14.65	17.03
Protein %	36.22	21.57	19.29	17.99	17.78	17.13
Carbohydrate (Lactose) %	-	33.86	30.29	28.24	27.91	26.90
Ash%	-	5.26	4.70	4.39	4.34	4.18
Fat %	-	0.85	0.76	0.71	0.70	0.68
Total solid %	96.60	96.16	92.58	92.61	94.68	94.32
Moisture %	3.38	3.83	7.42	7.39	5.32	5.68
Total %	100	100	100	100	100	100
Production Yield %	74.54 ± 5.06	74.14 ± 2.65	76.92 ± 4.1	78.89 ± 2.93	79.51 ± 3.46	80.64 ± 3.26

Usually, drying is used to preserve perishable foods which contain high amounts of water or moisture that provides an excellent media for microorganisms to grow in. Milk is one of these foods which can be preserved for significant periods of time by spray drying. Dried milk powder contains fat which is preserved from oxidation (Faldt and Bergenstiihl, 1995; Millqvist-Fureby et al. 1999).

Carbohydrates play a great role in changing the encapsulation efficiency of milk proteins. Lactose as an example is used with sodium caseinate to increase the encapsulation rate of soybean oil (Faldt et al., 1996). High dextrose equivalent (DE) maltodextrin improved the spray dried soybean in sodium caseinate due to the formation of a strong interfacial layer around the oil droplets, preserving the casein micelles (Hogan et al., 2001). Hydrophilic interactions, hydrogen bonding and electrostatic interactions between carbohydrates and proteins are the major driving forces to form the complex of the biopolymers around the oil droplets (Doublier et al., 2000). The complex improves the emulsification properties of those compounds that are controlled by charge density effects (Mattison et al. 1999). Allison et al.(1999) stated that small molecular weight carbohydrates are able to protect the secondary structure of the proteins during drying.

The stable particles are those which contains less than 50% core material; this is obtained in powders prepared with skimmed milk and MD. In the current study powders prepared with sodium caseinate contained EOs above acceptable levels may affect the stability and the encapsulation efficiency.

Spray drying is an efficient method of drying encapsulated flavour and EOs which are heat sensitive because drying occurs over a relatively short time, and the temperature inside the atomised particles remain relatively low because the evaporation of the water on the surface adsorbs latent heat from the interior (Fang and Bhandari, 2012). It produces fine particles and a free flowing powder with high dispersity in an aqueous medium. The presence of MD increased the production yield of the spray dried emulsions and produced less hygroscopic powders with a lower risk of caking (Turchiuli et al., 2014).

Karl Fischer apparatus used for measuring the moisture content of the powders as conventional oven method was not reliable due to evaporation of most encapsulated EOs which is volatile with water and giving false results. It should also be noted that a trial attempt to freeze drying SM3 emulsion also carried out to compare it with spray drying technique, is also included in the discussion in next section.

4.4.3.2 Particle morphology of thyme EOs powders formed by spray dryer and freeze dryer

As shown in (Figure 4.16 a and b) the powder particles were spherical and small in spray dried powder while those in freeze dried powder were distinguished by having a large particle size with irregular shapes “broken glass". For further investigation and

visualisation of spray dried powders and identification of the EOs droplet location CSEM was used. An example of the resulting images are shown in (Figure 4.17 a and b). In the first picture, it is clearly seen that EOs droplets are forming a multi-core, embedding themselves inside the powder particle walls by forming microcapsules. No aggregation or caking was observed between particles when MD was present in the system. While, particles formed without MD, such as DSM0 showed a higher degree of stickiness and agglomeration with visible defects on the surface of the particles (Figure 4.17 b) and the type of particles identified were microspheres. The size of the EOs droplets inside the powder particles remained small (approximately 200 nm) similar to that in initial emulsion.

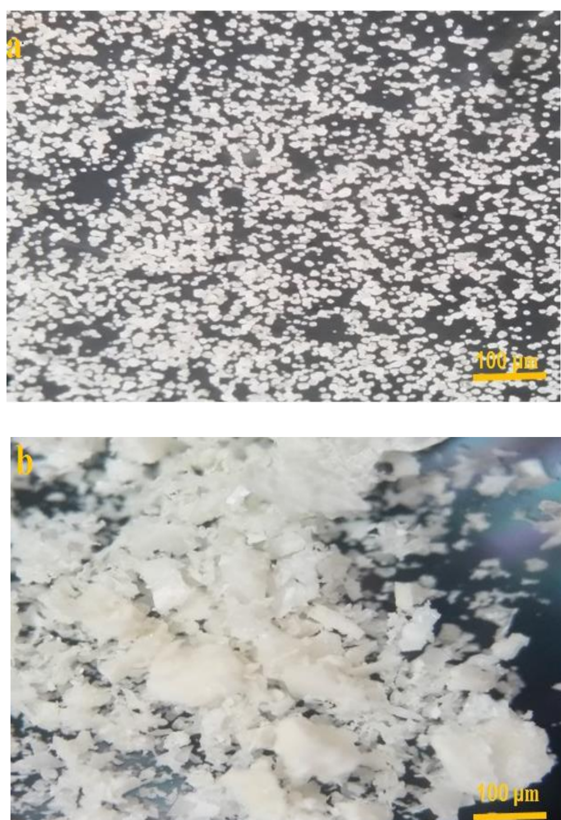
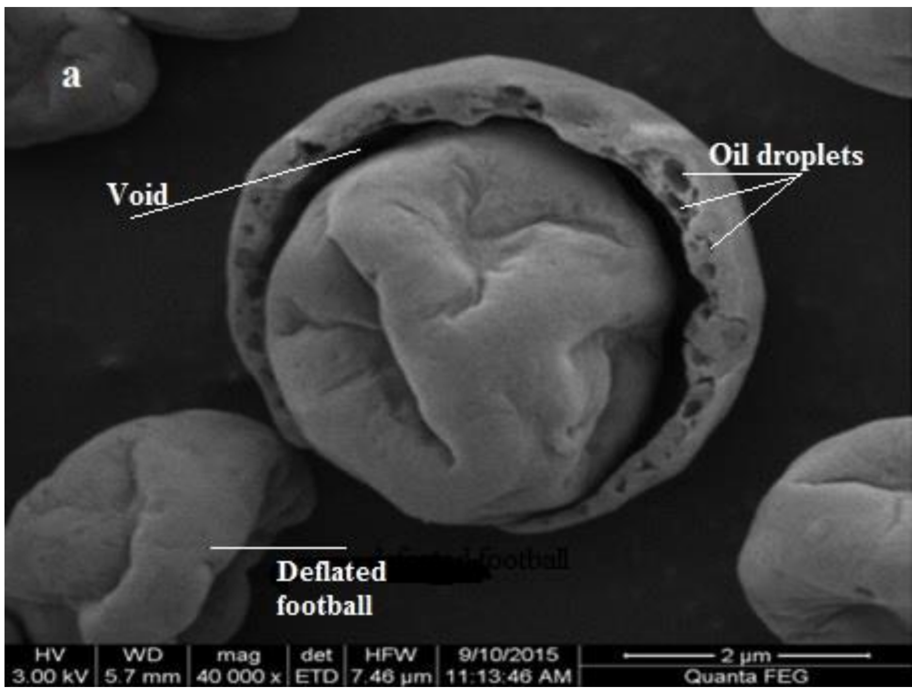


Figure 4.16: Light microscopy images of (a) spray dried and (b) freeze dried powders.



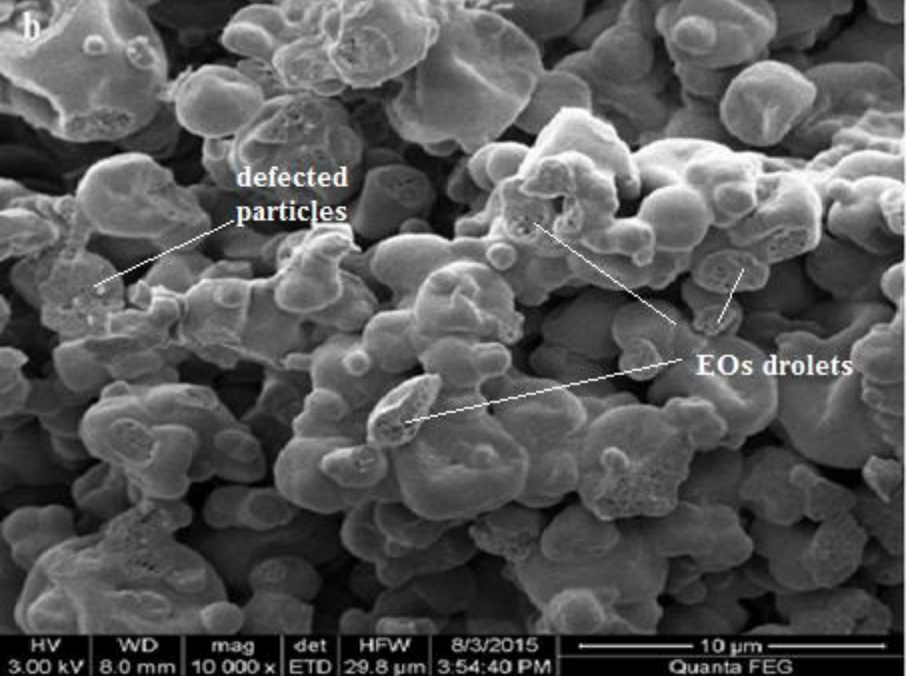


Figure 4.17: CSEM images are showing the internal and external microstructure structure of thyme EOs encapsulated in (a) skimmed milk + 3 % w/w MD (b) skimmed milk without MD.

The images obtained from CSEM was very reliable and clear which showed all minute details about the morphology and the distribution of the EOs droplets in the capsules which is not possible with other microscopy techniques. The morphology of the particles is important regarding the flowability and the release of the EOs from the spray

dried particles. The powder particles are characterised by having an interior void due to the expansion of the droplet during heating and the formation of hard crust around the atomised droplets during drying. The presence of a central void is usually observed in spray dried particles (Soottitantawat et al., 2003). Maltodextrin and lactose formed an amorphous state around the particles as a result of high drying temperature and rapid drying of the surface. When lactose experienced the high temperature, the water evaporated very rapidly. Thus crystallisation of lactose did not occur instead it turned to an amorphous state. As long as the T_g remains low (below 107 ºC for pure lactose) the powder remains stable against deterioration such as Maillard reaction, caking and oil oxidation (Vega and Roos, 2006). During drying in the chamber, the particle travelled to the bottom and further migration of the droplets from the centre of the particles towards the surface occurred. Consequently, droplets containing oil trapped and accumulated beneath the hard crust of the particle and a void left behind when the water evaporated from the droplets, the particle diameter increased as a result of air and water expansion inside the particles. The particle size decreased again as the water evaporated from the droplets (Pierucci et al., 2007). As the particles moved to the cyclone and collecting vessel most of the water inside the particles evaporated and the particle size decreased forming a spherical wrinkled particle with a void inside these are called as “deflated footballs” due to the presence of concavities on the surface of particles (Figure 4.17a). A narrow mean diameter distribution of particle size was obtained with a mean diameter of 9 ± 5.48 µm as measured by Morphologi instrument. In both images, it is clearly shown that the small EOs droplets distributed within the wall matrix. The similar structure of particles observed by many researchers such as (Reineccius, 1995). Because, the mean diameter of the oil droplets in the initial emulsion were small, they remained small in the microspheres after drying, this is due to preservation of the oil droplets during atomisation as they passed through the 0.4mm nozzle hole. Therefore, the droplets did not experience rupturing or shearing immediately after leaving the nozzle. Moreover, using high pressure (0.75 bars) to atomise the feed produced fine particles that dried rapidly in the chamber because the drying time is proportional to the surface area of the atomised droplets. Another explanation of the narrow particle distribution is that the very fine particles travelled further to reach the filter next to the cyclone and the large particles if they were present they would deposit on the walls of the drying chamber and the cyclone (Threlfall-Holmes, 2009).

The size of the powder particle is determined by both the physical properties of the feed for instance the viscosity, the total solid content and also by the condition of the spray dryer such as flow rate, temperature and the atomisation (Reineccius, 2004; Jafari et al., 2007; Masters, 2002; Finney et al., 2002; Fang et al., 2006). Moreover, the size of the particles depend on the type of the atomiser as Finney et al. (2002) reported; they noted that spinning atomisers produce smaller particles than nozzle atomisers, and the effect of atomisers type is greater than that of temperature during spray drying on the size of the particles. However, the operational temperature influences the particle size, small particles can be produced with low inlet temperatures and when there are large differences between inlet and outlet temperatures. However, this led to slow drying and shrinkage of the particles as the particles take a longer time to form and to stabilise their structure. The feed's total solids also govern the powder particle size, the higher the total solid, the larger the particles as they tend to dry very rapidly when compared with the lower total solid feed (Reineccius, 2004). The influence of the particle size on encapsulation efficiency is clearly understood. Some researchers such as Blackebrough and Morgan (1973) and Fang et al. (2006) have stated that the lower surface oil and higher encapsulation efficiency are achieved when large particles are produced. In contrast, (Reineccius and Coulter, 1969) and Finney et al. (2002) reported that the encapsulation efficiency was not affected by the size of the particles rather by the solid content of the feed. Additionally, (Soottitantawat et al., 2005a) stated that the encapsulation efficiency is more affected by the emulsion size and they also noted that the particle size does not have a major influence on retention of flavour. The retention was low for the large particles compared to the intermediate particles, as reported by (Chang et al., 1988) when encapsulating orange oil in modified starch.

No stickiness observed in spray-dried powders containing MD due to using moderate inlet temperature within the required ranges below T_g this depend on the moisture content of the inlet air and the moisture content of the resulting powders. Lower content of moisture is an indication of successful spray drying process as no stickiness, agglomeration or caking was observed in the samples. The presence of MD aids the spray drying of the droplets and prevent the stickiness (Bhandari et al., 1997). However, particles of the powder produced from skimmed milk have a different morphology compared to powders that contained MD. There was a lack of voids and in addition droplets were distributed evenly inside the particles in contrast to particles containing

maltodextrin. Many reasons are explaining the formation of particles in this shape these can be explained by the differences between particles in the shell and/or skin formation depending on the peclet number, which is the rate of evaporation divided by the speed of diffusion. Where no MD is available in the droplets, there was a lack of hard shell around the particle because the diffusion rate was faster than the evaporation rate resulting in low peclet number. While the shell formed in particles containing MD rapidly, therefore the evaporation rate was higher than the diffusion rate which formed hollow particles with oil droplets locating themselves beneath the hard shell of the particles. The peclet number and its relation to the particle morphology are well described by (Vehring, 2008). As a result of slow shell formation the retaining of the core material was reduced, as shown in the next sections. The presence of MD is preferred in atomisation and spray drying as it increases the total solid of the feed without a dramatic increase in viscosity (Pierucci et al., 2007). Pierucci and co-authors reported that the addition of MD and carboxyl methyl cellulose-CMC enhanced the retention of tocopherol a phenomenon which is also explained by the above mechanism.

Although large particles are not desirable regarding the flavour retention, they are often preferred as they are easier to disperse in water; small particles tend to form lumps which are non-dispersible in cold water (Buffo et al., 2002; Turchiuli et al., 2005). Large particles are produced to enhance the dispersity, wettability, flowability, and solubility of the powder alternatively fluidised bed agglomeration techniques can be used to attach small particles together.

4.4.3.3 The surface oil content of different powders of thyme EOs

The analysis of the non-encapsulated surface from the powders by GC-MS are shown in (Figure 4.18 and Table 4.6). The concentration of surface oil was highest (4.93%) for the powders prepared with sodium caseinate as the wall material. The difference was significant with other types of powders. The amount of surface oil increased in the SC powder and reached (6.94%) over storage time. The lowest surface oil was observed on SM3 both at the beginning and at the end of storage 0.79% w/w and 1.25% w/w respectively. No significant differences in the surface oil concentration were identified between DSM3 and DSM2.5. While both were varied significantly with DSM0, DSM1 and DSM2. As a general trend, the amount of surface oil declined with respect to an

increase in maltodextrin levels and increased with storage time in all samples except SM2, which showed no significant differences during the storage period.

The surface oil calculated depending on the amount of thymol on the surface as it was the dominant component in the thyme oil in the current study. The percentages of surface thymol are shown in (Table 4.7). There was a different quantity of thymol on the particle surface determined by the MD concentration. Lower thymol levels were detected when high levels of MD used, while powders prepared with skimmed milk without MD showed higher thymol levels on their surfaces. On the other hand, p-cymene on the surface was lower in comparison to thymol. However similar trends were observed in all powders, and lower percentages of p-cymene noticed in the powders containing high amounts of MD.

The increase in surface oil is related to the pressure of the homogeniser, the size of the droplets of the primary emulsions used for the spray dryer, and the number of passes through the homogeniser as described by (Minemoto et al., 2002; Keogh et al., 2001). The lower surface oil is then related to the higher stability of the emulsion droplets during atomisation and drying; smaller droplets remain intact when they leave the atomiser while larger particles may disrupt as a result of shear effects. Smaller particles also have a larger surface area which forms the hard crust faster than the larger particles.

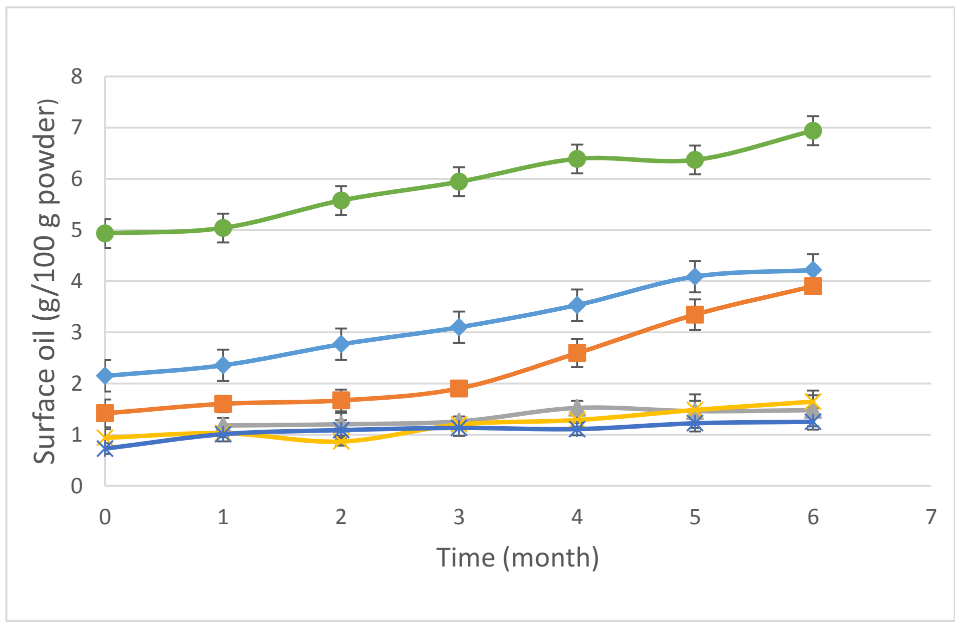


Figure 4.18: The surface oil (g/100g powder) in different powders during 6 months of the storage at room temperature , —◆—DSM0, —— DSM1, —▲—DSM2, —×— DSM2.5, —✳— DSM3 and —●—DSC.

The total surface oil calculated regarding the presence of thymol as the predominant compound on the surface.

Table 4.6: The surface oil of various types of powders according to thymol.

Type of powder	DSC	DSM0	DSM1	DSM2	DSM2.5	DSM3
Surface oil (g/100g powder)	4.93[a*] ± 0.25	2.15 [b] ± 0.43	1.42 [b]± 0.27	1.80 [b] ± 0.14	0.94 [b] ± 0.17	0.73 [b] ± 0.10

*Different letters indicate the significant difference between means at ($P < 0.05$)

Table 4.7: The percentage of the migrated thymol and p-cymene on the powder particle surfaces.

Type of powder	DSC	DSM0	DSM1	DSM2	DSM2.5	DSM3
Surface thymol %	8.21	6.21	4.53	4.00	3.20	2.57
Surface p-cymene %	1.05	0.22	0.16	0.11	0.09	0.08

In a study by (Chang et al., 1988) it is reported that particle size affected the surface oil of the particles, they found that greater surface oil and lower core material were obtained when the particle size increased. This is in contrast to results found by (Finney et al., 2002) who stated that the surface area of large particles in relation to the volume are smaller than the small particles, resulting in a decrease in surface oil. Soottitantawat and co-workers explained that the rise of surface oil in medium and large particles were due to the longer time required by large particles to form a film and dry. As a result, there was a greater loss of the core materials during spray drying. A further explanation for low retention in large particles is related to the particle morphology, as the large particles require a longer time to dry, the particles may damage (shrink or defect) which increases the particle's surface area and increases the loss of the flavours and surface oil.

Atomisation is the first stage in spray drying and loss of volatiles occurs directly as a thin sheet of the emulsion is formed prior to breaking into droplets, the thin sheet has a high surface area. The sheet is then sprayed into the drying chamber together with

turbulent air. The retention of volatiles is related to the pressure in the nozzle and the speed of the centrifugal atomiser. High pressure leads to a rapid breakup of the emulsion sheets to form droplets, the longer this process takes, the greater the loss of the volatiles due to the high surface area of the emulsion in sheet form. When the pressure is increased incrementally from 1.83 to 3.55 and 7 MPa the retention of propyl acetate is increased respectively from 31 to 35, and 45 %. Using high pressure draws more hot air into the drying chamber producing smaller droplets which tend to form thin films very quickly due to their high surface area (Judson. K., 1995), moreover, the droplets dry faster when contact with hot air is increased. The pressure used in the current study was ~ 0.75 bars, and the nozzle orifice was 0.4mm which produced small droplets allowed retention of higher amounts of EOs.

The drying temperature is another factor which determines the surface oil content of the particles. The low concentration of surface oil in the powders with maltodextrin is attributed to the concentration of the maltodextrin and increase in the total solid of the initial emulsion. A further explanation is that a thicker layer of maltodextrin on the surface may prevent the volatiles from evaporation as the crust formed rapidly in the drying chamber, in the higher total solid, compared to less total solid droplets. The rate of load percentage of the EOs in the capsules is reduced when the maltodextrin level is increased, because the higher core load results in increased loss of the volatiles (Hogan et al., 2001b; Rosenberg et al., 1990; Hogan et al., 2001a; Bertolini et al., 2001; Tan et al., 2005). This observation is in agreement with Yoshii et al. (2001) and Danviriyakul et al. (2002) who found that migrated core materials in the stored powder decreased with increasing maltodextrin concentration. Retention of volatiles depends on the contact between hot air and the atomised droplets. The mass transfer of the liquid inside the droplets can be improved by increasing the droplet contact with hot air and rate of heat transfer from the droplets (Coumans et al., 1994). The humidity of the inlet air also affects the rate of drying. Rapid drying of the droplets can be achieved when dryer inlet air is used (Reineccius, 2004).

The enhancement of protein functionality as an emulsifier by a combination of proteins with carbohydrates as wall materials showed greater retention of the core materials for oils and flavours. Milk proteins (whey& casein) are widely used in the preparation of emulsions including spray dried emulsions. Casein outperforms whey protein (WP) due to its resistance to denaturation by heat, decreases in the level of oil on

the surface and smaller droplet size. On the other hand, Fäldt and Bergenståhl (1996a) found that whey protein concentrate (WPC) was less efficient for the encapsulation of soybean oil when compared with SC. SC together with lactose lowered surface oil on the powder particles of 30% w/w emulsion from 30% to less than 5%; this may be due to the ability of lactose to produce a hard shell (glass-like) around the atomised droplets when dried rapidly in the drying chamber. The presence of lactose with SC in the emulsions may be another explanation of high retention rate of EOs as shown in the current study. Soy protein with MD was used to encapsulate volatiles by (Re and Liu, 1996); moreover, whey protein concentrate (WPC) and skimmed milk powder (SMP) blended with MD was used to enhance caraway EOs retention in capsules by (Bylaitë et al., 2001), they found that mixing WPC with MD increases the retention of caraway EOs, while blends of MD with SMP showed less retention.

Compounds identified on the surface of the particles tend to be those with lower molecular weight such as thymol, carvacrol, p-cymene (MW= 150) while other higher molecular weight compounds such as γ-Himachalene, copaene, caryophyllene, caryophyllene oxide and humulene (MW=204) have not been detected. This phenomenon can be described by the hypothesis of selective permeability of the crust formed during spray drying which allows smaller molecules to diffuse through with water and prevents larger molecules (Bertolini et al., 2001). The polarity of the compounds in the core also governs the diffusion through the wall materials. Accordingly, the amount of thymol which contains a hydroxyl group, on the surface of the different powders, was more than that of the hydrocarbon p-cymene. The solubility of thymol in the aqueous phase determine thymol diffusion with water during atomisation and drying (Rosenberg et al., 1990; Voilley, 1995).

Spray drying is useful for encapsulation of volatile compounds as retention of the core material is achieved as a result of minimal overheating of the particles (Zuidam and Shimoni, 2010). The phenomenon of selective diffusion, described by Thijssen in 1971, explains how water diffuses rapidly from droplet surfaces as it is heated by hot air, this results in drying the surface area; selective diffusion then plays a role and the diffusion coefficient of the core material is reduced and is less than that of water. However, with volatile compounds, the rate of core diffusion depends on molecular diameter rather than molar volume. It has been observed that monoterpenes with the same molecular weight and solubility have different retention rates during spray drying, this is related to the

various molecular structures of monoterpenic compounds and their boiling points (Bertolini et al., 2001).

4.4.3.4 Encapsulation efficiency of different powders containing thyme EOs

The encapsulation efficiency (EE %) of different powders was measured by recovering the authentic encapsulated oil using Clevenger apparatus with respect to surface oil. The effectiveness of the microencapsulation process was determined by the oil present on the surface of the particles and recovered EOs from the capsules directly after powder preparation. This was also repeated following six months of storage at room temperature. The SM3 emulsion was supposed to contain 5% EOs, but the recovered oil from emulsion showed that around 4% of the added oil had been lost during emulsion preparation both in the high shear mixer and homogeniser which decreased the EE % of the emulsion to 95.73%.

The maximum encapsulation efficiency was achieved when thyme EOs are encapsulated in skimmed milk containing 3 % w/w maltodextrin (DSM3) and on the first day of storage. The powder was able to retain 97.38 % of the authentic oil present in the initial emulsion. The lowest retention of the EOs was detected in the DSC after 60 days of storage, and the retention reduced to 70.10% at the end of storage (Table 4.8).

There was a significant difference ($P < 0.05$) between the EE% of powders produced with sodium caseinate in comparison with powders formed from skimmed milk combined with different maltodextrin concentrations. Powders prepared with sodium caseinate were presumed to contain 60.37 % w/w of EOs as core material (Table 4.5). However, during atomisation and spray drying some oil may evaporate from the atomised droplets and some oil migrated onto the surface of the particles 4.93g/100g powder (Table 4.6).

Thus, the recovered oil content reduced in the spray dried powder, and the encapsulation efficiency was 84.90%. During the first four months of storage, the powder did not show any significant change in EE%. Conversely, the EE% reduced significantly to 69.42 and 70.10 % after 5 and 6 months storage, respectively.

Table 4.8: The encapsulation efficiency and the standard deviations of different powders during six months of the storage.

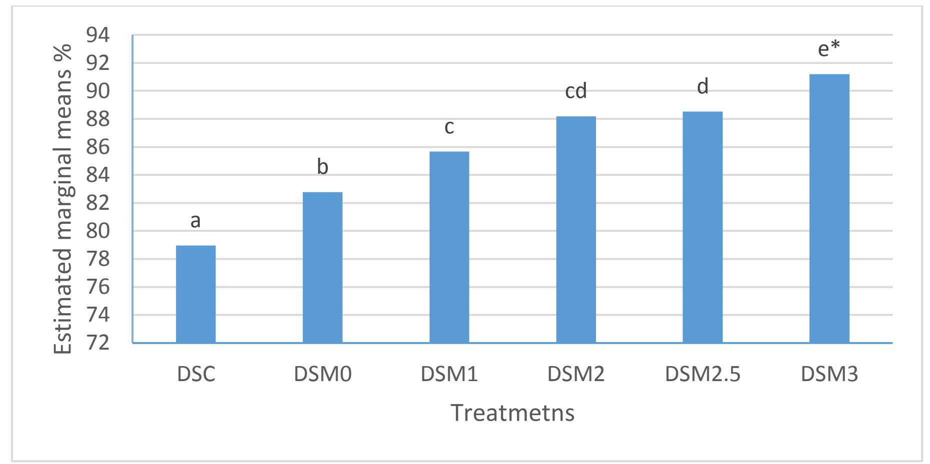
Encapsulation Efficiency %							
Type of powder	**Storage time (months)**						
	0	**1**	**2**	**3**	**4**	**5**	**6**
DSC	84.90[a] ±3.15	83.65[a] ±3.69	82.92[a] ±2.84	81.25[a] ±3.22	80.50[a] ±2.06	69.42[b] ±1.90	70.10[b] ± 0.48
DSM0	86.55[a] ±4.82	85.07[a] ±2.80	84.58[a] ±3.93	82.44[a] ±3.30	82.28[a] ±3.92	80.60[a] ±3.22	77.96[a] ±2.92
DSM1	89.32[a] ±3.62	88.44[a] ±3.09	87.75[a] ±4.20	85.33[a] ±3.22	84.88[a] ±4.86	83.78[a] ±2.43	80.17[a] ±1.88
DSM2	92.30[a] ±2.28	91.32[a] ±1.46	91.17[a] ±2.65	88.40[ab] ±3.50	88.14[ab] ±4.64	84.02[ab] ±4.03	81.85[b] ±3.00
DSM2.5	94.42[a] ±0.96	92.21[ab] ±3.13	91.27[ab] ±4.49	88.41[abc] ±2.29	87.67[bcd] ±1.36	83.44[cd] ±2.29	82.25[d] ±1.92
DSM3	97.38[a] ±3.03	94.53[ab] ±1.79	93.15[abc] ±1.61	92.39[abcd] ±1.83	88.21[bcd] ±3.35	87.29[cd] ±3.00	85.43[d] ±2.42

*Different letters shows the significant differences ($P<0.05$) between means of the same type of powder along with the storage time.

The retention of the EOs in powders prepared from skimmed milk was significantly different from the powder prepared in SC. Immediately after powder preparation, the EE% of DSM0 was 86.55 %, no significant changes were observed during 6 months storage. Encapsulation of EOs in a blend of skimmed milk and maltodextrin enhanced the retention of the volatile EOs are shown in (Figure 4.19). It is clearly shown that there is a linear correlation between the concentration of the maltodextrin and the retention of

thyme essential oils in the powder particles. The EE% significantly differs between DSM0 82.87 % and DSM1 85.67 %, which was produced from an emulsion containing 1% MD. Highly significant differences observed between DSM0, DSM2 88.17 %, DSM2.5 88.53 % and DSM3 91.20 %.

In a further comparison of storage times, high retention of the EOs was observed immediately after spray drying of the emulsions. The levels then declined with storage of the powders. The differences were not significant between the beginning of storage and after three months of storage in all samples. However, significant differences are observed after three months. The lowest level was obsereved after six months of storage. As a general trend, the retention of the core material decreased with storage time.



* Bars with different letters indicate significant differences at ($p < 0.05$).

Figure 4.19: The comparison between different powders encapsulation efficiency. The values are the mean of overall EE% regardless the storage time.

Clevenger is the precise apparatus to recover all encapsulated and non-encapsulated EOs from the powders. The effect of the different emulsion formulation on the EOs retention in spray dried powders was investigated. Powders prepared using sodium caseinate showed high retention of the core material when spray dried directly after emulsion preparation. However, incorporation of maltodextrin into liquid skimmed milk improved the properties of the powder with reference to surface oil and encapsulation efficiency. The higher retention of EOs in emulsions containing higher levels of maltodextrin may be attributed, to the increase in the total solid of the atomised droplets of the feed this slightly increased the viscosity and decreased the internal circulation and diffusion of the EOs volatiles. It may also promote the rapid formation of the semi-

permeable membranes during spray drying, retain more volatiles as a result (Ré, 1998, McNamee et al., 2001, Rulkens and Thijssen, 1972, Coumans et al., 1994, Soottitantawat et al., 2005b, Minemoto et al., 2002).

The retention of the EOs after spray drying and storage depended on the concentration of maltodextrin and the total solid of the feed as reported in many studies such as (Jafari et al., 2007; Rajabi et al., 2015). The total solid of thyme oil emulsion prepared with SC was 8% w/w and as mentioned earlier the powder made from this emulsion was characterised by lower EE% and retention of the EOs during storage as compared to powders contained MD. The higher retention of the EOs was proportional to the amount of MD in the feed. The EE% of the DSM0 was 86.55% which contained 0 % w/w MD, at the same time DSM3 which contained 3% w/w MD was able to retain higher amounts of the EOs during spray drying and was characterised by higher EE% which reached 97.38%. The increase in EE% with total solid content is confirmation of the hypothesis that the reduction in core circulation and diffusion in liquids with high solid content also the higher solid content decreased the surface oil of the powder particles as clearly shown in (Table 4.6). The results are in agreement with (Liu et al., 2000) and (Soottitantawat et al., 2005b; Vega and Roos, 2006) who found the increment in retention occur with increasing the initial solid concentration. However, excess total solids may result in high loss and low retention for the following reasons: firstly adding more solid beyond the solubility limit reduces the encapsulated core and secondly it increases the viscosity of the emulsion. Total solid content should be optimised to allow effective encapsulation of the required core material (Rosenberg et al., 1990; Sankarikutty et al., 1988). The total solid in the emulsions containing MD, in the current study did not exceed 17 % w/w as shown in (Table 4.5) this may totally dissolve and enhanced the retention of the EOs. These results were in agreement with Liu et al. (2001) who demonstrated that the retention of ethyl propionate and ethyl butyrate improved with an increase of the total solid concentration of the feed. In contrast, they also reported that the retention of d-limonene (95%) was not affected by the total solid content rather than the physiochemical properties of the core material.

Secondly the rapid formation of the hard crust, in feeds containing higher total solid content, around the droplets to form a semi-permeable membrane, which prevents large molecules from diffusing through the wall material, while allowing smaller molecules such as water to diffuse (McNamee et al., 2001). The lack of emulsifying properties of

maltodextrins prevents their use as emulsifying agents alone(Kagami et al., 2003). However, combination with different proteins (Gharsallaoui et al., 2007) in skimmed milk improved the properties of the powders containing maltodextrins because both are high molecular weight compounds which proved to increase retention of the core material (Voilley, 1995). Maltodextrin as spray drying aid reported in many studies. Apintanapong and Noomhorm (2003) they found that MD reduced the moisture content of a microencapsulated powder of 2-acetyl-1-pyrroline (flavour used in aromatic rice) comparing to that of gum acacia. The retention of some flavours by maltodextrin was investigated by (Bangs and Reineccius, 1982); they found that the DE of 10 retained the flavour more than that of the higher DE of 36.5. The retention of ethyl butyrate increased during storage when the maltodextrin concentration was increased (Yoshii et al., 2001). When maltodextrin with high DE is used it inhibited the oxidation of orange peel oil and β-carotene (Anandaraman and Reineccius, 1986; Desobry et al., 1997). Other researchers stated that higher DE resulted in higher encapsulation efficiency and decreased oxygen permeability in microencapsulated powders (Sheu and Rosenberg, 1998; Hogan et al., 2003; Anandaraman and Reineccius, 1986; Hogan et al., 2001a). The improved encapsulation efficiency of high DE may related to the ability of shorter oligosaccharide to form less porous shells around the encapsulated material and more uniformity can be observed when they dried forming a hermetic shell which prevented the encapsulated EOs to move outside the particle during drying and storage(Vega and Roos, 2006). The surface fat decreased with increasing DE of the maltodextrin achieving 98% encapsulation efficiency (Danviriyakul et al., 2002). However, the encapsulation efficiency declined to 67% when maltodextrin with DE 10 was used to encapsulate Allylguaiacol compared to modified starch 94% (Re and Liu, 1996). The low viscosity at a high concentration of maltodextrin makes it ideal for use in spray dried emulsions (Mongenot et al., 2000).

Thirdly, the drying temperature is another factor controlled the retention of the volatiles inside the powder particles because thyme oil components are volatile and liable to evaporation during drying. Thus higher temperatures resulted in lower volatiles retention, however in the current study the inlet temperature was120 °C which considered low. The effect of temperature on the polyphenol retention is reported by (Sansone et al., 2011) who found significant differences in the retained polyphenol level when the temperature increased from 120 °C to 200 °C. The spray dryer conditions also influence the retention of core materials. The principle variables include feed flow rate, inlet and

outlet air temperature, the speed of the centrifugal atomiser, the size of the orifice and pressure in the nozzle, the air flow and the size of the powder particles which are formed.

The effect of inlet air temperature on the encapsulation efficiency has been well studied by several researchers such as (Shiga et al., 2004; Lee et al., 2005; Bangs, 1985; Rosenberg et al., 1990; Bhandari et al., 1992; Liu et al., 2000). They found that the optimal retention of flavours could be obtained with temperatures between 160-220 ºC, due to the rapid formation of a crust membrane on the droplet surfaces. The particles may be damaged if higher temperatures are used due to internal liquid boiling and bubble formation which can lead to a breakdown of the droplet membrane causing rapid evaporation of volatiles. This phenomenon is referred to as ballooning it is affected by both the design of spray dryer and types of wall materials used (Drusch et al., 2006). In contrast, Bhandari et al. (1992) did not observe any ballooning when they used a high temperature of 400 ºC in a Leaflash spray dryer to encapsulate a mixture of linalyl acetate and citral in maltodextrin and gum Arabic; they were able to retain 84% of the flavour compounds in the powder. Similarly, Shiga et al. (2004) also stated that the retention of shiitake flavour is increased with increasing air inlet temperature and solid content. In contrast to the previous findings, Anker and Reineccius (1988) and Finney et al. (2002) reported that the air temperature had no effect on the retention of orange oil and diacetyl but that higher temperature decreased the surface oil level. Finney et al. (2002) stated that the surface oil level is increased when higher inlet temperatures are used. Nevertheless, Bhandari et al. (1992) indicated that the surface oil decreased when higher temperatures were used due to the rapid formation of the hard crust around the droplets preventing volatiles from draining out of the surface. The conclusion must be that the effect of high inlet temperature on volatile retention is different according to the type and physiochemical properties of volatiles. The retention of ethyl caproate increased to some extent when the temperature was increased from 40 to 100 ºC while the d-limonene retention did not change with temperature (Liu et al., 2000). It was also related to the high stability of the emulsions of d-limonene and ethyl caproate during spray drying comparing to the emulsions of ethyl butyrate ($C_6H_{12}O_2$) or ethyl propionate ($C_5H_{10}O_2$). When the temperature is increased to 115 ºC, the droplets tend to break and the loss of these compounds increases. Rosenberg et al. (1990) observed that ethyl caproate ($C_8H_{16}O_2$) retention increased at high inlet temperature and high concentration of solids. However, when high inlet temperatures were used the crust around the droplets formed

rapidly which prevented the volatiles from evaporating increasing the encapsulation efficiency.

Generally, the encapsulation efficiency depends on many factors some related to the core material others related to the feed properties and the drying conditions. The molecular weight, polarity, interaction with wall materials and the degree of volatility of the core material have a great role in the retention rate of the core material (Madene et al., 2006). High molecular weight compounds have larger size molecules decreasing their diffusion rate through the wall material of the atomised droplet and the semi-permeable shell of the capsule. The retention of strawberry flavours in gum Arabic was investigated by (Voilley, 1995); he found that the encapsulation efficiency of ethyl hexonate with high (MW=144) was greater than that of ethyl butyrate (MW=116).

Fourthly, the vapour pressure of the compounds in the core materials, which is known as relative volatility with respect to water, governs the retention of volatiles before the semi-permeable surface has formed (Rulkens and Thijssen, 1972). In a study on the spray dried flavour compounds octanol, octanone and octanal by (Bangs and Reineccius, 1982) in MD, casein, soy protein and WPI, it was found that lower relative volatility compounds had higher retention in capsules than those with high relative volatility.

Additionally, the polarity of the encapsulated compounds plays a great role in their level of retention in the capsules during the drying time as water diffuses through the semi-permeable membrane. Highly polar compounds are more soluble in water than nonpolar compounds. As a result, the loss of these compounds with water is higher than for nonpolar compounds (Ré, 1998; Rosenberg et al., 1990). Moreover, the concentration or load of the core materials also influences retention and encapsulation efficiency. The minimum quantity of wall material with higher core retention is favourable economically; high core load resulted in low retention and high surface oil and lower EE% (Hogan et al., 2001b; Rosenberg et al., 1990; Hogan et al., 2001a; Bertolini et al., 2001; Tan et al., 2005). Hogan et al. (2001a) found that the EE dropped from 89% to 18.8% when the ratio between soy oil to SC rose from 0.25 to 3. The ratio of 1:4 for the core to wall such as GA and modified starches was found to be an optimal ratio (Madene et al., 2006; Desai and Park, 2005; Soottitantawat et al., 2005a). Carmona et al. (2013) found that the retention of orange essential oils encapsulated by WPC and MD was strongly affected by the total solid concentration of the emulsion.

The properties of the feed is another factor determining the encapsulation efficiency and in determining the retention of the volatiles, the droplet size, stability, total solid and viscosity of the feed emulsion has a significant role. Increasing the dissolved total solid content improves the retention of volatiles by promoting the formation of the semi-permeable membrane. Similarly increasing the viscosity of the wall material reduces circulation in the droplet reduces the time for the skin to form (Ré, 1998; McNamee et al., 2001; Rulkens and Thijssen, 1972; Coumans et al., 1994; Soottitantawat et al., 2005b; Minemoto et al., 2002). However, excess total solids may result in high loss and low retention for the following reasons: firstly adding more solid beyond the solubility limit reduces the encapsulated core and secondly it increases the viscosity of the emulsion. Total solid content should be optimised to allow effective encapsulation of the required core material (Rosenberg et al., 1990; Sankarikutty et al., 1988). In contrast, the retention of core material in some systems depends on the physiochemical properties of the core materials more than the on the amount of the total solids. For example in a study conducted by Liu et al. (2001), they found that the dissolved total solid did not affect the retention of d-limonene (95%) while the type of flavour and the emulsion stability played a major role in flavour retained during spray drying. In contrast, the retention of ethyl propionate and ethyl butyrate improved with an increase of total solid concentration.

Feed temperature is an additional factor affecting the encapsulation efficiency of the volatiles. The investigation of feed temperature is found in several studies, for example, Rulkens and Thijssen (1972) used different spray drying condition to examine their effects on volatile retention; they used methanol, n-pentanol and n-propanol in maltodextrin. They stated that volatile losses occur principally from the point when the emulsion leaves the nozzle until the droplet is formed. A high feed temperature reduces the viscosity of the feed. The result is a reduction in volatile losses and more rapid formation of droplets. High feed temperature also increase the solubility of solids allowing higher loading. (Sivetz and Foote, 1963) commented in their book on coffee processing they identified that low feed temperature resulted in high retention of the aroma compounds; this was attributed to an increase in the viscosity of the feed. High viscosity feed increases the droplet size and limits circulation inside the droplets while improving the retention of the volatiles.

The viscosity of the emulsion has a pronounced effect on the retention of volatiles as discussed above. The viscosity of the emulsion can be increased by adding thickeners

such as alginate, gums, carboxyl methyl or cellulose without increasing the total solid content above 1%w/w of the wall materials. Sodium alginate with MD was used by Silva and Re (1996) to encapsulate Allylguaiacol. Sodium alginate with GA was employed by Rosenberg et al. (1990) to encapsulate ethyl caproate. They found that the retention of flavour compounds is proportional to the viscosity increment, They found, the optimum viscosity was 105mPa in the first study and in the second study with the addition of sodium alginate it was 125 to 250 mPa. Gelatine with GA optimised the retention of ethyl butyrate; an effect which is explained by the improvement in crust formation (Liu et al., 2001). In contrast to previous works when xanthan gum is used no significant improvements are observed in flavour retention (Reineccius and Coulter, 1969). It is not practical to increase the viscosity of the initial emulsion beyond the optimum because it influences droplet formation and results in loss of the volatiles during atomisation, there is a danger that larger particles with irregular shapes will be formed (Ré, 1998; Bhandari et al., 1992; Silva and Re, 1996).

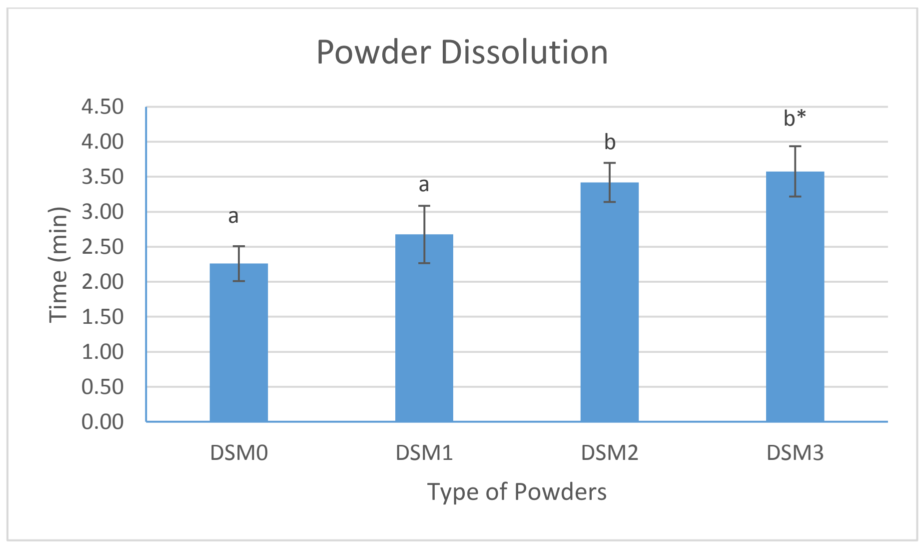
The stability of the initial emulsion plays a role in retaining the flavour during spray drying; stable emulsions have higher encapsulation efficiency (Rosenberg and Sheu, 1996; Danviriyakul et al., 2002; Minemoto et al., 2002). Hogan et al. (2001a) and Hogan et al. (2001b) stated that the retention of soy oil encapsulated in SC decreased when the emulsion droplet size increased by using low pressure during the homogenisation process. Liu et al. (2000) and Liu et al. (2001) also found a correlation between emulsion stability and retention of volatiles when using a blend of GA and MD to encapsulate ethyl butyrate, ethyl propionate and d-limonene, which confirmed the loss of volatiles when emulsion droplets are destroyed during spray drying. d-limonene showed higher stability during spray drying comparing to ethyl butyrate emulsion, and the droplet size was increased 20-40 times. Similar results were reported by (Soottitantawat et al., 2003) when they found that 80-95% of d-limonene was retained in the capsules while only 40-60% and 40-50% respectively of the ethyl butyrate and ethyl propionate were retained. The stability of the current emulsions may be one of the factors aided higher retention of the EOs in the powder particles.

Another important factor is the small size of the emulsion particles in the current study is believed to enhance the encapsulation efficiency of the powders. The size of emulsion droplets was found to influence the retention of volatiles and encapsulation efficiency (Sheu and Rosenberg, 1995; Shiga et al., 2001; Soottitantawat et al., 2003; Re

and Liu, 1996; Soottitantawat et al., 2005a; Liu et al., 2001; Minemoto et al., 2002). The size of the emulsion droplets also governed the retention as smaller size particles (< 240 nm) as they did not experience high shear force during atomisation and passed through the 0.4 mm nozzle pore without defects; this resulted in a higher retention of volatiles. Many studies confirmed this hypothesis which reported that the size of emulsion droplets was found to influence the retention of volatiles and encapsulation efficiency (Sheu and Rosenberg, 1995; Shiga et al., 2001; Soottitantawat et al., 2003; Re and Liu, 1996; Soottitantawat et al., 2005a; Liu et al., 2001; Minemoto et al., 2002). The stability provided by smaller droplet emulsions (< 1µm) further improves retention of the volatiles during spray drying. The stability of linoleic acid against oxidation is increased by decreasing the droplet size; this may be due to the reduction of surface oil on spray dried particles (Minemoto et al., 2002). The same results were also confirmed by Soottitantawat et al.(2001) and Liu et al. (1999) when they demonstrated that the retention of d-limonene was improved with finer emulsion droplets (< 2 µm) this attributed to the stability of the smaller droplets during both atomisation and spray drying. They also found that the emulsion droplet size does not affect the size of the spray dried particles as it is shown in (Figure 4.17a) the oil droplets remained small (< 250 nm) while the powder particles are large (~ 9 µm). The difference in retention rate depends on the solubility of the core materials; hydrophilic compounds such as ethyl butyrate ($C_6H_{12}O_2$) and ethyl propionate ($C_5H_{10}O_2$) showed lower retention even with a small droplet size when compared to d-limonene which is hydrophobic. This phenomenon can be explained as smaller droplets have a larger surface area which may facilitate the diffusion of hydrophilic compounds through the wall material (Soottitantawat et al., 2003). The amount of surface oil is related to the emulsion droplet size, the larger the droplets, the greater the amount of surface oil on the powder particles (Soottitantawat et al., 2003; Sara and Gary, 1988; Danviriyakul et al., 2002)they stated that larger droplets lost more volatiles during atomisation due to shear effects. Larger particles lose volatiles as a result of droplet disruption during atomisation and evaporation of the volatiles during drying and/or insufficient encapsulation of large oil droplets after breaking the large emulsion droplets. Thus, the surface oil is more prone to oxidation and off-flavour formation which shortens the shelf-life of the powder. Both DSC and DSM2.5 and freeze dried powders were not further investigated due to the lower EE% of the DSC and freeze dried (EE% = 73.43), and relatively similar properties of DSM2.5 and DM3.

4.4.3.5 The dissolution of different powders in water

Dissolution test for the three different powders was carried out in the ultrasound velocity metre (UVM) by adding 1g of powder into 70 mL water (~14 mg/mL). The dissolution of the powders was very rapid occurring in a short time after introducing them to the water. The dissolution time is shown in (Figure 4.20). Statistically, there was a significant difference between DSM0 with DSM2 and DSM3, while no significant differences were observed between DSM0 and DSM1, and between DSM2 and DSM3 as showed in.



* Bars with different letters indicate significant differences at ($p < 0.05$).

Figure 4.20: The required time of the dissolution of different powders at 25 °C at 300rpm stirring.

For microscopic images, the DSM3 powder was used as a model. The images clearly show the rupture of particles and the dissolution of the powder. As shown in (Figure 4.21 b) the particles swelled when they absorbed the water, the size of the particles were increased from around 9 µm to more than 30 µm. The particles disintegrated and broken up into small pieces after 2-3min of introducing to water (Figure 4.21 c), subsequently, after 10min of the dissolution the powder fragments disappeared and small colloidal particles appeared in the solution similar to the initial emulsion (Figure 4.21 d).

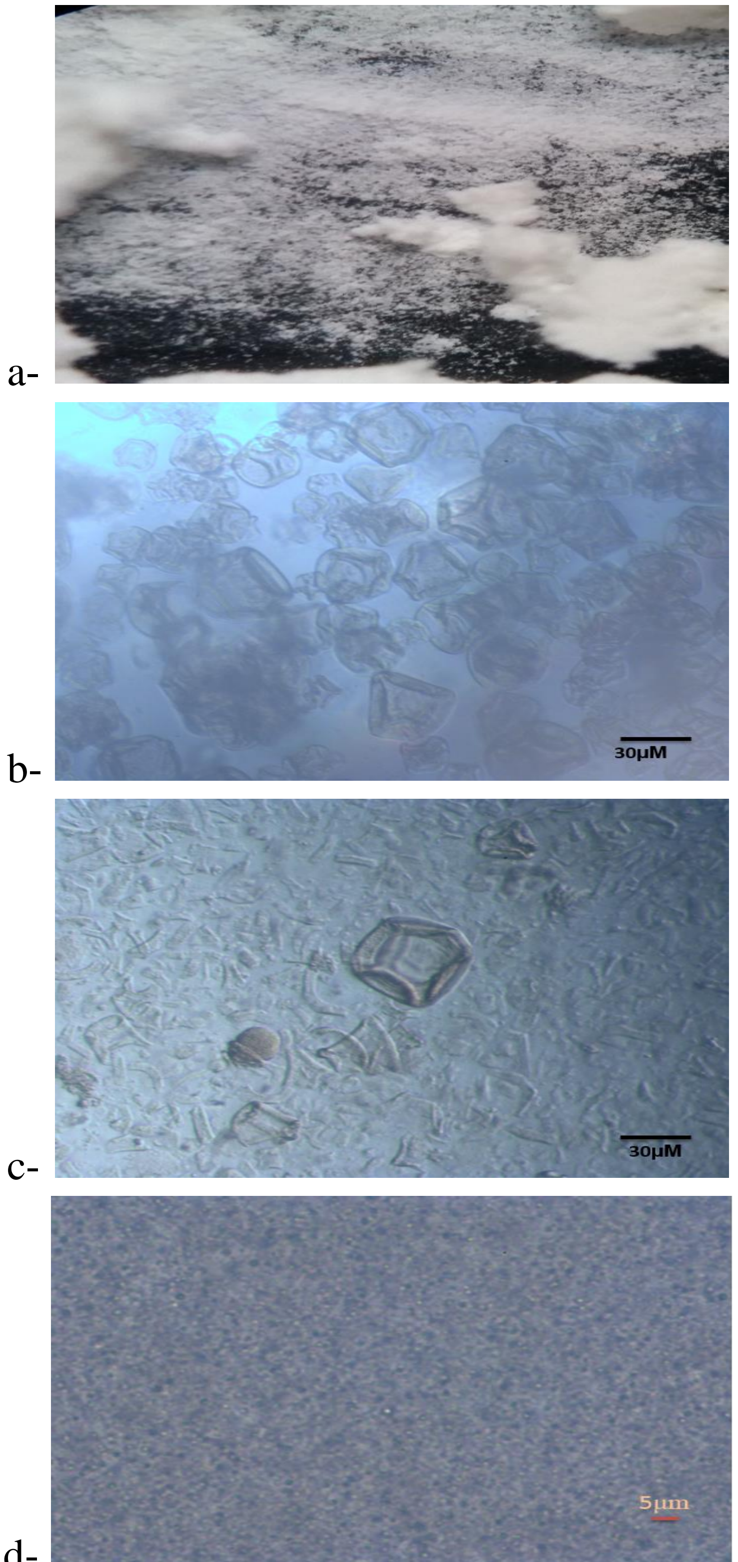


Figure 4.21: Different steps in powder hydration and dissolution. (a) Powder particles, (b) after adding water, (c) after (5min) and (d) after 10 min of the addition to water.

The dissolution of the wall material resulted in core material liberation and release. The complete dissolution of the powder was confirmed when Cryo-SEM was used to monitor the dissolution (Figure 4.22).

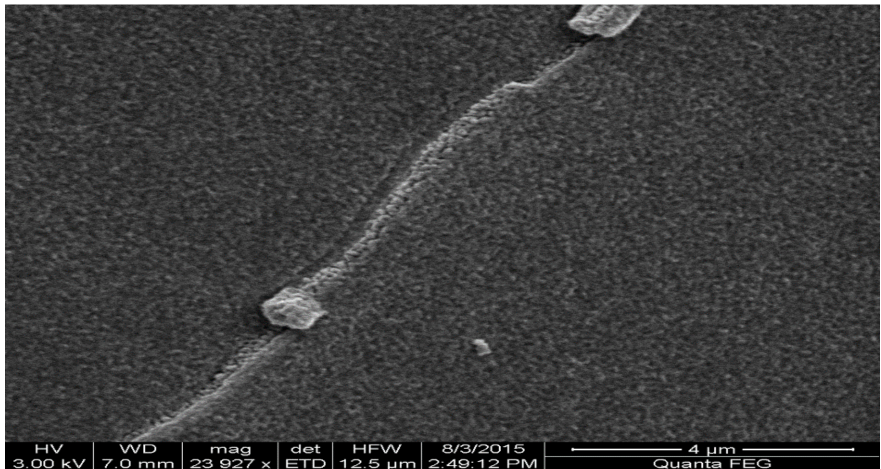


Figure 4.22: CSEM photogram of DSM3 powder after 10 min of reconstitution showing small droplets of the EOs.

Powders showed rapid dissolution which considered favourable if the rapid release of the core material is required in aqueous food products. The powders also can be applied to dry or semi-solid food products where slow release is required such as cheese and bread. Precise results obtained by using UVM, because the sound velocity signal disappears until all powder agglomerates dissolute and turn back to droplets of the same size of the initial emulsion where no further changes in the velocity of sound is detected. This technique was simple, fast and reliable. The dissolution detection of powder as the opaque solution is not possible with conventional methods such as using spectrometric techniques which depend on the absorbance. The dissolution of the powder occurred by erosion of the powder particles in a short time; this took place in all powder types in less than 4 minutes. Dissolution depends on the many factors including the wettability of the powder which directly depends on the concentration of surface oil on the powder particles, particle size, morphology, surface area and surface charge (Vega and Roos, 2006). The surface oil on the powder types was 2.15, 1.42, 1.80 and 0.73 (g/100g powder) for DSM0, DSM1, DSM2 and DSM3 respectively. Secondly, it depends on the properties of the wall materials regarding their solubility in the aqueous phase and finally on the diffusion coefficient of the particle constituents in the dispersion phase. However, the dissolution time slightly decreased for the powders with slightly higher surface oil content

comparing to those with low surface oil. The reason may be due to the presence of higher levels of and a thicker layer of maltodextrin in powders with higher MD concentrations. Therefore the dissolution is more dependent on the amount of MD rather than the surface oil content as there is not a large difference between powders in surface oil content. Alternatively, because these amounts of surface oil are considered low with no effects on the dissolution rate. The presence of oil also fond to decrease the wettability markedly as shown by (Kim et al., 2002) who observed the decrease the wettability time of whole milk and cream powder from 15 min and more to 15s and 100s respectively when they washed the powder with petroleum either. Rapid solubility may result from the interactions between water and amorphous particles which are formed due to the presence of MD and lactose (Hancock and Parks, 2000). Moreover, lactose is hygroscopic with a high wettability rate due to the formation of small angle contact with water (Fäldt and Bergenståhl, 1996b; Kim et al., 2002). Therefore, the presence of hydrophilic carbohydrates increased the wettability of the powders and increased dissolution rates, both MD and lactose worked synergistically to improve the solubility of the powders containing them. Moreover, high DE maltodextrin (DE =19) was used in the current study which is in agreement with the finding stating that carbohydrates with high DE are more soluble than the lower DE carbohydrates (Vega and Roos, 2006). The morphology of the powder particles is one of the other factors that facilitate dissolution due to larger surface area of the irregular particles with concavities (Vehring, 2008). As the powder particles obtained in this study contained an interior void (Figure 4.17 a), the surface area exposed to water increases significantly when the particle breaks because it became in contact with water on both sides increasing the rate of dissolution and release of the core material. The instant rupture and dissolution of the particles are demanded in this study because the prompt release is required when the powder is applied to aqueous food products such as milk and juice. Therefore water soluble materials had been chosen, as the speed of swelling and release of the capsule’s content depends on the degree of water absorption by the wall material.

The results from the mean size distribution of the reconstituted droplets revealed that the droplets of the initial emulsion did not coalesce or aggregated during high shear atomisation in the spray dryer. Therefore, the size distribution of the particles in the reconstituted powder represented approximately the same size as the droplets in the original emulsion (~240 nm).

4.4.3.6 The release rate of some terpenes from different powders

The release rate of the thyme EOs principal components, from different types of powders during 60 min of dissolution, are shown in (Figures 4.23 a, b, c, and d). The release pattern showed a similar trend in all powder types with only minor differences. Most of the components were released after 15min of dissolution reaching their highest concentration. The reason for the rapid release is because powders dissipated during the first 5 min of the reconstitution. However, the released samples withdrawn after 15 min for analysis, at this point the released compounds were at their optimum concentrations. Thus, the concentrations of the released compounds were remained roughly constant for 60 min of dissolution.

From the detected compounds in thyme essential oils, most of the compounds were released from the capsules at different quantities of their initial concentrations. Some compounds were undetected due to low concentration in the solution. The concentrations of the components were calculated by the GC-MS analysis every 15 min intervals from the reconstituted powder samples. In all powder types thymol as the major compound was released exceptionally with a release rate over (> 85%) of the original thymol amount in the powders from the beginning of dissolution experiment until the end. In contrast, it is clearly shown that carvacrol released in lower amounts of its original concentrations and at its best its release rate did not exceed 18%. On the other hand, p-cymene as the second thyme EOs components released in lower rates in all powder types and did not excess 9% during dissolution period.

The release of thyme oil components depends on their concentration, physiochemical properties of the individual components in thyme oil, the wall material and particle morphology. All three components were released rapidly from the capsules at the beginning of powder dissolution. Thymol had similar release pattern in all powder types and reached over 85%. The higher release rate of thymol may be due to the presence of hydroxyl group which facilitate its diffusion and release to the aqueous phase. Nevertheless, around 10-15% of thymol was entrapped in the capsules and did not release. Even though, the concentration of carvacrol in thyme EOs was lower than p-cymene

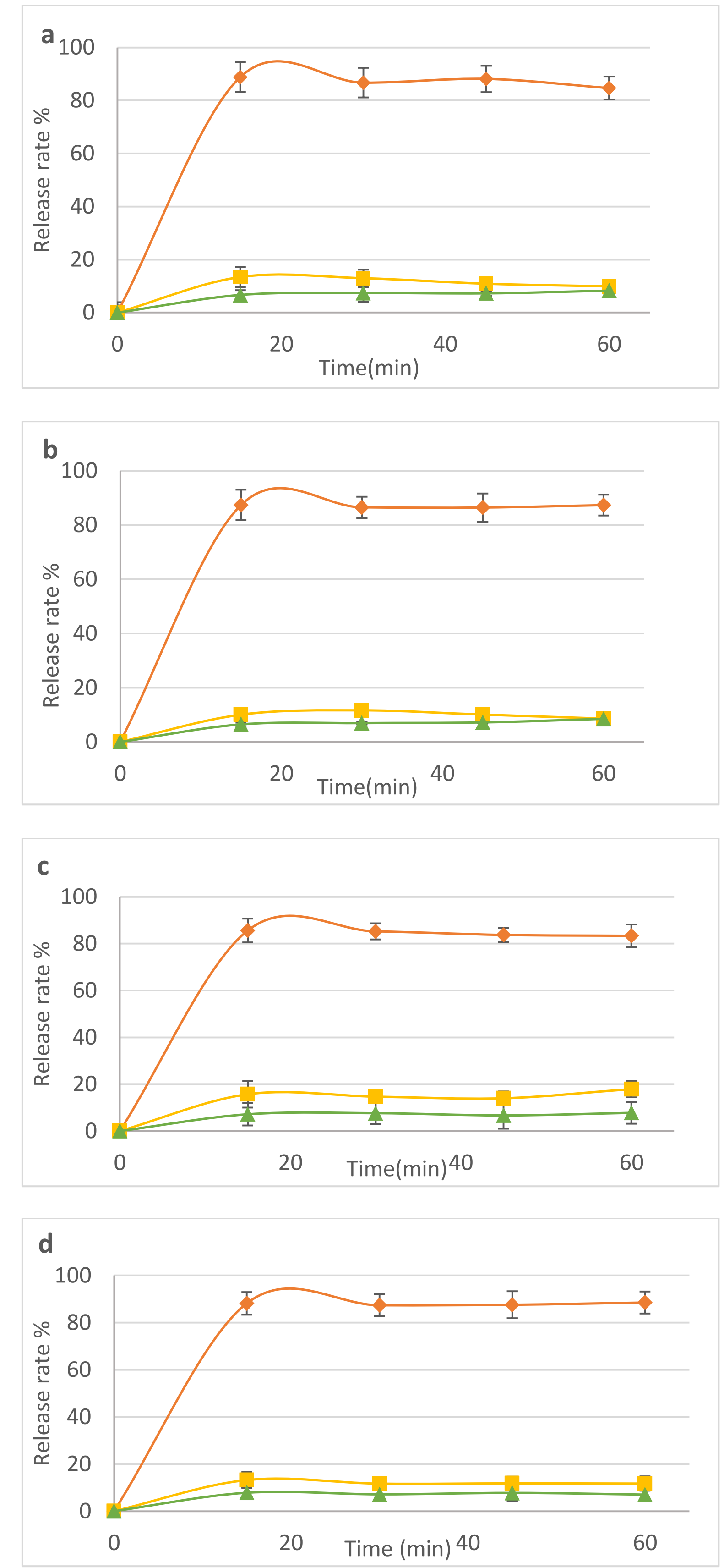


Figure 4.23: The release kinetic of various compounds in (a) DSM0, (b) DSM1, (c) DSM2 and (d) DSM3 at 25 °C. thymol , carvacrol and p-cymene.

concentration, the release rate of carvacrol was higher than that of p-cymene in all powders. The polarity of carvacrol from the attached hydroxyl group may aid its release, as occurred with thymol. The interactions between capsules which contain various components may result in retaining some of the components. In a recent experiment, the withdrawn samples were passed through 0.4µm syringe filter which required high pressure to filter the samples. Using large force may ruptured and damaged the intact capsules and increase the release rate of thyme EOs components. However, if this was the case, then all components would have to release at the same rate. To acquire reliable results, it is considered that normal Whatman filter should be used without using pressure to infiltrate the samples or using a dialysis bag and then analysing the filtered samples.

The release profile simply occurred by solvent release. Therefore most of the core material releases rapidly this is in contrast to other release mechanisms such as diffusion, osmotic, pH or temperature and they normally result in slower release rates.

4.5 Conclusion

Nowadays, consumers and industries demand products that contain natural components due to health consequences of synthesis chemicals. However, the cost, availability and processing are the limitations facing the industry. Therefore, the objective of this chapter was to produce and a delivery system composed of natural elements with low cost to encapsulate thyme EOs. Results show that skimmed milk was an excellent emulsifier and generated nano-sized particle with extending stability of the formed emulsion. Moreover, the addition of polysaccharide into thyme oil emulsions in skimmed milk improved the emulsion systems. Spray drying successfully employed to dry the emulsions which produced small particles without any alteration to the original oil droplets with high encapsulation efficiency. The decrease of surface oil, and the retention of the volatiles during spray drying and storage enhanced when the MD concentration increased. Powders contained thyme EOs were characterised by instant dissolution when added to water which is favourable when using in aqueous food products. Most of the bioactive compounds were released from the capsules when introduced into water in short time which was recommended when rapid release of compounds is required.

Chapter 5 The Assessemant of Antibacterial Activity of Free and Encapsulated Thyme Essential Oils

5.1 Abstract

Essential oils and their constituents have been used in cosmetic, pharmaceutical, agriculture and food industry as natural sources of antibacterial agents. Their activity in food products is inadequate due to the limitation of dispersity and solubility of these compounds as they are mostly hydrophobic. Moreover, their activity is hindered by different food components such as proteins and fats when added to food products. Therefore, enhancing their activity using encapsulation technique is of a great interest and beneficial for industry and consumers. The objective of this study was to evaluate the role of encapsulation on the antibacterial activity of thyme EOs in both food products and *in vitro*. Results showed that the antibacterial activity enhanced when EOs are encapsulated rather than being used in bulk. The minimum inhibitory concentration (MIC) of the encapsulated EOs was 0.3 mg/mL, which was significantly different ($p < 0.05$) when compared to free EOs. Their activity was also improved in food products, the shelf-life of skimmed milk increased by 18 days beyond the best-before-date when 0.75 mg/mL encapsulated EOs was used. Whereas free EOs were able to extend the shelf-life for only eight days. On the other hand, when non-pasteurised whole milk treated with encapsulated EOs the shelf-life decreased to 7 days and 4 days in refrigerator and room temperature, respectively. Furthermore, it was shown that thymol availability decreased by food components especially fat which affected the activity of EOs negatively. Using thyme EOs in combination with low temperature as combined hurdles was efficient to extend the shelf-life of raw milk.

5.2 Introduction to the antimicrobial activity of thyme EOs

Plant essential oils are produced from secondary plant metabolites products, and therefore they will not participate in plant growth and development, but they are performing some biological functions in the plant (Pichersky et al.; Gershenzon and Dudareva, 2007; Vickers et al., 2009). Recently essential oils applied in healthcare products, foods, agriculture, and cosmetics. In food, many studies have been conducted to evaluate the EOs ability as natural preservatives to extend the shelf-life and provide safer food products (Fisher and Phillips, 2008; Gyawali and Ibrahim, 2014; Prakash et al., 2015). The consequences of using chemical preservatives in food to control pathogenic and spoilage microorganisms emphasised the importance of using natural preservatives (Faleiro, 2011). Natural preservatives are those extracted from plant or

derived from animals or microorganisms without any alteration to their composition or properties (Li et al., 2011). Usually, natural antimicrobial are derived from sources already used as part of the human diet and generally are recognised as safe (GRAS) (Chalova et al., 2010; Callaway et al., 2011). Furthermore, essential oils are considered effective in human health progress as antioxidant, antimicrobial, anticancer, anti-inflammatory and analgesic. They have the ability to extend the shelf-life of the agricultural products due to their fungicidal, bactericidal and insecticidal activity. In the food industry, essential oils or the plant used for enhancing the flavour (Ballabeni et al., 2004; Tognolini et al., 2007; Huang et al., 2007).

However, the direct addition of essential oils into food products is limited due to organoleptic changes to food products and inefficiency in their activity due to their limited solubility and dispersity in aqueous systems. The objective of this experiment was to compare the effect of encapsulation on thyme EOs antibacterial activity against gram-negative *E.coli* and gram-positive *Staphylococcus aureus*, *in vitr*o and to assess the role of EOs in extending the shelf-life of pasteurised skimmed milk and non-pasteurised whole milk milk.

5.3 Materials and methods

5.3.1 Materials

The materials used in this experiment include nutrient agar which was obtained from (Fluka), nutrient brot from (MERCK), sulphuric acid (H_2SO_4, 1%), ($BaCl_2.2H_2O$,1.175%) and NaCl from (Sigma Aldrich). Pasteurised skimmed milk was purchased from local supermarket (Tesco) and raw unpasteurised whole milk was obtained from (Delph House Farm, Huddersfield, UK).

5.3.2 Methods

The evaluation of the antibacterial activity of thyme EOs powders

There are various methods for measuring the antibacterial activity of the EOs including, the zone of inhibition, disc diffusion, turbidity, minimum inhibitory concentration (MIC) and impedance. All methods mentioned above are affected by many factors such as the composition of the EOs, the strain, the growth conditions and size of inoculum of the tested bacteria, and finally by the growing method and enumeration technique of the surviving bacteria after treatment with the antibacterial agents. For *in vivo* experiments of the thyme EOs in different forms pasteurised skimmed milk, whole raw milk (unpasteurised) and baobao juice were tested.

Two main sets of experiments were performed to test the antibacterial activity of bulk and encapsulated thyme essential oils *in vitro* and *in vivo*. Cultures of two types of foodborne bacteria isolates of *Escherichia coli* O157: H7 *(gram-negative)* and food poisoning *bacteria Staphylococcus aureus (gram-positive)* were used to assess the antibacterial efficiency of thyme EOs in different formulations.

5.3.2.1 Minimum inhibitory concentration

The protocol described by Andrews (2001) and NCCLS (2006) were used with some modification to assess the minimum inhibitory concentration (MIC). Bacterial isolates of *E.coli* and *Staphylococcus aureus* were obtained from the School of Biology/ University of Leeds. Both cultures were maintained on nutrient agar and kept in the refrigerator for further subculturing.

0.5 McFarland standard preparation and suspension concentration adjustment

0.5 McFarland standard was prepared by adding 9.95 mL of sulphuric acid (H_2SO_4 1% w/v) and adding 0.05 mL of anhydrous barium chloride ($BaCl_2.2H_2O$ 1.175% w/v) dropwise to the sulphuric acid solution and stored in airtight screw cap tube in a dark place at room temperature. The reaction produced a precipitated barium sulphate which gave the solution a turbidity. The absorbance of turbid solution was checked by spectrophotometer and adjusted to read 0.063 at a wavelength of 600 nm. The solution was stored in a dark cupboard at room temperature. Prior to use, the McFarland standard was vortexed to ensure the desired turbidity. The standard can last for 6 months.

Inoculum preparation

A quantitative method of MIC was chosen for the determination of EOs antibacterial activity *in vitro*. For each tube containing EOs, a certain concentration of bacterial suspension prepared by transferring the growth culture (prepared 18-24 hours agar plate) into nutrient broth using sterilised loop and incubated overnight at 37°C until turbid suspensions were formed.

Preparing of thyme oil concentrations

The concentrations of thyme EOs used in all MIC experiments for all formulations were (0.05, 0.15, 0.2, 0.3, 0.35, 0.4, 0.45, 0.5, 0.6, 0.70, 0.8, 1, 1.5 and 2 mg/mL) and were introduced into the inoculum in the following formulations described below:

Essential oils solution (ES)

Free essential oils were dispersed in deionised water to obtain 6 mg/mL stock solution. The required concentrations (0.05 - 2 mg/mL) were prepared from the stock solutions and 2 mL of each solution was added to the test tubes which contained 2 mL of bacterial suspension (~ 1×10^6 CFU/mL) to obtain a final bacterial suspension of ~5×10^5 CFU/mL This step was applied for both *E.coli* and *Staphylococcus aureus* for all EOs formulation.

Essential oils non solution (ENS)

Individual concentrations of the free EOs were prepared by adding a certain amount of EOs directly into test tubes to obtain the required concentrations, then bacterial culture was added to obtain ~5×10^5 CFU/mL suspension.

Reconstituted essential oils (REO)

A dispersion of powder containing essential oil was prepared by dissolving 24 g of encapsulated EOs (DSM3 - 25% w/w EOs) in 1 L of deionised water to obtain a stock solution of 6 mg/mL. Subsequently, a serial dilution was prepared from the stock to achieve the required concentrations of the EOs. Similar bacterial suspensions were added to every test tube containing REO to obtain the same suspension concentration as mentioned above.

Non-reconstituted essential oils (NREO)

Different amounts of DSM3 powder were added directly into test tubes and disintegrated by adding 2 mL nutrient broth to give the required EOs concentrations, then bacterial culture was added to obtain ~5×10^5 CFU/mL suspension.

MIC suspension preparation

The bacterial suspension was prepared as described above and diluted in saline solution (0.8%) to achieve the turbidity of 0.5 McFarland standard by comparing the bacterial suspension with 0.5 McFarland standard visually using a white background with black lines in a good lighting. At that turbidity, the concentration of the bacterial cells is approximated to be less than 150×10^6 CFU/mL. The suspension was then diluted in saline solution (1:150) to obtain 1×10^6 CFU/mL.

For each test tube containing 2 mL of the EOs solution two millilitre of the diluted suspension of bacteria cultures (ca. 1×10^6 CFU/mL) were added to obtain the final concentration of ~5×10^5 CFU/mL, vortexed followed by incubation for 24 hours at 37 ^{o}C. On the following day the samples were checked visually for definite turbidity and any turbidity considered as bacterial growth, the endpoint of turbidity in the row was inferred as the lowest concentration of EOs which inhibited the visible growth. One millilitre from the test tube with lowest EOs concentration which did not show any turbidity was serial diluted (from10^{-1} to 10^{-8}) in sterile saline solution (0.8%). The last three dilutions were plated on nutrient agar and incubated at 37 ^{o}C for 24 hours. On the following day, samples were examined for any colony and enumerated.

Preparation of total count plate

Nutrient agar was prepared by dissolving 28 g of agar powder in 1L deionised water then sterilised in the autoclave at 121 ^{o}C for 15 min. The molten agar was placed in a

water bath at 45 ºC to cool it down and to prevent solidification prior to pouring into the plates. After adding 1 mL of the diluted samples (10^{-6}, 10^{-7} and 10^{-8}), molten agar poured into plastic petri dishes to about 0.3cm thickness and moved gently to mix and spread the sample in the agar. After the agar media dried and solidified at the room temperature, the petri dishes were inverted and placed in the incubator at 37 ºC for 24 hours. On the following day, only the plates which contained between 25-300 CFUs were calculated for enumerations.

5.3.2.2 The activity of different forms of thyme EOs in food products

To evaluate the activity of four different forms of the thyme EOs in food systems, three types of foods were chosen: unpasteurised whole raw milk, pasteurised skimmed milk and baobao juice.

The evaluation of thyme EOs antimicrobial in Skimmed milk

Pasteurised skimmed milk was obtained from local supermarket 5 days prior to its expiry date (best before date) was stored in the fridge. Thyme EOs in different formulations as described previously and required concentrations were added to skimmed milk on the last day of the best-before-date. The skimmed milk and different formulations of the EOs were mixed in sterile 50 mL test tubes vortexed for 2 min to obtain concentrations of EOs ranged (0-0.75 mg/mL) and were stored at 7 ºC for 20 days (Steven, 2009). On the day of the experiment (every day), samples of skimmed milk were examined for total plate count (TPC) of post-pasteurisation psychrotrophic prepared by serial dilutes in sterile saline solution (0.8%) and plating the last three dilutions (10^{-6}, 10^{-7} and 10^{-8}) on nutrient agar with incubation for 48hours at 37 ºC. On the following day, the incubated plates which contained colonies more than 30,000 CFU/mL were recorded as the end of shelf-life according to the regulation by Food Standard Agency 2013. This procedure was repeated for the different samples of stored milk at every 24 hours intervals when no countable colonies detected on the agar plates. However, samples showed distinct colonies were further investigated to evaluate and compare the growth kinetics of the microorganisms in the presence of different EOs formulations and concentrations.

The assessment of the antibacterial activity of thyme EOs in unpasteurised whole milk

Unpasteurised whole raw milk was obtained from (Delph House Farm, Huddersfield, UK) transferred into the lab using the cool box to maintain low temperature and directly used when delivered to the lab. To investigate the activity of the different EOs formulations on prolonging the shelf-life of the unpasteurised whole milk, two sets of different concentrations of EOs (0-2 mg/mL) were prepared in whole raw milk in sterile 50 mL tubes. The first set stored at room temperature and another set stored at 4 ^{o}C. Serial dilution of the raw milk in (0.8%) saline solution on the first day prepared and last three dilutions (10^{-6}, 10^{-7} and 10^{-8}) were plated on the nutrient agar, incubated at 37 ^{o}C for 24 hours and were checked for the total count on the following day. The same procedure was used to assess the microbial growth in the stored milk samples with different concentrations of EOs, at room and refrigerated temperatures every 24 hours.

The antimicrobial activity of the encapsulated thyme EOs in Baobab juice

To assess the activity of encapsulated thyme EOs in juices. Baobab juice was chosen. The preparation of the juice was carried out by dissolving 10% of fruit pulp powder in water, the mixture was homogenised using kitchen blender for 10 min. The mixture then centrifuged for 10 min at 4000 rpm to obtain clear juice. The pH of the juice was about 3. Different concentrations ranged (0-0.75 mg/mL) encapsulated thyme EOs were added to the Baobab juices and stored in room temperature for 2 weeks. After wards the microbial content of the stored samples assessed using BacTrac (model 4300, SY-LAB Geräte GmbH, Germany) which is automated technique uses the change in impedance of the medium to draw a growth curve if microorganisms are present in the medium.

The available thymol in milk samples

The main component responsible for antimicrobial activity in thyme EOs in this study was thymol, therefore, an experiment was carried out to find the amount of thymol available in the milk samples. A number of concentrations (0.3, 0.5 and 0.75 mg/mL) of the EOs in different formulations (ES, ENS, NREO and REO) were added to skimmed milk and full-fat milk in 10 mL test tubes. The mixture was mixed and vortexed for 2 min. After 60 min the samples were withdrawn using a syringe and filtered through 0.45µm syringe filters into Eppendorf tubes centrifuged at 4500 rpm for 5 min and the supernatant were withdrawn and stored in the freezer. On the day of the analysis samples

allowed to thaw at room temperature and 0.5 mL was added to 14.5 mL solution of methanol: water (50:50) in glass vials and analysed using GC-MS according to the procedure described in Section 3.3.2.

5.4 Results and discussion

This section contains various results from the antibacterial activity of free and encapsulated thyme EOs. The minimum inhibitory concentration (MIC), the activity in pasteurised skimmed milk and in raw milk is reported.

5.4.1 Antimicrobial evaluation

Thyme EOs were used in four different formulations to evaluate their antimicrobial activity. The treatments are denoted as ENS for free thyme EOs (non-solution) which directly added to the inoculums according to the required concentration. ES for the solution of free EOs in deionised water which required concentrations prepared from diluted stock solution. NREO for encapsulated EOs (non-reconstituted) powder added to the inoculum directly. REO for reconstituted encapsulated EOs in deionised water which prepared from a stock solution of the powder. The quantitative and qualitative assessment of the antibacterial activity in our study were assessed by absence or presence of the microbial growth in the nutrient broth media and subsequently incubated plates.

5.4.1.1 The minimum inhibitory concentration

To investigate the antimicrobial activity of the different formulations of EOs, gram-negative *Escherichia coli* O157: H7 as common foodborne and gram-positive *Staphylococcus aureus* toxic-forming bacteria were selected. The MIC's of the various formulations of the EOs against both bacteria are listed in (Table 5.1). The results using duplicate samples showed that 0.43 mg/mL of oil ENS is required to act as MIC against *E.coli*. The MIC value then increased to 1.12 mg/mL when a solution of free EOs (ES) used which was approximately 2.5 times higher than ENS. In another comparison between reconstituted REO and non-reconstituted NREO powders, it was found that the MIC concentration was 0.3 mg/mL when the powder was directly added into the test tubes containing bacterial suspensions. The required concentration for the MIC then increased to 0.35 mg/mL when the REO was used with no significant differences between the two types. From these results, it is shown that enhancement of antibacterial activity of the EOs occurred by means of encapsulation; lower concentrations of the encapsulated EOs were required compared to the free EOs.

Several methods are available to test the antimicrobial activity of essential oils against both gram-negative and gram-positive bacteria. Agar dilution, broth dilution, and disc

diffusion are the main methods to test *in vitro* susceptibility of the bacteria. In the broth and Agar dilution methods, the Minimum inhibitory concentrations (MIC) can be determined. The MIC is the lowest concentration of antibiotic that is able to inhibit the growth of a known concentration of the test microorganisms. While in the disc diffusion method the diameter of the inhibition zone (the area around the paper discs where no bacteria growth is detected after incubation) is determined. The method is conducted using a paper disc which is saturated with antimicrobial agents and placed on inoculated Agar plate, the magnitude of the diameter of inhibition zone on the inoculated plate indicates the degree of antimicrobial effectiveness. The greater the diameter, the more efficient the essential oil. Since the essential oils are hydrophobic their diffusion through the Agar is limited therefore this method is not suitable. While in the broth and Agar dilution methods this problem can be resolved by mixing the essential oil with an appropriate solvent or surfactant at low concentrations to enhance the dispersity and solubility of the essential oil in the aqueous media. However, the information obtained from the zone of inhibition in the disc diffusion technique is less useful than the data offered by the Agar or broth dilution methods. This is due to less information being available on the amount and concentration of the essential oil in the final product in comparison to the agar and broth dilution method (Burt, 2004, Lahlou, 2004).

The different treatments showed relatively different activity against *Staphylococcus aureus,* as gram-positive bacteria. In comparison with *E.coli,* slightly lower concentrations of EOs were required to inhibit any visual growth in the inoculum which contained ($\sim 5 \times 10^5$ Cfu /mL) of *Staphylococcus aureus*. The ENS was found to be active at 0.4 mg/mL which was slightly lower than the concentration (0.43 mg/mL) against *E.coli* with trivial differences.

A similar trend was observed when REO used against *Staphylococcus*. The higher concentrations (0.75 mg/mL) of ES was required to act as MIC this was significantly different from the other formulations. ES had a lower MIC against gram-positive than gram-negative bacteria. NREO affected both types equally while there were slight differences between the activities of REO against both types of bacteria, with non-significant differences ($P < 0.05$). The order of the antibacterial efficiency against introduced bacteria was NREO > REO > ENS >ES.

Table 5.1: Minimum inhibitory concentration (MIC) of different treatments.

Type of Bacteria	MIC concentration (mg/mL)			
	ENS	**ES**	**NREO**	**REO**
Escherichia coli	0.43[a*] ± 0.03	1.12[b] ± 0.38	0.3[a] ± 0.05	0.35[a] ± 0.08
Staphylococcus	0.40[a] ± 0.10	0.75[b] ± 0.16	0.3[a] ± 0.05	0.33[a] ± 0.07

*The differences between values with the same letter is nonsignificant within same row ($P < 0.05$).

Essential oils used as antibacterial agents against most gram-positive, and gram-negative bacteria are those that have the MICs levels below 1% (V/V) or (10000 ppm or 10 mg/mL), examples are tea tree, oregano, lemon myrtle, lemongrass, and clove. This is in comparison with conventional antibiotics where the MIC susceptibility concentration is in µg/ml is around 1000 times less than the concentration of essential oils that are able to perform the same antibacterial action. The purpose of many studies is to test the activity of the essential oils on bacteria which are encountered within the food industry and consequentially play a role in human health (Holley and Patel, 2005, Burt, 2004, Carson et al., 2006).

The gram-positive Vancomycin-resistant *Enterococcus* and methicillin-resistant *Staphylococcus aureus* (MRSA) which are responsible for severe infections in human have been assessed for susceptibility to essential oils in many studies. Among these studies is the study by Tsao and Yin (2001) who investigated the activity of both Chinese Leek and Garlic essential oils on MRSA *in vitro* in broth microdilution, they found that the MICs were 48 mg/L and 32 mg/L respectively. The lower MICs concentration related to the higher concentration of diallyl disulphides in garlic. In a further study by Adukwu et al. (2012) lemongrass and citral showed inhibition of three of methicillin sensitive and two methicillin resistance *Staphylococcus aureus* at concentrations of 0.03% (v/v) and 0.06% (v/v). They reported that lemongrass showed anti-bacterial effects at a concentration of 0.125% (v/v). In the same study, the EOs of grapefruit showed less efficiency than those of lemongrass. In these trials the MICs were between 0.5% and 2% (v/v) and the bactericidal concentrations increased between 2% and 4% (v/v). The activity of tea tree essential oils against MRSA has also been investigated by many researchers. The MICs by the agar or broth dilution methods were found to be between

0.125-1% v/v (Nelson, 1997, Elsom and Hide, 1999, Hayes and Markovic, 2002, LaPlante, 2007, Mayaud et al., 2008, Loughlin et al., 2008). The MIC concentrations of different formulations used in the current study were below the suggested MICs by the previous researchers. Fourteen different essential oils were studied by (Mazzarrino et al., 2015) against different strains of *Salmonell*a and *L.monocytogenes*. Samples tested by both MIC and disc diffusion methods produced results which showed that the order of essential oils efficacy was oregano > cinnamon=clove > thyme > tea tree.

It is clear that the encapsulation of thyme essential oils in the current study enhanced the antibacterial activity against assessed bacteria. Many researchers announced similar results; they found that the encapsulated thyme EOs were more effective than free or bulk EOs against *E.coli, S. dysenteriae* and *S. Typhi* bacteria (Moghimi et al., 2016a; Moghimi et al., 2016b). The emulsion of the essential oils was also found to improve the antibacterial activity of the EOs in many other studies (Xue and Zhong, 2014; Zahi et al., 2015; Maté et al., 2016). The results of this study were in contrast with other studies where they found there was no improvement regarding the antibacterial activity when using emulsions of EOs. Chang et al. (2012) used corn oil and medium chain triglycerides (MCT) to inhibit Ostwald ripening and noticed no differences between the bulk and emulsion against the tested bacteria. Reduced activity of the thyme essential oils may be due to using carrier oils such as corn and MCT. Because the oil acts as a solvent for the thyme EOs preventing them from reaching the bacteria cell wall (Ziani et al., 2011; Chang et al., 2012). Additionally, Xue et al. (2013) used a conjugate of maltodextrin with whey protein and polypropylene glycol (PG) to encapsulate thymol and preserve milk with different fat content and noticed the variation in antibacterial efficiency in different kinds of milk according to fat content.

Among the treatments used in the MIC experiment, variations between samples were observed, solutions of the EOs that showed MIC higher than 1% were considered unfavourable while lower concentrations are thought to be useful in food product applications (Holley and Patel, 2005; Carson et al., 2006). From the results of the current work, it is suggested that the type of formulation of the EOs added to the inoculum affects activity against bacteria in the inoculum. For example, when essential oils are added into inoculum directly (ENS) it was more active than adding prepared concentrate from a solution of essential oils (ES). The reason for the different activity between the two formulations may be due to the separation of the oil when preparing a stock solution of

oil and water as they are immiscible, loss of EOs during subsequent dilution of the stock solution may also have an effect. As a result, the oil did not distribute equally in the solution, and lower concentrations of EOs is presented in the solutions were added into the inoculum. On the other hand, direct addition of the EOs assured the presence of the exact concentration of the EOs in the inoculum such as in the ENS and NREO samples. However, when encapsulated EOs are used, lower concentrations are required to achieve the MIC, for instance, non-reconstituted encapsulated EOs (NREO) showed higher activity and lower MIC concentration (0.3 mg/mL) when directly added to the inoculum against *E.coli* and *Staphylococcus* respectively. However, the same scenario was repeated with the encapsulated EOs; when a solution of encapsulated EOs (REO) used and showed lower activity but the differences was insignificant statistically. The similar activity of NREO and REO when tested against gram-negative and gram-positive bacteria may be due to improvement in the dispersity and solubility of the encapsulated EOs in the aqueous media compared to ENS and ES. The high value of MICs of ES negatively affected other values in which no statistically significant differences were observed between them despite the differences in their values.

It is clear that the EOs activity improved against both types of bacteria when EOs are encapsulated. The similar activity of the encapsulated thyme EOs against gram-negative and gram-positive bacteria can also be explained by the interactions between hydrophilic surfaces of the capsules containing EOs and the porin protein presented on the outer membrane of gram-negative bacteria which allows the hydrophilic solutes to pass through (Nazzaro et al., 2013). The ability of the EOs to reach the periplasm of the gram-negative bacteria and increase the extracellular ATP is also reported by (Helander et al., 1998a). Because thymol is the dominant constituent in thyme EOs which are used in this study, therefore according to Burt (2004) the gram-negative wall membrane could be broken by thymol resulting in a cytoplasmic permeability increase and ATP release. This result showed that encapsulation of the essential oil increased and improved the activity with both gram-negative and gram-positive bacteria. This improvement in EOs activity is an advantage of the current delivery system when compared to another study where they used bulk oil in this they found differences in susceptibility of both types of bacteria. These results were in agreement with (Lis-Balchin et al., 2003) who reported that there was no difference in activity of EOs on both types of bacteria, furthermore (Smith P. et al., 1998; Bhargava et al. 2015), found no differences in the activity of citrus essential

oils on both types of bacteria. Moreover, they explained that the improvement in performance of the EOs emulsion is due the reduction of hydrophobic property of the EOs using emulsification.

Many other studies have shown that essential oils are more active against gram-positive bacteria than gram-negative (Smith-Palmer et al., 1998; Marino et al., 2001; Delaquis et al., 2002; Pintore et al., 2002; Burt, 2004; Di Pasqua et al., 2005; Mayaud et al., 2008; Chorianopoulos et al., 2004). The peptidoglycan, which is made from sugars and amino acids forms 90-95% of the gram-positive hydrophobic cell wall (20-80nm) and 90% of bacteria dry weight, may help the interaction with essential oils which is also hydrophobic (Soković et al., 2010). In contrast, the complex cell wall of gram-negative bacteria contains a thinner peptidoglycan layer (2-3nm), which consists only10% of bacteria dry weight, and has an outer membrane formed of a double layer of phospholipids linked by peptidoglycan layer and LPS on the outer membrane. The later are responsible for repealing or tolerating foreign compounds such as hydrophobic essential oils (Kim et al., 2011; Ravichandran et al., 2011; Nazzaro et al., 2013; Bajpai et al., 2013). The sensitivity of gram-negative and gram-positive bacteria is found to be different when carvacrol and thymol are used against them (Dorman and Deans, 2000). The ATP release raised as a result of cytoplasmic membrane permeability increase when carvacrol and thymol are used against gram-negative bacteria due to breaking down of the wall membrane (Burt, 2004).

Furthermore, when EOs dispersed in the water, the MIC concentration increased and reached the highest value 1.12 and 0.75 mg/mL for *E.coli* and *staphylococcus* respectively. The decrease in NREO and REO compared to ES and ENS is contributed to the higher dispersity of the powder containing EOs more than the oil in the solution which may experience phase separation in the broth medium due to hydrophobicity effect of the EOs components. While powder particles are able to disperse all over the broth medium that contains bacterial suspension without separation or sedimentation. The higher dispersity of the encapsulated EOs aided the oil to distribute all over the solution and become closer to the bacterial cell wall. Therefore, the EOs components were partitioned into phospholipid cell wall causing disruption of their functionality. In addition, the less efficiency of the EOs solution also linked to the limited solubility of the EOs in the aqueous solutions. Therefore, more EOs required to inhibit the activity and growth of the microorganisms. In contrary, lower concentrations of the EOs required when the

encapsulated EOs were used. The MIC concentration reduced for both *E.coli* and *Staphylococcus* respectively compared to the free EOs in both forms with encapsulated EOs.

The range of the MIC concentrations of the encapsulated EOs used in this study was slightly lower compared to the thyme oil emulsion in SC and lecithin employed by Xue and Zhong (2014). Moreover, an emulsion of *Thymus daenensis* EOs emulsified by Tween 80 and lecithin against *E.coli* and *S. Enteritidis* which was 0.4 mg/mL by (Moghimi et al., 2016b). The enhancement of EOs activity against bacteria using emulsification is well reported in many studies. Such as improvement of d-limonene activity against both types of bacteria by (Zhang et al., 2014) and improvement of oregano oil emulsion on lettuce against foodborne bacteria by (Bhargava et al., 2015) in comparison to the free oil. The enhancement in emulsion activity can also be explained by the decrease of the oil droplet size, the increase of the surface area which resulted in the improvement dispersity of the oil droplets. In addition, where microorganisms grow in colonies or producing films the inner bacteria protected by the outer cells from large EOs droplets of the emulsion (Burt, 2004).However, the EOs droplets in the current study are small compared to bacteria cells (800-2000nm) or films, which make them more diffusible into colonies and cells. On the other hand, free oil can be easily separated when added to the aqueous phase (Bhargava et al., 2015). These results also in agreement with results found by Pan et al. (2014), where they discovered that the antilisterial activity of thymol enhanced by encapsulation using sodium caseinate as wall material. They stated that thymol as hydrophobic compounds has more affinity to partition in phospholipids on bacteria cell wall than casein. Therefore casein did not affect the activity of thymol. Furthermore, (Wattanasatcha et al., 2012) reported that the activity of thymol against *E.coli* and *Staphylococcus aureus* extended in cosmetic lotion by encapsulation in ethylcellulose/methylcellulose.

The activity of the EOs against microorganisms is linked to the increase in permeability and disruption of the cell membrane. The release of proteins, potassium ion and nucleic acids from the cells observed when *E.coli* suspension treated with sage emulsion (Moghimi et al., 2016a).

The best MIC concentration in the present study was 0.3 mg/mL when NREO used, and it was less than the MIC in other studies. For instance, when Adukwu et al. (2012) evaluated the antibacterial efficiency of lemongrass the results showed inhibition of three

of methicillin sensitive and two methicillin resistance of *Staphylococcus aureus* at concentrations of 0.06% (v/v) which about double of the current concentration. It was lower than the 8 spices of thyme oil which ranged between 0.05-0.78% as reported by (Oussalah et al., 2006). The MIC of different EOs in different food products is described in detail by (Burt, 2004). In contrast (Jamil, 2009) found that the MIC is 0.015% when used free *Thymus syriacus* against *E.coli* ATCC 25922 this may be due to high concentration of thymol and carvacrol which reached 74.1% and 9% respectively. The activity of thyme EOs depended on a number of phenolic compounds content. In this study, thymol consisted about 37% of the thyme EOs which considered low compared to other studies as mentioned earlier there is a variation in EOs composition according to many factors. One can choose the thyme EOs that contain higher amounts of phenolic compounds to reduce the added EOs into food products without changing the organoleptic properties of the food. The lower solubility and limited hydrogen bonding of hydrocarbons and terpenes limited their activity as antimicrobial agents, while the oxygenated terpenes showed higher activity against a broad spectrum of microorganisms. Hydrogen bonding and the size of the molecules also play a role in their activity, particularly on gram-negative bacteria, it is found by Griffin et al. (1999b) that smaller molecules showed better activity than the larger molecules. The degree of partitioning coefficient of the EOs in bacteria cell membrane directly affects their antimicrobial activity, the higher the partitioning coefficient, the more active the EOs.

The variety of essential oil constitutes led many researchers to investigate and identify the main active compounds against microorganisms. In clove oil carvacrol and eugenol (Chaieb et al., 2007), terpinen-4-ol in tea-tree (Carson et al., 2002), thymol and carvacrol in oregano (Lambert et al., 2001) were found to be responsible for the antibacterial activity. Moreover, studies stated that the different chemical constitutes in the essential oils have different antimicrobial activity. The greater antimicrobial activity is referred to phenolic compounds and aldehydes than non-phenolic alcohols. This followed by hydrocarbons which have the least activity against bacteria (Inouye et al., 2001; Delaquis et al., 2002; Carson et al., 2006; Mayaud et al., 2008; Hyldgaard et al., 2012). The chemical structure of the essential oils constitutes has a significant role in their activity. Carvacrol is found to enlarge liposomal membranes; its effect reaches a maximum at concentrations of 0.5 μmol/mg. It should be noted that EOs are characterised by their ability to reach periplasm through porin protein in gram-negative bacteria in

contrast to other antimicrobials (Helander et al., 1998b). Another explanation or mechanism is that hydrophobic hydrocarbon tends to accumulate and disassociate the interactions between proteins and the lipids on the cell wall and interactions between hydrophobic parts on the protein with the hydrocarbons occur (Juven et al., 1994)

The potential for synergistic effects between EOs constituents also improves the antimicrobial activity as studied by (García-García et al., 2011). They found that the combination of carvacrol and thymol were more active than thymol-eugenol and carvacrol – eugenol combinations. The binary combination of thymol and carvacrol reduced the concentrations to 62.5 and 75 mg/kg respectively. A mixture of eugenol, thymol and carvacrol inhibited the growth of *Listeria innocua*, in concentrations of 56.25, 31.25 and 75 respectively. Carvacrol and p-cymene also showed a synergetic effect against *B. cereus* this concluded by the swelling effect of p-cymene on bacteria cell walls which facilitate the penetration of carvacrol into the cell (Ultee et al., 2002). The combination of a monoterpenoid alcohol such as linalool and menthol with a monoterpenoid phenol such as eugenol showed a significant antibacterial activity (Bassolé et al., 2010). The current thyme essential oils contained a variety of compounds as illustrated earlier which may work synergistically in eliminating the microbial growth

5.4.1.2 The evaluation of the antimicrobial activity of the different forms of the EOs in Pasteurised Skimmed milk

All four formulations of the thyme EOs were evaluated as the antimicrobial agents to extend the shelf-life of the pasteurised skimmed milk. The final concentrations of thyme EOs for all forms in the milk samples adjusted to obtain 0.3, 0.5, and 0.75 mg/mL in addition to a control sample which contained 0.00 mg/mL of thyme EOs. The shelf-life of the skimmed milk at 7 ºC after adding different concentrations of thyme oil in various forms are summarised in (Table 5.2).

There was no sign of microbial growth in treated samples with thyme EOs neither in untreated samples within 6 days of the storage behind the best-before-date when stored at 7 ºC. However, samples containing 0.3 mg/mL EOs preserved the milk for longer time according to the form of the EOs used compared to non-treated samples. Moreover, no microorganisms detected for 9 days when 0.3 mg/mL NREO and REO used, while samples with ES showed microbial growth after 7 days of the storage when they used at the similar concentrations. When the concentration of the EOs in the samples increased

to 0.5 mg/mL the shelf-life of the samples prolonged to 14 days with NREO while remained 7 days with ES formulation. The longer shelf-life of the skimmed milk was 18 days at the concentration of 0.75 mg/mL of NREO without any detection of colony forming units when samples investigated.

The growth kinetic of the total count in skimmed milk treated with different concentrations of EOs in various forms are illustrated in (Figure 5.1). The untreated skimmed milk samples were more prone to microbial growth and contamination. On the other hand, samples treated with thyme EOs showed less and slower rate increase in the number of the microorganisms. Samples with 0.3 mg/mL EOs in different forms showed longer shelf-life compared to the control samples. The rate of reduction was concentration dependent, the higher the concentration, the less the number of the viable cells in skimmed milk were detected.

Table 5.2: The extended shelf-life of pasteurised skimmed milk in the presence of different levels of encapsulated and free thyme EOs in various forms stored at 7 ºC.

Thyme oil (mg/mL)	0.00	0.3	0.5	0.75
The required time for growth (days)*				
ENS	6	8	9	11
ES	6	7	7	8
NREO	6	9	14	18
REO	6	9	12	15

*The shelf-life after best-before-date.

The bacteriostatic effect of the EOs controlled the number of the microorganisms in the treated samples even after they started to appear in the samples. The number increased relatively at a lower rate compared to the untreated samples. The results from the current experiment suggested that the bactericidal effect of the EOs prevent the bacteria from growth for different periods according to the formulation and concentration of EOs used. However, most psychrotrophic are able to form spores so that they may remained in the treated milk and flourished again after certain period depending on the concentration of

the EOs used at the later stages of the storage. The magnitude of reduction in different formulations and concentrations were different. In untreated samples, the number of the viable microorganism was increased and reached above (1.00e+10). On the other hand, when EOs in different forms was used, the number of the viable microorganisms reduced dramatically and reached below (1.00e + 08) with NREO at 0.75 mg/mL. The growth rate associated to the formulation and the concentration of the EOs used. There are obvious differences between all treatments as shown in (Figure 5.1 a, b and c).

The availability of thymol of the various EOs formulations is shown in (Figure 5.2). The concentration of the available thymol in skimmed milk (SM) depended on the concentration of the added thymol initially. In addition, the formulation in which the EOs introduced into the skimmed milk also played a great role in determining the availability of thymol in the milk. Higher availability observed for NREO followed by REO, ENS and ES. These results are inline with the results of the antibacterial activity of the different formulation as illustrated earlier.

For example, when 0.3 mg/mL EOs in forms of free oil (ES) added to the skimmed milk it was supposed to contain 0.11 mg/mL thymol. While it was found that the amount of thymol in these samples reduced to 0.072 mg/mL which comprises only 66% of the initial thymol concentration. Reduction of thymol also observed in the other formulations. The highest availability of thymol in skimmed milk detected when NREO added at all three different concentrations followed by REO, ENS and ES.

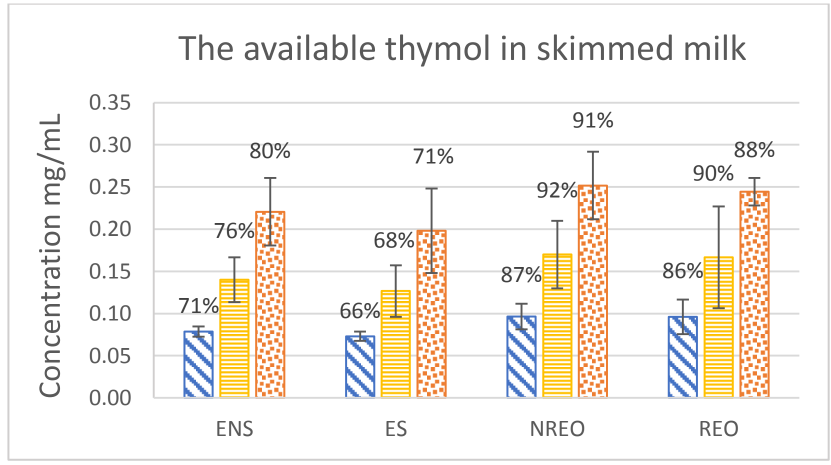


Figure 5.1: The available concentrations and the rate of the thymol in skimmed milk for different EOs formulations of actual concentrations of ▧ 0.3 mg/mL, ▤ 0.5 mg/mL and ▩ 0.75 mg/mL.

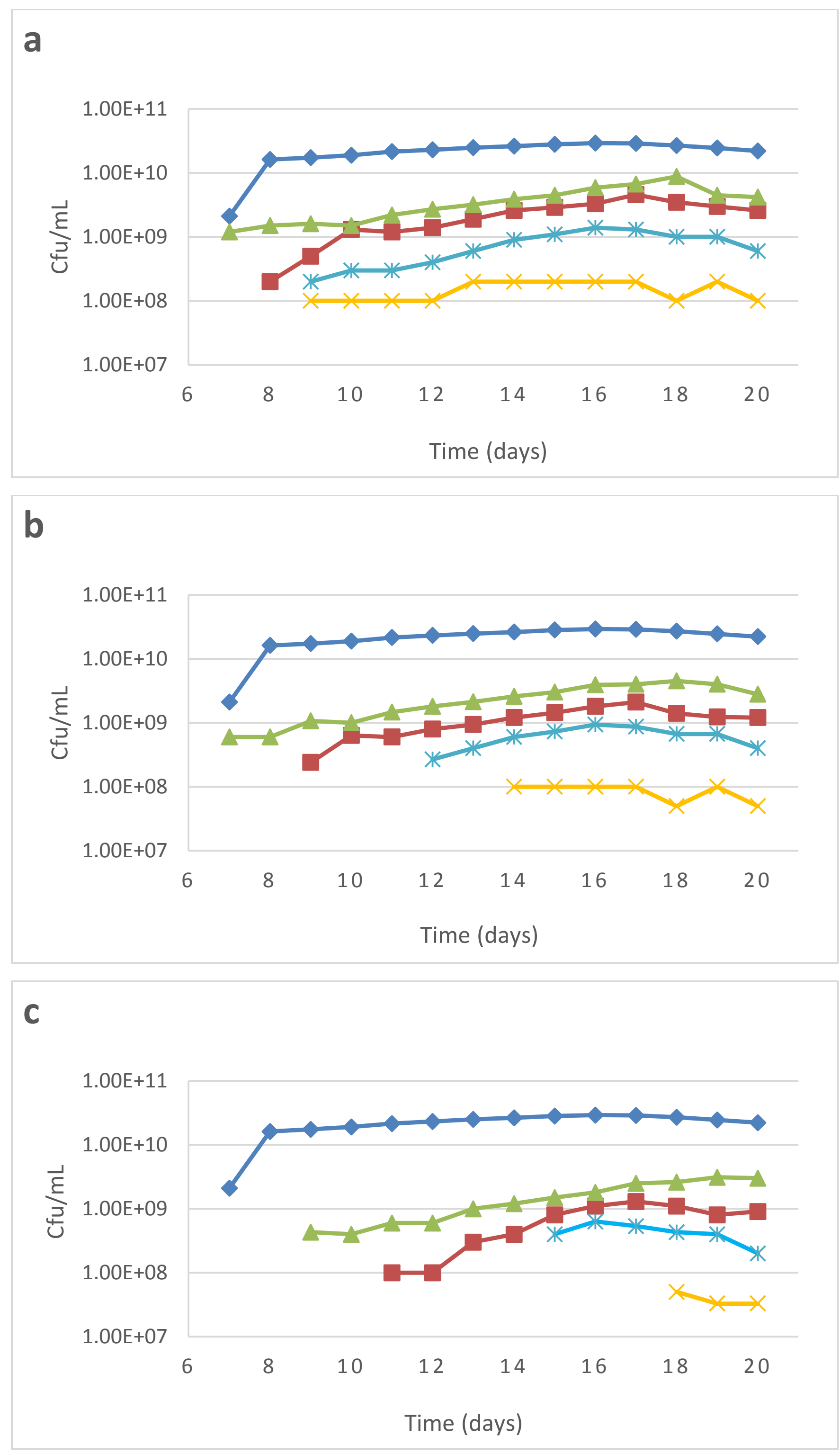


Figure 5.2: The growth kinetic of the total count of skimmed milk psychrotrophic during 20 days of the storage at 7 ºC (a) at 0.3 mg/mL (b) at 0.5 mg/mL and (c) at 0.75 mg mL EOs in different formulations, Control, NREO, REO, ENS and ES.

Results showed that the pasteurised skimmed milk was free from any microbial growth during 6 days of storage at 7 ^{o}C after its best-before-date. This result is an indication of a following good hygienic practice when the milk is prepared and distributed. The required shelf-life by the market is around 14 days or more which can be attained by practising a safe handling and processing of the milk, cleaning and a standard sanitation program after pasteurisation. The pasteurisation of the milk destroyed most of the bacteria in the milk, the initial total count of microorganism in pasteurised milk should be less than 500 CFU/ml. However, some psychrotrophic spoilage bacteria able to form spores and survive pasteurisation process and appear again in refrigerator storage leading to post pasteurisation contamination and limit the shelf-life of the pasteurised milk, particularly when gram-negative bacteria is presented in the milk (Barbano et al., 2006). The chance of gram-negative bacteria such as *Pseudomonas spp.* to present in milk is limited by practising hygienic process. However, some gram-positive bacteria such as *Bacillus spp.* and *Microbacter* also have the ability to survive the heat treatment of the milk which limit milk shelf-life. Samples containing more than 30000 CFU/mL is considered as unsafe for human consumption.

From the current experiments, it is evident that adding thyme EOs contributed in prolonging the shelf-life of the pasteurised skimmed milk, regarding the level and the formulation of the EOs used as natural preservatives. Higher activity of the essential oil observed when encapsulated EOs is directly added to the milk samples. However, it is more practice in the food industry to prepare a solution of the antibacterial agents then dispersing it into a large amount of the food during processing. Thus according to the results of the current study, it is found that the reconstituted powder containing EOs is the proper choice to be employed to preserve food with natural antimicrobials. The EOs was able to extend the shelf-life of pasteurised skimmed milk for around 18 days beyond its original best-before date, and the concentration is below the limited dose which considered safe for human consumption. It is potential to apply to milk, cheese, bread, juice, ready to eat meals, fruit, vegetable or any other perishable foods to provide safe food, decrease the food waste and improve the quality. Moreover, the addition of EOs enhances the quality of food by retarding the oxidation in foods and other beneficial effects of some compounds on the human health after consumption as reported in many studies which acts as an antioxidant, anti-inflammatory and anti-cancer agents.

The activity of EOs in different formulations is directly linked to the concentration of the thyme oil components in the continuous phase. In this study, thymol took into account, as an indication of EOs availability in the aqueous medium, as it is the dominant compound and responsible for the antibacterial activity of thyme EOs. The higher availability of thymol in samples containing encapsulated EOs is related to the higher dispersity of the powder particles in the aqueous medium than the free oils. Lower availability of thymol in samples containing solutions of EOs is linked to the limited solubility and dispersity of the different formulations of the EOs in skimmed milk samples. Milk Samples contained a lower concentration of thymol were less effective against microorganisms. The decreased concentration of thymol is may be due to interactions between different compounds in skimmed milk such as proteins with thymol through different intermolecular interaction. Therefore, in skimmed milk higher concentrations of thyme EOs was required to inhibit microbial growth than the required concentration of MIC. These outcomes were in agreement with results demonstrated by (Xue et al., 2013). The small particle size of the reconstituted powder (< 250 nm) may be the key factor to their high activity, due to their ability to penetrate the bacteria cell wall and reach their phospholipid membrane. Another explanation is that the water solubility of the oil droplets enhanced by decreasing the droplet size (Moghimi et al., 2016a). However, it is not probable to compare the results of the current study with other studies which used encapsulated thyme oil. Due to many factors such as harvest location, genetic, chemotype, wall material, and the encapsulation techniques (Hyldgaard et al., 2012) in addition to the dissimilarity of microorganisms and the conditions of preparation and incubation.

Greater EOs required when added to milk samples than the MIC samples; the reasons are the binding of the active components with other components inside milk (Hyldgaard et al., 2012). Moreover, as suggested by (Gill et al., 2002) microorganisms are able to repair damaged cells when they present in food products using the available nutrients.

5.4.1.3 The *in vivo* test of whole unpasteurised milk stored in refrigerator and room temperature

All forms of thyme EOs were used to evaluate their activity to preserve and extend the shelf-life of unpasteurised whole milk. Samples stored at different temperatures to investigate the combination effect of temperature and the EOs. The shelf-life of the whole

milk treated with the various concentrations of thyme EOs and in different forms are shown in (Table 5.3 and 5.4). The initial total count of the fresh sample was around $4x10^5$ CFU/mL which considered acceptable according to Food Standard Agency regulations. Samples stored at room temperature showed short shelf-lives which verified by detecting viable colonies growth in the incubated plates of the samples on the nutrient agar. As shown, samples exhibited microbial growth after 24 hours of the storage in the control samples (without EOs) and the CFU/mL increased to $2.3X10^7$. Similarly, samples of milk contained 0.75 mg/mL of ENS and ES and stored in the refrigerator were unable to eliminate microbial growth after 24 hours of the storage. While the encapsulated EOs when used at the same concentration was able to preserve the raw milk for more 2 days. As the concentration of the EOs was increased, all types of the EOs showed an extended activity and prolonged the milk samples shelf-lives without any detected microbial growth. ENS could extend the shelf-life for 2days while the milk samples stored with REO and NREO extended the shelf-life for 4 and 5 days respectively at 1 mg/mL EOs. At a concentration of 2 mg/mL the longer shelf-life observed with NREO which reached 7 days followed by REO, ENS and ES.

Table 5.3: The in vivo test of whole unpasteurised milk stored in refrigerator.

Thyme oil (mg/mL)	0.00	0.75	1	2
The required time for growth (days)				
ENS	1	1	2	3
ES	1	1	1	3
NREO	1	2	5	7
REO	1	2	4	5

Same formulations of the EOs were used in the whole milk and stored at ambient temperature. The EOs in all forms and at a concentration of 0.75 mg/mL were unable to protect the milk from microbial growth and spoilage. Whereas, when the concentration

increased to 1 mg/mL NREO and REO showed further activity, no colonies were detected for 3 and 2 days respectively. At 2 mg/mL of EOs, the milk remained clear from any microbial growth for 3, 2, 4 and 3 days for samples containing ENS, ES, NREO and REO correspondingly.

The concentration of the available thymol in unpasteurised whole milk is shown in (Figure 5.3). When EOs added to the samples a dramatic reduction in the available thymol was detected in all formulations and at all different concentrations. In comparison with thymol in skimmed milk, it is clearly shown that the concentration is reduced by more than 4 folds in the whole milk. For instance, when 0.75 mg/mL ES used in both experiments, the concentration of the available thymol decreased to 0.052 mg/mL in whole milk while it was 0.22 mg/mL in the skimmed milk. The thymol availability rate also fell by around 2-3 folds for other EOs formulations compared to the rates in skimmed milk.

The similar decrease also occurred in all other formulations which are reduced by various percentages. The rate of the reduction of the available thymol from the original content is very high. The lowest recovery was for ES at 0.75 mg/mL thyme oil since it lost around 83% of the initial concentration. The higher the concentration of the EOs used the most available thymol in milk were detected as illustrated in (Figure 5.3).

Table 5.4: The in vivo test of whole unpasteurised milk stored at room temperature.

Thyme oil (mg/mL)	0.00	0.75	1	2
The required time for growth (days)				
ENS	1	1	1	3
ES	1	1	1	2
NREO	1	1	3	4
REO	1	1	2	3

Despite the increase of thyme EOs concentration, the shelf-life of the whole milk dramatically decreased in comparison to skimmed milk. The longer shelf-life was observed for samples containing 2 mg/mL NREO, which reached 7 days. However,

samples containing 0.75 mg/mL NREO were able to extend the shelf-life of the skimmed milk for a longer period of 18 days above the expected best-before-date. In another comparison, samples stored at room temperature had shorter shelf-lives compared to the samples stored in the refrigerator. In all samples, NREO was superior regarding the inhibition of the foodborne microorganisms.

If samples stored at ambient temperature is compared with those stored in the refrigerator it is clearly shown the differences between the shelf-lives among the samples even when the same concentrations of the EOs were used. At 2 mg/mL the longer shelf-life obtained when NREO used and stored in the refrigerator which extended for 5 days. Then the shelf-life of the milk decreased to 3 days when the same concentration of NREO added to whole milk samples and stored at room temperature because the temperature is one of the vital factors regarding bacterial growth and maintenance. Similar trends were observed for other formulations as well.

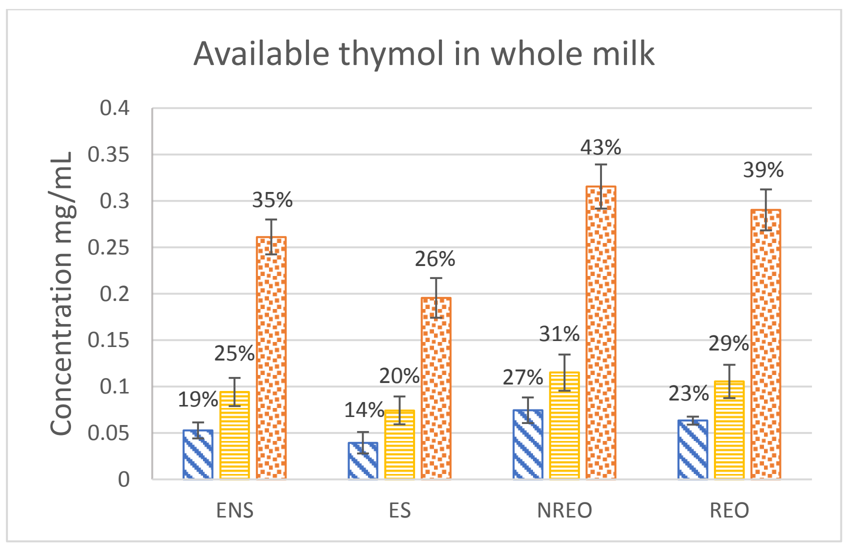


Figure 5.3: The concentration and rate of the thymol in whole milk for different EOs formulations of 0.75 mg/mL, 1 mg/mL and 2 mg/mL.

The shelf-life of the whole milk was dramatically reduced compared to the shelf-life of the skimmed milk. This occurrence may be due to the presence of the fat in the raw milk which hinders the activity of the EOs entirely or partially (Mejlholm and Dalgaard, 2002) by protecting the bacteria from EOs (Tassou et al., 1995). The concentration of the EOs in the different formulations play a great role in determining the activity of the EOs. The fractionation of the free oil and encapsulated particles in the milk fat reduced the availability of the EOs and its bioactive compounds in the milk serum. The decrease of

the antibacterial activity is directly related to the availability of thymol as it is the dominant compound of thyme EOs in this study. It is worth mentioning that proteins in milk found to limit the antibacterial activity of carvacrol against *Bacillus cereus* when used by (Pol et al., 2001). The antibacterial activity of thyme EOs decreased in the samples containing less concentrations of thymol. As illustrated earlier, the reduction of the thymol in whole milk samples is a good indication of probable temporary binding between thymol with milk protein and fat globules. Most of the thymol antimicrobial activity was hindered due to the presence of fat and protein inside the milk. In addition, to the reasons above, the initial microbial content of the two different kinds of milk played a role in determining the effective concentration of the thyme EOs. However, using encapsulated EOs reduced the antagonism effect of milk components and enhanced their activity.

However, depending on the availability of thymol in the whole milk with increasing the concentration of the EOs, the available EOs in the milk increased and showed some antibacterial activity as can be seen at 1 and 2 mg/mL when the encapsulated EOs were used. Nevertheless, samples contain 2 mg/mL EOs affected the organoleptic properties of food which may not be possible to apply to food products which are not compatible. It is evident from this experiments that the encapsulation of the essential oils enhanced the dispersity of the oil and distributed them in the milk which was able to preserve the milk from microbial contamination for the extended period compared to the free EOs due to increasing the availability of the bioactive compounds.

Furthermore, it understandable that the combination of two preserving methods such as EOs and low temperature is an excellent approach to producing safe food without reaching the limited doses of the antibacterial agents as increasing EOs concentration leads to organoleptic alteration of the food and health consequences. The current results are in agreement with others where they used a combination of hurdles to preserve and extend the foods shelf–life. For example, the antibacterial effect of thymol and carvacrol against *Salmonella Typhimurium* increased when combined with organic acids such as acetic acid and citric acid. Whereas, no significant change was observed when lactic acid was combined with them. In their conclusion Zhou et al. (2007) revealed that to achieve an adequate antibacterial activity of thymol and carvacrol, their combination with organic acids offers an effective solution.

In their study Friedly et al. (2009) compared the antibacterial activity of five citrus EOs alone and in combination with organic acid against *Listeria spp*. They found that the EOs had the MIC of 0.55% and MBC of 1.67 % if used alone while when it combined with 0.12% organic acid caused the reduction of both MIC and MBC to 0.04%. They also concluded that the synergic effect of the organic acids helped EOs to be used in lower concentration by ten folds to inhibit bacterial growth. Reduction of *Salmonella* and *Campylobacter* which are mesophilic bacteria was observed when a combination of thyme and orange EOs were used to preserve skinless chicken breast in marinade at concentrations of 0.5%. Non-significant effect of the combination observed when whole wings with skin-on were used, which may suggest the need to use higher concentrations of the EOs (Thanissery and Smith, 2014). In another study by Ilhak and Guran (2014), fish patty treated with a combination of thymol 0.1 w/w and sodium lactate 1-2% v/w stored at 4 ºC for five days. They found that the combination was ineffective against *L.monocytogenes* nevertheless it was effective of *Salmonella Typhimurium*.

The concentrations of thyme EOs used in food samples were below the toxic dosages which considered acceptable to the health of the consumers. In contrary, some of the essential oils are toxic at effective doses for antibacterial activity. However, they may be used as antiseptic products to prevent pathogen spread by skin contact. Slower respiration rates and reduced locomotor activity were observed in mice when they were exposed to EOs of thyme at concentrations of 0.5-3.0g/kg body weight (Qureshi et al., 1991). However, the European Commission has accepted the use of some EOs constitutes such as thymol, carvacrol, linalool eugenol, cinnamaldehyde, vanillin, citral and limonene as food flavours. Also, these compounds and the EOs of clove, thyme, cinnamon, nutmeg, basil and mustard are classified by the Food and Drug Administration (FDA) of the USA as (GRAS) (Hyldgaard et al., 2012).

5.4.1.4 The antimicrobial activity of encapsulated thyme EOs in Baobab juice

Encapsulated thyme EOs was able to inhibit microbial growth at 0.3 mg/mL as showed by impedance curves of Bactrac instrument (data not shown). Whereas samples incubated without adding EOs powders showed a growth curve as indication of microbial growth in Baobab juice. Unfortunately, due to the lack of calibration curve no cell enumeration was carried out. However, the antimicrobial concentration was lower in Baobab juice compared to both skimmed and whole milk. This was occurred due to the

lack of proteins and fat in addition to low pH 3 which increased the bioactivity of thyme EOs, due to increasing in solubility of EOs at lo pH (Burt, 2004; Friedly et al., 2009).

5.5 Conclusion

Thyme essential oils are hydrophobic therefore their antimicrobial activity is limited when added to aqueous food products in addition to the interactions with food matrices. The encapsulation of essential oils found to reduce these unfavourable effects. In this study, the antibacterial activity of encapsulated and free thyme EOs was assessed in both in vitro and in vivo systems. Results showed that the antibacterial activity was significantly increased when encapsulated thyme oil used in comparison to free oil in both systems. It was also revealed that the encapsulated EOs inhibited both gram-negative and gram-positive similarly. The shelf-life of pasteurised skimmed milk and non-pasteurised whole milk prolonged for some extent when thyme EOs added in different forms. Moreover, the availability of thymol remarkably affected the antibacterial activity of both encapsulated and free thyme EOs. The activity lower antibacterial activity was observed in whole milk than in skimmed milk due to the presence of fat which reduced thymol availability. The combination of thyme EOs and low storage temperature significantly affected the shelf-life of the whole milk.

General Conclusion

This research showed that thyme EOs contained different compounds at different concentrations which affect the physiochemical and biological properties of the oil and the emulsion system. Skimmed milk in liquid formulation showed to act as a novel emulsifier for thyme EOs. The stability of the produced emulsions for 60 days with small particle sizes (< 240 nm) is of considerable advantage, since the preparation is simple and requires only a homogenisation and spray drying. In contrast, other techniques used in emulsification entail lengthy, complex and expensive procedures in the preparation of the capsules. The addition of 3 % w/w maltodextrin enhanced the retention of EOs in the capsules and reduced the surface oil due to increased total solids. The maximum encapsulation efficiency (97.38%) was achieved when the concentration of MD increased from zero in the initial emulsion to 3 % w/w.

This novel encapsulation technique could be of great commercial benefit, as it produces capsules from safe food material in the form of a powder which can be applicable in many industries such as food, medicine, pesticide, and cosmetics. This technique may be applied to incorporate functional foods, flavours, probiotics or colourants into food products or pharmaceuticals. The encapsulation technique is able to preserve the core materials from undesirable changes and release them at the target destination and appropriate time. The preservative properties of the capsules may decrease the waste in the food sector and provide safer foods free from synthetic additives. As discussed in earlier sections, this novel technique needs only two straightforward preparation steps which produce powders containing high amounts of thyme EOs (97 %) of the initial amount which is considered cost effective in addition to their improvement in their activity and extended the shelf-life of the core material.

The physiochemical properties of the EOs components were found to play a crucial role in determining the properties of the emulsions and the powders. Terpenoids showed solubility in water to some extent and they also showed partitioning in both octanol and water phases. The location of the polar group greatly affected the solubility and partitioning of these compounds in the water phase. The amphiphilic compound tended to self-associate and produce weak emulsion due to the packing of the benzene rings on their molecules via *pi* stuck intermolecular interactions. Some terpenoids in thyme EOs showed some degree of surface activity and were able to reduce the surface tension of the deionised water, and they reached the CMC below their solubility limit. This property

helped to lower the free energy of the system, which is favourable to obtain thermodynamically stable emulsions. It was confirmed that the interaction of EOs with other components at the interface increased the stability and decreased the droplet size of the emulsions in addition to their effect on surface oil content and the release from the capsules. This suggests that the amphiphilic compounds in EOs enhanced the emulsion properties by locating themselves at the interface between water and oil droplets. Moreover, they may interact with different sites of proteins and water via a variety of bonds including van der Waals, dipole-dipole, pi-pi interactions and hydrogen bonds. On the other hand, sunflower oil emulsion in the same system of emulsification showed larger particle and lower stability due to the weak van der Waals interaction of oil at the interface.

The dissolution of the powder occurred quickly, and the UVM technique was found to be rapid enough to determine the rate of powder dissolution, especially when compared to more conventional techniques. Moreover, the release experiment showed a high release rate of the active compound of thymol which makes this encapsulation technique useful regarding the release of the components into food products at the short time as recommended for rapid release in some foods. However, the release rate depended on the type and concentration of EOs components in the core. Polar molecules which have a smaller size such as thymol released at higher rates than nonpolar compounds such p-cymene and large molecules such as β-caryophyllene.

The enhancement of biological activity was in strong accordance with the method of EOs application to food or bacteria suspensions. The maximum activity was found for encapsulated EOs, and lower activity observed when free oil used. However, the activity of the EOs significantly decreased when added to food products due to the binding of thyme EOs active components with food components. The availability of thymol in the food system directly affected the antibacterial activity; encapsulated techniques increased the availability of thymol. Therefore, the encapsulated EOs was able to increase the shelf-life of pasteurised skimmed milk for 18 days beyond its best-before-date of skimmed milk which make them excellent natural preservatives to be used in dairy products. The encapsulated EOs also showed antibacterial activity against the presented microorganisms in unpasteurised whole milk and was able to extend the shelf-life of the milk when combined with low temperature storage. It is recommended to use the same preservative technique on cheeses which uses raw milk. The MIC experiment showed

that there was a slight difference between the gram-positive and gram-negative when free oil used while no differences observed when encapsulated oil used which suggested higher dispersity of encapsulated EOs that make them available closer to the cell membrane of bacteria.

Recommendations and future work

It is worth testing the antibacterial activity of the EOs emulsions in skimmed milk and MD in both *in vitro* and in the food products. Mixing the emulsions with aqueous food products and passing it through high pressure homogeniser may enhance and increase the activity of the EOs. This emulsion system may then be applied to encapsulate other food additives such as flavours, colourants, enzymes, probiotics and nutraceuticals to enhance the bioavailability. The encapsulated EOs may be added to bakery, soft cheese, meat products or fish to evaluate their role in preventing microbial growth and extending the shelf-life. It is also possible to use this encapsulated system in cosmetic products and evaluating their activity. Further investigation of the effect of the trace compounds is recommended to clarify their role in the antibacterial activity of thyme EOs. It is also recommended that skimmed milk from different sources be used to compare and evaluate their ability to encapsulate essential oils. Moreover, analysing the protein structure of skimmed milk powder (SMP) and liquid skimmed milk using techniques such as electrophoresis is highly recommended. This is because spray drying of SMP may affect the proteins functional properties and stability of the emulsions prepared with them. It worth investigating the effect of different spray drying inlet temperatures on the encapsulation efficiency of the EOs emulsions in skimmed milk and maltodextrin. Testing other carbohydrates with skimmed milk may also result in better emulsions or powders of EOs. Finally, sensory evaluation of the foods with added thyme EOs is highly recommended to investigate consumer acceptability of the product.

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