Microencapsulation of Natural Antimicrobial Agents to Minimize Loss from Food Packaging Films

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ABSTRACT

The inherent volatility and/or heat sensitivity of many natural antimicrobial (AM) additives can be detrimental to their widespread use in commodity polymer packaging film formulations. In this study, beta-cyclodextrin (β-CD) inclusion complexes with naturally-derived AM agents: thymol, carvacrol, and linalool were prepared using a co-precipitation technique. The complexes were optimised and then characterised by techniques including thermal analysis. They were then incorporated into low-density polyethylene (LDPE) films with AM agents added directly for comparison. The subsequent release of the AM agents into food simulants was studied followed by an investigation of the efficacy of the films *in vitro* against selected bacteria. The films were later tested on real foods to assess their potential for controlling microbial growth and lipid oxidation.

In the initial experiments, conditions for synthesising the β-CD/AM agent complex including solvent composition, temperature, reaction time, and total solvent volume were investigated to optimise the inclusion efficiency (IE) and yield. Electrospray ionization mass spectrometry and gas chromatography were used to confirm the formation and quantify the amount AM agents that were encapsulated, absorbed onto the surface, or remaining in the filtered solvent. The systematic optimisation of the conditions improved both the yield of the complex and the IE of the AM agents. Using a 1:1 mole ratio of the AM agent to β-CD, the optimised parameters resulted in maximum yields of 87, 84 and 86% (w/w) for thymol, carvacrol and linalool respectively with IE's close to 100% (w/w) for each agent.

The kinetics of the thermal decomposition of the optimised β-CD and complexes of the three AM agents were then investigated using thermogravimetric analysis. Under a linear temperature ramp and in the degree of conversion, α, domain: $0.1 \leq \alpha \leq 0.8$, the major decomposition steps of the β-CD, and complexes with carvacrol, linalool and thymol occurred at *ca*. 300°C and followed Avrami-Erofeev kinetics with apparent activation energies, E_a, of: 156 ± 6, 107 ± 7, 96 ± 3 and 110 ± 3 kJ mol^{-1} respectively. Below *ca*. 300°C there were staged mass losses from each of the complexes that were not observed for the neat β-CD. These were attributed to lower energy binding interactions

and accounted for a little over half of the available guest species in the complex in each case. Lower temperature mass losses for β-CD complexes with carvacrol (*ca*. 140 to 230°C) and linalool (*ca*. 95 to 150°C) were analysed and found to be adequately fitted by second-order kinetics with apparent E_a values of: 37 ± 1 and 69 ± 6 kJ mol^{-1} respectively. The results suggest the optimized complexes are generally thermally stable and would potentially be suitable for high-temperature extrusion processes with acceptably low losses.

The next experiments involved the incorporation of the AM agents into LDPE films either directly or encapsulated in β-CD. Quantification of the AM agents was performed immediately following thermal processing, then six and thirty days after the film samples were stored in an open atmosphere. After six days, no AM agent was detected in the films where the agent was added directly to the film whereas the films containing encapsulated agents showed only small decreases in the concentrations of the agents up to 30 days. The migration of AM agents from LDPE films into 95% (v/v) ethanol/water mixtures food simulants at 4°C was adequately described using first-order kinetics and Fick's second law of diffusion. For the AM agents added directly to the film, the initial release rates were between four and eight times greater than those of the encapsulated agents. Similarly, the diffusion coefficients of the free agents were *ca*. four to five times greater than the encapsulated agents.

The free and encapsulated natural AM agents incorporated into LDPE film were tested against *Escherichia coli* (ATCC 25922) in order to assess the potential of the AM inclusion complexes for use in food packaging films. The direct incorporation of the complexes in the film formulations resulted in little inhibition of the target bacterium as assessed by the agar diffusion method even with AM levels as high as 5% (w/w). In comparison, levels of 2% (w/w) of free thymol and carvacrol added directly to the film demonstrated inhibition. The addition of glycerol to the film formulations was investigated as a means of facilitating the AM agent from the complex. A concentration of 1% (w/w) of glycerol in the film formulation was found to result in microbial inhibition which increased with additional glycerol. The use of 2% (w/w) glycerol resulted in a more pronounced inhibition of targeted microorganism. Upon the addition of glycerol, all of the films showed AM activity against the target bacterium with the exception of those containing linalool in either the free or encapsulated forms. Upon the addition of 2%

(w/w) of glycerol to the film formulation, encapsulated thymol at a concentration of 2% (w/w) was more effective than encapsulated carvacrol at a concentration of 3% (w/w) against *E. coli* with zones of inhibition of 30.70 ± 0.72 and 29.61 ± 0.86 mm respectively.

In the final experiments, the LDPE films containing encapsulated thymol were tested on real food systems. The level of thymol was 1 to 3% (w/w) relative to the LDPE and glycerol was added in order to obtain the optimum controlled release. In the case of packaged minced beef inoculated with *E. coli*, no inhibition was observed when the concentration of encapsulated thymol was 3% (w/w) with 2% (w/w) glycerol, however, the same film reduced *E. coli* growth by 0.7 $\log_{10}$ CFU g^{-1} on chicken breast fillets compared with the control during storage at 4°C for 12 days. The growth of *E. coli* was found to be affected by the temperature at 4°C whereby the bacterial counts remained relatively low with slow growth over the test period under refrigeration. However, when the temperature increased to 10°C it was also found that the presence of coliforms interfered with growth of *E. coli* and, in general, the films containing encapsulated thymol effectively reduced coliform growth. Analysis of the antioxidant (AO) activity of films using the diphenyl-picrylhydrazyl (DPPH) radical assay showed a 71% reduction in DPPH concentration for the LDPE/thymol/β-CD films containing 3% (w/w) thymol. Furthermore, a film comprised of 1% (w/w) thymol with glycerol stored at room temperature for 20 months showed a reduction by 23% in DPPH concentration confirming that the films are suitable for extended storage. Analysis of the formation of thiobarbituric acid reactive substances on packaged minced beef showed decreases in lipid oxidation of 60 and 75% for films containing 1% and 3% (w/w) thymol in the film respectively. The films therefore show promise for the dual purpose of AO and AM activity in order to prolong the shelf-life of selected food products.

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DECLARATION BY AUTHOR

I, Ghofran Al-nasiri, declare that the PhD thesis entitled “Microencapsulation of Natural Antimicrobial Agents to Minimize Loss from Food Packaging Films” is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature:  Date: 6/02/2019

ABBREVIATIONS

β-CD	β-cyclodextrin
AM	Antimicrobial
AO	Antioxidant
AP	Active packaging
a_w	Water activity
BHT	Butylated hydroxytoluene
CoQ10	Co-enzyme Q10 (ubiquinone-10)
DE	Dextrose equivalent
DSC	Differential scanning calorimetry
EE	Encapsulation efficiency
EOEs	Essential oil extracts
EOs	Essential oils
ES/MS	Electrospray ionization mass spectrometry
GA	Gum Arabic
GC	Gas chromatographic
HLPC	high-performance liquid chromatography
HP-β-CD	hydroxypropyl-β-cyclodextrin
HPMC	Hydroxypropyl methyl cellulose
IE	Inclusion efficiency
IEP	Isoelectric point
LDPE	Low density polyethylene
MAP	Modified atmosphere packaging
MD	Maltodextrin
MDA	Malondialdehyde
MS	Modified starch
OSA	Octenylsuccinic anhydride
PBS	Phosphate-buffered saline
PLA	Poly(lactic acid)
PVA	Poly(vinyl alcohol)
RH	Relative humidity
SDS	Sodium dodecyl sulfate
SPI	Soy protein isolate
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TEAC	Trolox equivalent antioxidant capacity
TEP	Tetraethoxypropane (malondialdehyde precursor)
TG	Thermogravimetric
WPC	Whey protein concentrate
WPI	Whey protein isolate
ZP	Zeta potential

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1 Introduction

1.1 General Introduction

Over recent decades, there has been a steadily growing interest in the development of new and novel food packaging techniques to meet global food demands. New techniques are vital in order to extend the shelf-life of food by delaying food spoilage; to maintain food safety, integrity and quality; and to reduce environmental impacts and economic costs associated with packaging wastes (Appendini and Hotchkiss, 2002). Active packaging (AP) is an innovative approach to address these issues and encompasses a wide range of techniques where the packaging, the product, and the environment interact beyond primary packaging functions (Ahvenainen, 2003). Food characteristics that can be enhanced by various AP technologies include physiological processes (e.g. respiration of fresh fruit and vegetables), chemical processes (e.g. lipid oxidation), physical processes (e.g. staling of bread, dehydration), infestation (e.g. by insects) and microbial aspects (e.g. spoilage by micro-organisms) (Ahvenainen, 2003). Examples of common AP technologies are listed in Table 1.1 and in some cases, two or more systems might be used together to optimize the packaging environment (Fang *et al.*, 2017). Several reviews of AP technologies and their applications have been reported over the years including those by Suppakul *et al.* (2003), Kerry *et al.* (2006), and more recently by Fang *et al.* (2017) and Silva *et al.* (2018b). Of the AP technologies that have been developed, those that reduce the need for the addition of chemical preservatives directly into foods are increasingly popular.

1.2 Antimicrobial Packaging Systems

Antimicrobial (AM) packaging is one of the more promising forms of AP technologies where AM agents incorporated directly within the packaging or coated onto the film to kill or inhibit the spoilage and pathogenic microorganisms that can contaminate foods (Quintavalla and Vicini, 2002). Effective AM packaging system can be achieved by different modes including: (i) incorporation of AM agents into a sachet/pad inside the package; (ii) directly incorporating AM agents into the packaging film; (iii) coating AM agents on the packaging material; or (iv) the use of polymers that have intrinsic AM properties (e.g. chitosan, poly-L-lysine, calcium alginate, etc.) (Fang *et al.*, 2017).

Table 1.1: Examples of active packaging technologies

AP Technology	Advantages	Limitations	References
O_2 scavengers	• Prevent rancidity in oils and fats • Minimize the loss of nutrients such as vitamins and minerals • Kill insects and worms and their eggs in rice, wheat and soybeans • Inhibit the growth of moulds and aerobic bacteria that limit shelf-life	• Water activity levels higher than 0.92 enable many microbial pathogens to grow such as *Clostridium botulinum* under anaerobic conditions • Temperatures of food packaging must not be lower than 0°C • In gas packaging, the growth of aerobic microorganisms can occur depending on the oxygen level	Vermeiren *et al.* (2003)
CO_2 generators	• Inhibit microbial growth	• High concentrations can stress the packaging resulting in deterioration and/or destruction of packaging due to increased internal pressure • High concentrations favour higher production of *C. botulinum* toxins	Suppakul *et al.* (2003) Vermeiren *et al.* (2003) Fang *et al.* (2017)
Modified atmosphere packaging (MAP)	• Control microbial growth by changing environment (i.e. from aerobic to anaerobic) • CO_2 is lower density and relatively inexpensive	• Different gas formulations require specialized and expensive equipment • In some products, temperature control is required • CO_2 generates carbonic acid leading to acid effects such as colour changes, off-tastes and aromas • CO_2 is an oxidant that can cause a bleaching effect, and oxidation of volatiles and flavour components	Spencer (2013)
Ethanol generators	• Inhibit microbial growth	• High concentration not accepted by consumers	Suppakul *et al.* (2003)
AM packaging	• Inhibit microbial growth • Controlled release mechanism can extend activity • Generally, low concentrations are needed	• Potential issues with the solubility, organoleptic properties and toxicity of AM agents • Physical stability of AM agents is affected by thermal stability and mechanical energy • Physical and mechanical integrity of packaging materials can be altered • The use of some AM agents is regulated	Han (2003)

There are a number of advantages of incorporating AM agents directly into packaging film compared to traditional methods of combining additives directly into food components or by dipping or spraying processes. These advantages include: (i) only a small amount of AM agent is required to have the desired effect; (ii) microbial growth occurs primarily on the surface of the food rather than inside; and (ii) slow release of the additives from the packaging film can protecting the surface of the food packaging components which consequently prolongs the shelf-life (Appendini and Hotchkiss, 2002, Guarda *et al.*, 2011). A disadvantage of the direct incorporation of volatile AM additives to the foods rather than in the film is that such additives might be rapidly lost or diluted by the food components below the active concentration levels. As a consequence, the shelf-life of the packaging can be significantly affected by the migration of the AM additives into the food matrix rather than on the surface where microbial spoilage can occur. In addition, the incorporation of natural AM agents directly into food requires higher concentrations in order to inhibit or delay the growth of microorganisms and such high concentrations can adversely affect the flavour of the packaged food products (Suppakul *et al.*, 2006, Sung *et al.*, 2014, Aloui and Khwaldia, 2016).

Some critical aspects that are considered significant in determining the type of the packaging film that can be used in AM packaging systems include factors such as the initial food composition and quality; the shape and size of the product; distribution methods; the required shelf-life of the product; and the process of disposal of the packaging material (Brown, 1992). For “edible” coatings or packaging, different factors are considered including coating characteristics (composition, chemical structure, viscosity of the coating solutions, coating thickness, degree of crosslinking); coating processing conditions (temperature, pH, type of solvent); and the type and concentration of additives (emulsifiers, plasticizers, or cross-linking agents) (Aloui and Khwaldia, 2016). Barrier properties related to the transport of moisture, oxygen, and carbon dioxide, the chemical composition and structure of the coating materials, as well as the storage conditions, determine the effectiveness of edible coatings in preserving food quality and extending the shelf-life (Aloui and Khwaldia, 2016).

Moisture availability can provide a suitable environment for microorganisms to grow on the surface and affect food quality. Hydrophilic materials are sensitive to moisture and this can subsequently affect the gas and vapour permeability through

packaging films. Therefore, the control of moisture is considered an important aspect in protecting food components, especially in applications such as transpiration of horticultural produce, melting of ice during transportation of chilled foods (i.e. fish), and general fluctuations in temperature during storage (Rooney, 1995). Some manufacturing processes may incorporate anti-fogging additives within the polymers before processing which are activated by contact with the surface of the packaging. Such anti-fogging materials reduce the surface tension between water molecules and plastic films as they accumulate which results in transparent films thereby enabling the consumer to see the contents of the package (Rooney, 1995). However, anti-fog materials do not reduce the moisture content of the environment inside the packaging, and microorganisms can grow on the surface of the food if the conditions are favourable. As a result, anti-fog additives are considered to be a cosmetic form of AP rather than a technique to protect the packaging components themselves.

A wide range of synthetic polymers are widely used in packaging and one such common polymer used primarily in film applications is low-density polyethylene (LDPE). First commercialized in the 1940s (Brown, 1992), toughness, flexibility, melt process ability and good water vapour barrier properties as well as its cost-effectiveness in the form of blown films make LDPE a preferable polymer (Brown, 1992). It also offers an excellent matrix as a carrier for additives such as AM agents (Suppakul *et al.*, 2003, Suppakul *et al.*, 2006, Suppakul *et al.*, 2008, Suppakul *et al.*, 2011a). Another common synthetic polymer used to produce high-barrier packaging films is ethylene-vinyl alcohol which is also an excellent carrier for a range of additives (Muriel-Galet *et al.*, 2012a, Muriel-Galet *et al.*, 2012b).

1.2.1 Antimicrobial Packaging in Meat Industry

The Australian red meat industry is the world's largest exporter of beef and sheep meat with 20% and 36% of total world exports respectively. The industry is the second largest contributor to the Australian farming economy with a value reaching about 13.3 billion Australian dollars in the 2012-2013 financial year (Fang *et al.*, 2017). Therefore, in the meat industry, adequate storage and packaging of products is crucial for the safety and stability of fresh meat from the farm through to the consumer. According to Zhou *et al.* (2010), the definition of fresh meat "*includes meat from recently processed animals as well as vacuum-packed meat or meat packed in controlled-atmospheric gases, which*

has not undergone any treatment other than chilling to ensure preservation". Packaging materials and related AP technologies can contribute to the maintenance of freshness and safety by minimizing lipid oxidation and controlling populations of spoilage microorganisms (Fang *et al.*, 2017).

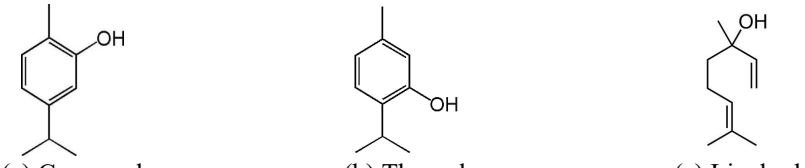
Microbial contamination, lipid and protein oxidation are the major modes of quality deterioration of meat products (Guyon *et al.*, 2016, Fang *et al.*, 2017). In 2009, spoiled meats resulted in approximately 48 million food-borne illnesses and 3000 deaths in the United States, and 5550 illnesses resulting in 48 deaths in the European Union (Guyon *et al.*, 2016). In general, people with compromised immune systems, the elderly and infants are the most susceptible to serious food-borne illnesses which can be caused by microbial contamination, by bacteria, yeast and moulds. Examples of common pathogenic microorganisms which can contaminate raw, undercooked or cooked whole meat and poultry or processed meats include: *Campylobacter jejuni*, *Salmonella spp, Staphylococcus aureus, Listeria monocytogenes, Clostridium perfringens, Clostridium botulinum,* and *Escherichia coli O157:H7* (Guyon *et al.*, 2016, Fang *et al.*, 2017). Since microbial contamination of meat occurs primarily at the surface, the incorporation of AM agents into the surface of the packaging polymer is perhaps the most efficient way to attain slow release without depleting the concentration of the AM agent into the food component (Quintavalla and Vicini, 2002).

Sensory properties including colour and odour are among the most crucial aspects consumers evaluate before making purchases (Guyon *et al.*, 2016). Meat discoloration is generally related to lipid oxidation due to the conversion of oxymyoglobin to metmyoglobin resulting from a decrease in heme redox stability rather than the oxidation of specific amino acid residues (Faustman *et al.*, 2010). The process of lipid oxidation contributes to the development of aromatic compounds that results in odour and off-flavours in meat products. Undesired organoleptic changes as well as microbial contamination on fresh meat products are generally increased due to their high moisture and nutrient content (Andrés *et al.*, 2017). In addition, the presence of high concentrations of oxygen molecules in the headspace of food packaging can lead to oxidation, colour change, and facilitate the deterioration of meat products (Suppakul *et al.*, 2003).

Minced meat usually has a far shorter refrigerated storage life in contrast to whole cuts of meat due to the greater potential for lipid oxidation of the former. This is a result of the mincing process which facilitates the incorporation of oxygen, mixes reactive components, and increases surface area as a result of particle size reduction (Fik and Leszczynska-Fik, 2007, Faustman *et al.*, 2010). Minced meat is highly sensitive to bacterial attack, especially pathogenic strains of *E. coli* (Arin *et al.*, 2017). The different techniques used to extend the shelf-life of meats and meat products are summarized in Table 1.2 (Zhou *et al.*, 2010). Recently, Silva *et al.* (2018b) reviewed the effectiveness of several preservation techniques in the control of poultry microbial contamination and the existing regulations of using these techniques.

1.2.2 Natural AM Agents

Many researchers in recent decades have investigated the effectiveness of incorporating natural sources of AM agents, including essential oils (EOs) derived from plants (Rodríguez *et al.*, 2006, Gutiérrez *et al.*, 2009b, Gutiérrez *et al.*, 2011, Sung *et al.*, 2014, Clemente *et al.*, 2016b), animal-based enzymes (such as lysozyme, lactoferrin), bacteriocins from microbial sources (such as nisin, pediocin), and biopolymers such as chitosan, for their AM potential in packaging films against bacteria, fungi, yeast, and mould. The main driving force associated with the choice of natural AM agents in these studies is that natural AM additives are generally considered to be free of side-effects on human health with many also used as antioxidants (AOs). Examples of natural AM agents that possess possibly the strongest AM activity against spoilage and pathogenic microorganisms include extracts of oregano, thyme, and basil. Carvacrol (5-isopropyl-2-methylphenol), thymol (2-isopropyl-5-methylphenol) and linalool (3, 7-dimethyl-1, 6-octadien-3-ol) are examples of natural AM agents extracted from these plant sources. Carvacrol is the major component (50-86%) of oregano and thyme (Guarda *et al.*, 2011) and thymol is also present in these herbs (Ponce Cevallos *et al.*, 2010), whereas linalool is the principle component of basil (Suppakul *et al.*, 2006). These natural agents are all monoterpenes and thymol and carvacrol are isomers as shown in Figure 1.1.



(a) Carvacrol (b) Thymol (c) Linalool

Figure 1.1: Molecular structures of natural AM agents

Table 1.2: Techniques used for processing meat products

Technique	Advantages	Disadvantages
Chilling	• Reduces carcass surface temperature • Enhances carcass drying • Decreases bacterial growth	• Difficulties in removing heat quickly from the deeper tissue of carcasses
Freezing	• No mechanical refrigeration equipment • Reduces microbial growth and chemical changes	• Can distort the product shape • Expensive
Super-chilling	• Microbial activity inhibited or terminated • Reduce labour, energy costs and weight losses • Protects meat from temperature rises in poor cold chains	• Chemical and physical properties can be changed or accelerated • Increase in product drip loss • Limited information around chilling times, chilling temperatures, air-flow and refrigeration loads
Ionising radiation	• Highly efficient inactivation of bacteria • Non-thermal • Preserves freshness and nutritional quality	• Colour changes
Chemical preservatives	• Inhibits microbial growth	• Consumer desire for natural preservatives • The use of ozone can be dangerous • Accelerates oxidation of fats • Effective against air-borne micro-organisms rather than those on meat
Natural preservatives	• Suitable for marketing 'green label' products • Inhibits the growth of microorganisms	• Can be expensive
High hydrostatic pressure	• Inactivates product-spoiling micro-organisms and enzymes at low temperatures • Non-thermal • Preserves sensory or nutritional characteristics of the product	• Undesirable fresh meat colour even at low temperature
MAP	• Extends the shelf-life of foods	• Better barrier against gas and moisture • CO_2 reacts with meat, changing the properties
AM Packaging	• Slow migration of AM agents from packaging material to the surface of the product, to maintain high concentrations (Quintavalla and Vicini, 2002)	• Concentration of AM agents' sensitive to extrusion and film processing • Interaction between AM agents and film surface are likely limited to enzymes or other proteins of micro-organism cell wall while being bound to the plastic • Fast migration dilutes AM agents, reduces it activity

For many years, these AM agents have been used as flavours in different types of foods and each of these agents possesses "Generally Recognised As Safe" status according to the American Food and Drug Administration (Kuorwel *et al.*, 2011b). Carvacrol and thymol are widely reported to possess AO and AM properties (Mourtzinos *et al.*, 2008). Various studies have reported that linalool works effectively as a fungistatic and antibacterial agent (Kuorwel *et al.*, 2011b). In addition, linalool has been used as a natural AO in meat products which resulted in reduced lipid oxidation relative to the control of packaged fresh beef by inhibiting the formation of metmyoglobin (Fang *et al.*, 2017). The shift in use from synthetic to these and other natural additives has resulted in many researchers investigating the effectiveness of these agents in inhibiting the growth of microorganisms. The efficacy of these agents depends on a number of factors that need to be considered when natural AM systems are to be designed, such as the carrier materials, the type of AM agents, the release mechanism of the AM additives, and factors that can impact on the activity of the AM agents (Cooksey, 2000).

1.2.3 Research Significance, Gaps, and Contribution to Knowledge

Consumer awareness of the possible adverse health effects from the use of synthetic additives in foods has increased developments of natural additives for food preservation. Over recent years, the addition of natural additives to packaging has become a favourable alternative to the direct addition to foods. Essential oils and their extracts as well as other plant-derived compounds are often used for this purpose due to their availability and efficacy as food preservatives (Valgimigli, 2012). The limitations of using natural AM agents include their excessive loss during processing of food packaging polymers, high evaporation rate, possible food tainting effects, low solubility as well as degradation over time (Bhandari *et al.*, 2001, Becerril *et al.*, 2007, Nerín, 2012, Sung *et al.*, 2014, Olmedo *et al.*, 2015, Gherardi *et al.*, 2016, Fang *et al.*, 2017, Soroush and Hossein, 2017). At present, these limitations render using many expensive volatile AM agents for packaging films commercially unviable. Thus, there is a need to focus on the development of AM packaging films with particular regard to optimizing processing conditions during film preparation (Del Nobile *et al*., 2009). Examples of some studies that have evaluated the retention of natural AM agents and/or their sensory evaluation in polymers are listed in Table 1.3. Although many natural AM packaging systems offer protection against many pathogens, the organoleptic effects due to the intense aroma has limited the application of these agents to meat products (Fang *et al.*, 2017).

Table 1.3: Evaluation of polymers containing natural AM agents

AM Agent	Polymer	% Loss	Evaluation	References
Linalool	LDPE	95	-	Suppakul *et al.* (2008)
Linalool, methyl chavicol	LDPE/ EVA	67 66	Taint in flavour not significantly detectable at low concentration by end of storage period 6 weeks	
Thymol, carvacrol, linalool	Starch	52		Kuorwel *et al.* (2011a)
Thymol, carvacrol	PP	56-75	-	Ramos *et al.* (2012)
Cinnamaldehyde	LDPE	15-45	Adversely affected with increase in concentration	Soroush and Hossein (2017)
Lemon	LDPE	-	Biscuits had the taste and aroma of lemon after 10 days of contact	Dias *et al.* (2013)
Allium sativum	LDPE/ethylene-vinyl-acetate	-	Maximum loss not reported, however 2,4, 6% (w/w) concentration showed no inhibition due to AM evaporation. The concentration 8% (w/w) showed inhibition.	Sung *et al.* (2014)

Microencapsulation can provide a means to protect volatile or otherwise unstable AM agents against harsh conditions encountered during thermal processing of the polymeric substrate so that the active AM agent can be successfully and efficiently incorporated in the food packaging material. Therefore, the significance of the current study is its propensity to overcome the abovementioned limitations by successfully incorporating encapsulated natural AM agents into food packaging film to produce film formulations which can be used as active AM packaging materials. The microencapsulation technique can: (i) provide a form of chemical protection to these natural extracts against degradation by decreasing the interaction of the encapsulated material with the external environment (oxidation, light-induced reaction, heat–promoted de-composition, etc.); (ii) control the extent of evaporation of the encapsulated material; (iii) facilitate the handling of the guest materials (e.g. converting liquid guest materials to a solid complex or physically isolating them), and (iv) stabilize the encapsulated agents (Reineccius, 1995, Lam and Gambari, 2014) preventing discolouring, thickening as well as masking unpleasant odours in food applications (Astray *et al.*, 2009).

There are currently few published studies on the use of microencapsulation techniques in the field of food packaging technology in which the active agent is encapsulated within another inert material (i.e. the wall). The wall hosts the molecule in order to provide protection against harsh conditions encountered during thermal processing so that the active AM agent can be successfully and efficiently incorporated in the food packaging material. The use of natural wall materials such as β-cyclodextrin (β-CD) is effective for providing protection against heat and evaporation (Bhandari *et al.*, 2001).

In addition, it is also critical to analyze complex food systems and include as many environmental variables and material characteristics as possible that are otherwise not possible to study *in vitro* (Corrales *et al.*, 2013). For example, Vermeiren *et al.* (2002) incorporated triclosan in LDPE films and showed strong AM activity against *L. monocytogenes in vitro* simulated vacuum-packaged conditions. However, the same films did not show activity *in vivo* on refrigerated vacuum-packaged chicken breasts stored at 7°C, thus confirming the complexity of real food systems.

The current study is also of significance to other scientific areas including those related to natural plant extracts and their constituents which are important raw materials for applications in the food, beverage, pharmaceutical, cosmetics, agriculture, sanitary and perfumery industries (Brown *et al.*, 2011). Many of these industries benefit from encapsulating such agents for various reasons and β-CD is a very common wall material used to achieve this. Given the high price of β-CD, it is therefore important to optimize the microencapsulation process to enhance the encapsulation efficiency, and yield.

1.3 Research Hypotheses and Aims

The hypotheses to be tested in this study are that:

- thymol, carvacrol, linalool can be successfully encapsulated in β-CD and incorporated successfully into LDPE packaging films
- the encapsulation process renders the film active as an AM material
- the encapsulation of the AM agents within β-CD reduces the loss of the active agent during processing

- the release of AM agent from the β-CD prevents the growth of spoilage microorganisms and minimizes lipid oxidation *in vitro* and on a real food product

To evaluate these hypotheses, this research aims to:

- Prepare and optimize the synthesis of AM/β-CD complexes by a co-precipitation method and quantify the amount of the AM agent encapsulated in the complex;
- Incorporate the complex into LDPE film and quantify the retained concentration of the AM agents after exposure to thermal processing required for film preparation;
- Evaluate the release of an AM agent from the β-CD cavity under thermal conditions, into an open atmosphere and into a food simulant;
- Test the activity of an encapsulated AM agent in inhibiting the growth of a microorganism in laboratory scale *in vitro* experiments;
- Investigate the feasibility of using film formulations containing an encapsulated AM agent in preventing oxidation and inhibit the growth of microorganisms in minced meat and chicken.

1.4 Thesis Outline

This thesis is comprised of the following chapters:

- Chapter 1 presents a general introduction with an overview of AM packaging systems focusing on the use of natural AM agents and linked to the current needs of the meat industry in particular. The research significance and aims of this work are also presented.
- Chapter 2 encompasses a literature review of microencapsulation techniques. It also discusses the fundamental properties of encapsulated bioactive compounds in terms of their stability, bioavailability and bioactivity, wall materials, controlled release of microcapsules and factors that affect encapsulation efficiency during preparation and that affect retention during the storage period.
- Chapter 3 investigates the experimental parameters that affect the inclusion efficiencies and the yield of β-CD inclusion complexes with the selected AM agents to optimize the production of the complexes.

- Chapter 4 explores the kinetics and decomposition of the prepared inclusion complexes by thermogravimetric analysis.
- Chapter 5 provides results relating to the stability of the AM complexes incorporated into a polymer film matric and examines the release of the AM agent into a food simulant at different temperatures.
- Chapter 6 investigates the use of glycerol to control the release of the AM agent from its polymer matrix into a food simulant and *in vitro*.
- Chapter 7 explores the use of the optimized AM complex incorporated into polymer films to inhibit microbial growth *in vivo* and to control lipid oxidation.
- Chapter 8 presents the conclusions of the research and possible future work.

Each of the experimental chapters (3-7) are presented with an introduction, materials and method section, results and discussion section, and a brief summary.

Chapter 2 Literature Review

2.1 Overview

This chapter explores the factors that affect the selection of encapsulation materials for a range of active agents with a focus on the factors that influence the capsule stability. The interaction between the wall and the bioactive agents and the conditions that govern the release and activity of the encapsulated agents is also discussed with a focus on AM and AO agents for various practical applications.

2.2 Introduction

A growing number of industries including those related to pharmaceuticals, nutraceuticals, agriculture and food packaging are increasingly interested in the use of a wide range of bioactive agents. These agents include pesticides, insecticides, and fertilizers, as well as anti-inflammatory, AM and AO compounds (Mourtzinos *et al.*, 2008, Lopez *et al.*, 2012, Guimarães *et al.*, 2015, Santos *et al.*, 2015, Avramenko *et al.*, 2016). In this context, a large number of studies have focused on the use of encapsulation techniques to offer protection by: (i) reducing the evaporation rate of encapsulated material to the outside environment (Shaikh *et al.*, 2006, Soliman *et al.*, 2013, Herculano *et al.*, 2015, Santos *et al.*, 2015), (ii) decreasing the interaction of these agents within the external environment such as pH range, relative humidity (RH), water activity (a_w), oxidation, light-induced reaction, and heat-promoted decomposition (Zhong *et al.*, 2009, Marcolino *et al.*, 2011, Paramera *et al.*, 2011, da Rosa *et al.*, 2015, Santos *et al.*, 2015, Wrona *et al.*, 2017); (iii) controlling the release rate to prolong the effect of encapsulated bioactive compounds at specific targeted sites and thereby enhance the biological, AM and AO functions (Keawchaoon and Yoksan, 2011, Paramera *et al.*, 2011, Wang *et al.*, 2011a, Déat-Lainé *et al.*, 2013, Hill *et al.*, 2013, da Rosa *et al.*, 2015, Yegin *et al.*, 2016).

Encapsulation can be defined as a mode in which an active agent or guest material is covered or coated within another material which is termed the shell, wall material, host molecule, carrier or encapsulate (Risch, 1995). The number of materials, methods and procedures available for encapsulation varies widely which are based on the requirements of the application itself including the specific release mechanism required, e.g. release occurs slowly over time or at certain point (Gibbs and Kermasha, 1999, Madene *et al.*,

2006, Fang and Bhandari, 2010, Nesterenko *et al.*, 2013, Xiao *et al.*, 2014b). The selection of a suitable wall material is dependent on the chemical properties of the bioactive agent, desired characteristics of the final product, and the microencapsulation method to be used. The encapsulation process itself is critical in order to improve the encapsulation efficiency and stability of the capsule in producing high-quality encapsulated materials (Liu *et al.*, 2016, Al-Nasiri *et al.*, 2018).

The selection of the wall material is based on the requirements that is has (i) good rheological properties at high concentration so that it is easily worked; (ii) the ability to disperse or emulsify and stabilize the emulsion with little or no chemical reactivity during encapsulation processing; (iii) the ability to provide protection during processing and storage; (iv) the ability to be easily removed from the solvent under drying; and (v) good solubility in the required solvents (Ray *et al.*, 2016). In addition, culturally-related customer concerns (e.g. Kosher/Halal and vegetarian requirements), safety-and health related issues (e.g. allergenicity) and cost constraints are also significant key factors for choosing the most appropriate materials (Xiao *et al.*, 2014b).

Encapsulation of these additives offers a solution to protect them from the harsh conditions of thermal processing and can facilitate the controlled release from the packaging environment. The most common compounds used for encapsulation (i.e. wall materials) include a wide range of carbohydrates and proteins and the selection of the most appropriate material depends on the properties of the active agent and the requirements of the application. This review considers encapsulation techniques and discusses the fundamental properties of encapsulated bioactive compounds in terms of their stability, bioavailability and bioactivity aspects. It also provides a general overview of the different wall materials that are widely used in encapsulation techniques and their behaviour in different systems. Furthermore, the controlled release of microcapsules in different systems has been given considerable attention to accommodate the need to readily identify matrices that provide sufficient protection and, at the same time, control the release of the active agent without suppressing its bioavailability and bioactivity. The factors that affect encapsulation efficiency (EE) during preparation and that affect retention during the storage period are also discussed.

2.3 Wall Materials

2.3.1 Carbohydrates

Carbohydrates are classified based on their molecular weight from small sugar molecules such as mono- and disaccharides e.g. glucose and sucrose; oligosaccharides e.g. oligofructose through to polysaccharides such as maltodextrin (MD), modified starch (MS), glycose syrup, and cyclodextrins. The latter are commonly used as wall materials to encapsulate a variety of active agents and are preferred due to their high amorphous content (Delarue and Giampaoli, 2006).

Starch

Starch is a polysaccharide with a semi-crystalline structure that is comprised of two polymeric forms, linear amylose and branched amylopectin with between 15 and 45% crystallinity (Delarue and Giampaoli, 2006). The starch repeating units are comprised of between 2 and 4 hydroxyl groups which can be substituted or reacted with other functional groups. Octenylsuccinic anhydride (OSA) is modified starch produced by substituting a hydroxyl group with a hydrophobic group to form stable oil-in-water emulsions (Mourtzinos *et al.*, 2008). This results in good microencapsulation capacity, especially when lipid-soluble bioactive compounds are encapsulated (Kenyon, 1995, Zhao and Tang, 2016). Starch can also be fully or partially hydrolysed by chemical or enzyme action which mainly affects the α-amylase structural components. Such reactions can result in a wide range of molecules that are classified based on their dextrose equivalent (DE) including: MD (DE > 20); glycose syrups (100 > DE > 20); and monosaccharides (DE = 100) (Voilley and Etiévant, 2006).

Maltodextrin

Maltodextrins are hydrolysed starch produced at a relatively low cost, and with neutral aroma and taste, low viscosity at high solids concentrations, good protection of flavours against oxidation (Fernandes *et al.*, 2014), and high water solubility (Saikia *et al.*, 2015). However, the low emulsifying capacity and low retention of volatiles (Runge, 2001) limits the ability of MD to be used alone without being incorporating with other wall materials (Fernandes *et al.*, 2014). The incorporation of a hydrophilic matrix can potentially reduce the oxidation of encapsulated materials due to the fact that oxygen is

hydrophobic and the presence of the matrix could decrease oxygen permeability (Bae and Lee, 2008).

Cyclodextrins

Cyclodextrins are widely used as microencapsulation materials to protect flavour and volatile compounds in food systems as they are chemically stable molecules. Alpha, beta, and gamma polysaccharides are three types of cyclodextrins that are comprised of six, seven, and eight glucose units respectively (Hedges *et al.*, 1995) with β-CD the most common wall material for encapsulation. The hydroxyl functional groups of the glucose monomers are located at the outer surface and as such give cyclodextrins their hydrophilic outer surface properties whereas the hydrogen atoms and glyosidic oxygen atoms of the glucose monomers give cyclodextrins their hydrophobic inner surface properties (Hedges *et al.*, 1995). These features play a role in complex formation with other active agents where polar molecules interact with hydrophilic groups to form hydrogen bonds and nonpolar organic molecules interact with hydrophobic groups. The interaction between the β-CD molecule and the active agent is influenced by several forces (Astray *et al.*, 2009) such as van der Waal forces, hydrophobic interactions, and dipole-dipole interactions. These forces are strong enough to form stable inclusion compounds without influencing the release mechanism of the guest material so that the latter becomes available for the desired application. With a shape resembling a hollow truncated cone, the structure of 'empty capsules' of β-CD provides effective protection for a wide range of organic molecules (Mourtzinos *et al.*, 2008, Astray *et al.*, 2009).

Chitosan

Chitosan is the second most abundant polysaccharide in the world (Xiao *et al.*, 2014b) and it is widely used to encapsulate bioactive compounds for controlled release. It is a polyelectrolyte and pH-sensitive polymer (Lacerda *et al.*, 2014) due to the presence of free amino groups that produce positive charges at pH values below 6.5 (Ocak, 2012). Chitosan is insoluble under neutral or basic conditions and undergoes complete solubilization in acidic media due to the protonation of its amino groups and swelling (Agnihotri and Aminabhavi, 2004).

Alginate

Alginate is another matrix that is widely used for the encapsulation of bioactive compounds. It is an anionic and hydrophilic polysaccharide and is one of the most abundant biosynthesized natural materials (Zia *et al.*, 2015). It is of interest as a wall material due to its unique colloidal properties, which include thickening, stabilizing, suspending, film-forming, gel-producing, and emulsion-stabilizing (Hambleton *et al.*, 2009). Alginate is also a polyelectrolyte and pH-sensitive polymer due to the presence of carboxylic groups within its structure. The general release when using an alginate matrix is low under acidic pH conditions and high under basic pH conditions (Chiu *et al.*, 2007).

2.3.2 Proteins and Gums

Protein materials are derived from a range of sources including gelatine, casein, and whey protein from animal products, with soy protein and zein obtained from vegetable sources. Nesterenko *et al.* (2013) have reviewed the effectiveness of vegetable proteins, industrial uses and future prospects in microencapsulation when used as wall materials for different applications. Protein wall materials have been shown to enhance the stability of bioactive agents (Rascón *et al.*, 2011, Gallardo *et al.*, 2013). Other advantages of using proteins as wall materials include biocompatibility, biodegradability, good amphiphilicity, good functional properties such as water solubility, and good emulsifying and foaming capacity (Nesterenko *et al.*, 2013). Corn zein is one of the more abundant plant proteins which is comprised of more than 50% hydrophobic amino acid residual surface groups and it is essentially insoluble in water at pH 2-8. Zein is positively charged at pH 2, slightly positive at pH 6, negative at pH 8 (Xiao *et al.*, 2011), and is only soluble in 55–90% aqueous alcohol (Chen *et al.*, 2015b). These properties can be utilised to precipitate micro-and nano-particles and encapsulate suitable guest materials.

Gums are used as thickeners in beverages and are widely used for the encapsulation of food products due to their good emulsification properties compared to unmodified carbohydrates (Runge, 2001). Typically gums are complex mixtures of glycoproteins and polysaccharides with gum Arabic one of the more common gum-based wall materials used in microencapsulation, particularly *via* spray drying (Fernandes *et al.*, 2014). It has been recognised as an effective encapsulating agent due to its ability to facilitate good volatile retention and stable emulsions over a wide pH range (Maisuthisakul and Gordon, 2012), as well as being compatible with most gums, starches, carbohydrates and proteins (Krishnan *et al.*, 2005). Other gums include xanthan gum

which is well known for its ability to increase the viscosity and subsequently increase the stability of emulsions (Khamis Ali *et al.*, 2009). Guar galactomannan is a water-soluble, non-ionic gum with excellent thickening properties and low production cost, however, it lacks emulsifying activity that can be crucial in microencapsulation of some constituents (Sarkar *et al.*, 2013).

2.3.3 Stability of Encapsulated Bioactive Compounds

One of the key functions of carbohydrate and protein wall materials is to provide stability to bioactive compounds against degradation reactions when the latter are exposed to one or more detrimental environmental factors such as light, temperature, moisture, high or low pH and/or oxygen. The efficiency of the encapsulation to provide this stability can be variable and depends on factors including: the wall material properties (physical and chemical structure, affinity of the bioactive agents to the wall); composition of the wall (particularly when two or more walls are blended); storage conditions including a_w, RH, and temperature etc.; and the addition of stabilising agents.

The oxidative stability of bioactive agents can be monitored using different techniques such as the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (Simon-Brown *et al.*, 2016), peroxide values (Bae and Lee, 2008, Wang *et al.*, 2011b, Wang *et al.*, 2016) p-anisidine values (Khamis Ali *et al.*, 2009), accelerated Rancimat test (Carneiro *et al.*, 2013, Gallardo *et al.*, 2013), thiobarbituric acid reactive substances (TBARS) (Avramenko *et al.*, 2016), and differential scanning calorimetry (DSC) (Karathanos *et al.*, 2007). It has been suggested that the oxidation process with respect to the peroxide value level in an edible food product should not be higher than 30 meq peroxide kg^{-1} oil (Gotoh *et al.*, 2007) and therefore only those studies that have reported a level below this threshold, and thus possess oxidative stability, have been considered in this review.

2.3.4 Wall Material Physical Properties

The physiochemical structure of the wall can strongly affect the barrier properties of the capsule and subsequently interfere with its quality and affect the degree of the wall matrix porosity. Consequently, the permeability to oxygen can result in increasing the oxidation process that affects the shelf-life of the final capsule (Bertolini *et al.*, 2001). Therefore, it is important to understand factors that can reduce surface oil content, and improve the retention of bioactive agents during long-term storage. Some studies have

revealed that the physical structure of the final encapsulated product can be related, as well as influenced, by one or more parameters such as emulsion stability, the type and the concentration of the wall, the ratio of the wall to the guest, the ratio of components in combined matrix wall systems, the concentration of the guest, the drying method, and the drying temperature (Bae and Lee, 2008, Najaf Najafi *et al.*, 2011, Silva *et al.*, 2014b).

In one study by (Najaf Najafi *et al.*, 2011), the authors showed that the type of wall had an impact on the physical structure of the product. It was found that Hi-Cap 100, a modified food starch, exhibited superior encapsulating properties and imparted a significant barrier effect against diffusion and loss of guest material in contrast with skim milk powder (Najaf Najafi *et al.*, 2011). However, the authors also found the drying method has greater impact on the physical structure than the wall type whereby encapsulated powder prepared by spray drying showed a higher stability of encapsulated cardamom oil during storage in contrast with freeze drying. Conversely, increasing the drying temperature during spray drying can initiate oxidation resulting in increased lipid oxidation during storage (Khamis Ali *et al.*, 2009, Wang *et al.*, 2011b, Wang *et al.*, 2016).

Moreover, the ratio of the wall to the guest also plays a significant role in the stability of the produced capsule. For example, high solids content (wall) and low oil concentration (guest) can lead to low lipid oxidation stability during processing and long-term storage due to the low level of surface oil on the surface particle (Tonon *et al.*, 2011, Herculano *et al.*, 2015). Increasing the solids content to an optimum ratio between the wall and the guest results in a high emulsion viscosity and lower droplet size. It also reduces the time that is required for the sample to be atomized during the drying process and, as a consequence, prevents lipid oxidation (Tonon *et al.*, 2011). Furthermore, the ratio of wall to the guest can contribute to improving the EE by reducing surface oil where the un-encapsulated oil is much more susceptible to oxygen that can initiate lipid oxidation during storage (Tonon *et al.*, 2011). Recently, Zhao and Tang (2016) found the UV- and heat-stability as well as prolonged storage stability of the enzyme CoQ10 when using different wall materials was largely related to the interfacial emulsifier concentration of their original emulsions.

The ratio of the components comprising blended walls is also crucial in determining the physical structure of the final product that can influence the capsule stability (Bae and Lee, 2008). The significance of the wall ratio may be due to the potential formation of intermolecular bonds which can create complexes between the

combined polymers (Calderón-Oliver *et al.*, 2017). For example de Oliveira *et al.* (2014) reported that thermal nanoparticle stability was improved when the alginate content was greater than that of cashew gum in a blend of the two components. The addition of a high amount of gum had resulted in destabilizing the alginate "egg-box" structure leading to a less stable new molecular arrangement.

2.3.5 Bioactive Agent Properties

The chemical properties of the guest material can further influence the molecular interactions within the wall (Chen *et al.*, 2015a). For example, wall materials that have naturally hydrophilic properties have little affinity for hydrophobic agents such as flavour oils (Shaikh *et al.*, 2006). Bule *et al.* (2010) found that GA as a sole wall material gave maximum CoQ10 retention after 6 weeks at 30 ± 2°C when compared to microcapsules prepared using a blend of GA-MD-n-OSA. Krishnan *et al.* (2005) reported the use of a combination of wall materials (GA, MD and MS) to encapsulate cardamom oleoresin and reported the mixture provided maximum protection at 25°C for 6 weeks which was even better than using GA alone. In another study, Shaikh *et al.* (2006) evaluated the GA and MS microcapsule stability of black pepper oleoresin extracts including piperine, the total volatiles and non-volatiles over a period of six weeks. The authors concluded that there was no major advantage of microencapsulation with respect to total volatiles over the test period in both matrices. However, piperine and non-volatiles were better protected by GA (Shaikh *et al.*, 2006). In addition, the authors reported GA provided better stability during storage than MS wall material.

The capacity of polysaccharides to provide protection may depend on the volatility, molecular size, chemical function, polarity and the chain length of the guest (Delarue and Giampaoli, 2006). The structure of GA is a highly branched heteropolymer of sugars, glucuronic acid and a small amount of protein covalently linked to the carbohydrate chain that offers good film-forming ability, is an excellent emulsifier and provides better retention and stability for non-polar substances (Barbosa *et al.*, 2005). The high molecular weight of CoQ10 and insolubility in aqueous solution may result in better protection in a GA matrix in contrast with the volatile contents in the latter two studies of Krishnan *et al.* (2005) and Shaikh *et al.* (2006).

The effects of chemical structure and functional groups have been demonstrated by the results of Bertolini *et al.* (2001) who investigated the retention of different monoterpenes citral, linalool, *β*-myrcene, limonene, and *β*-pinene using GA as a wall.

Alcohols are considered to be the best guest compounds that interact with polysaccharides followed by aldehydes and ketones (Delarue and Giampaoli, 2006). However, different observations were made by (Bertolini *et al.*, 2001) who found that monoterpenes of similar molecular weight encapsulated in GA were retained in the order of hydrocarbon > aldehyde > alcohol. The authors suggested the interaction with GA was governed by the polarity and the solubility of the encapsulated compounds in an aqueous medium. These two factors resulted in increasing the diffusion and higher losses during the formation of the capsules due to the polarity and the solubility. Therefore, aldehydes and alcohols undergo higher losses compared to hydrocarbons during the encapsulation process. The storage stability and shelf-life of these monoterpenes showed a reduction at a storage temperature of 50°C for 33 days, with high losses in the order of linalool > *β*-Myrcene > limonene > citral > *β*–pinene. Bertolini *et al.* (2001) concluded that GA was not a good matrix due to its chemical structure that is rich in hydroxyl groups. This resulted in the oxidation of different guests with different intensities due to the oxidative degradation of the guest and the resulting formation of rearranged products. Water-soluble flavours tend to diffuse faster through the wall matrix in contrast with insoluble flavours (Soottitantawat *et al.*, 2005).This is in agreement of the results of Simon-Brown *et al.* (2016) who found a greater than 50% loss for the ginger extract 6-gingerol encapsulated in MD, GA or their blends during spray drying.

The enhanced protection of bioactive compounds in a cyclodextrin matrix may depend on the guest, storage conditions, and the type of cyclodextrin (Santos *et al.*, 2015). Choi *et al.* (2009) reported eugenol encapsulated in 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CD) was significantly unstable during storage at 25°C under exposure to light irradiation in contrast to eugenol encapsulated in β-CD. The authors suggested that the hydroxypropyl side-chain of 2-HP-β-CD might interrupt eugenol inclusion within the cavity of the 2-HP-β-CD molecule. In another study, Marcolino *et al.* (2011) found that complexation of curcumin with β-CD did not improve its stability when stored exposed to light. However, this is contrary to another study by Mangolim *et al.* (2014) where the authors reported the photo-stability of curcumin in a β-CD matrix after complexation was enhanced against sunlight exposure when compared to free curcumin. The difference between the results of these two studies may be due to differences in the experimental conditions that were used. Marcolino *et al.* (2011) used an acetone/hexane mixture as an extraction solvent to extract and quantify curcumin in the complex whereas Mangolim *et al.* (2014) used ethanol with the latter a potentially more effective solvent in the extraction

process than acetone/hexane (Ferreira *et al.*, 2013). The above examples highlight the need for further optimization of the complexation process using CD wall materials and a need to understand the experimental parameters that may have led to the contrasting results in these similar studies. This is essential to generate the most suitable methods to produce microcapsules that possess high-quality characteristics in term of efficiency, stability, activity, and bioavailability.

2.3.6 Wall Compositions

In recent years there has been a progressive increase in the number of publications related to the combination of wall materials to overcome the disadvantages of some materials as summarised in Table 2.1. Several studies have investigated the combination of different classes of materials such as polysaccharides and proteins in different combinations in order to enhance the EE, capsule quality, structure, and stability of encapsulated bioactive compounds. Protein wall materials have much higher oxygen permeability than the wall matrix consisting of a mixture of protein and small molecular weight carbohydrates (Bae and Lee, 2008). In addition, the incorporation of sugars and starches with proteins is critical to produce a stable capsule that can withstand high temperatures and high pressure/shear conditions (Runge, 2001). Other studies have suggested the combination of polysaccharides with proteins can result in good oxidative stability during processing and storage (Bae and Lee, 2008).

Carneiro *et al.* (2013) and Zhao and Tang (2016) found the EE was highest for the wall combination of two polysaccharides compared with polysaccharide/protein combinations which showed the lowest. Conversely, capsules with the polysaccharide/protein combination of MD-whey protein concentrate (WPC) showed the best flaxseed oil lipid stability against oxidation at 40°C (Carneiro *et al.*, 2013). Zhao and Tang (2016) reported that a polysaccharide/protein combination showed slightly better heat stability than a combination of two polysaccharides whereas the UV stability was slightly higher for the latter. An exception to this finding was for the capsule combination of polysaccharide-protein (MD-defatted milk powder) that showed lower EE, UV and heat stability in contrast with all other capsules, although this capsule provided better stability and protection to CoQ10 than the free form.

Table 2.1: Summary of encapsulation of compounds in different wall materials

Wall	Guest	Application	Method	Comments	References
Alginate	Carvacrol	AM activity	Emulsion–extrusion	Stable for 5 h at 37°C, 30 d at 22°C in air-tight container, retained AM activity, rapid release at pH 7.4, slow at pH 1.5	Wang *et al.* (2009)
Alginate	Cinnamon oil	Antifungal, agriculture	Emulsion-extrusion	Sustained release, maintained activity at 28°C after 8 days with 30% fungal inhibition	Soliman *et al.* (2013)
Alginate	Clove oil	Antifungal, agriculture	Emulsion-extrusion	Sustained release, maintained activity at 28°C after 8 days with 50% fungal inhibition	Soliman *et al.* (2013)
Alginate	Nisin	AM activity	Vibrating technology	Best AM activity at pH 6, 4°C with stirring	Maresca *et al.* (2016)
Alginate	Thyme oil	AM activity	Emulsion, ionic gelation	Best encapsulation with 2% v/v oil, good AM activity	Benavides *et al.* (2016)
Alginate	Thyme oil	Antifungal, agriculture	Emulsion-extrusion	Sustained release, maintained activity at 28°C after 8 days with 50% fungal inhibition	Soliman *et al.* (2013)
Alginate, cashew gum	Pepper-rosmarin oil (*Lippia sidoides*)	Larvicide	Spray-drying	Addition of cashew gum improves hydrophilicity and release	de Oliveira *et al.* (2014)
Alginate, chitosan	Ground ivy, hawthorn, nettle, raspberry leaf, yarrow	AO activity	Electrostatic extrusion	Ascorbic acid degradation decreased AO over 4 weeks activity decreased from 32 to 85%	Belščak-Cvitanović *et al.* (2011)
Alginate, chitosan	Rifampicin	Drug delivery	Coacervation and impregnation	20% release at acidic pH in 2 h, 100% rapid release at pH 6.8	Lacerda *et al.* (2014)

Wall	Guest	Application	Method	Comments	References
Alginate, whey protein isolate	Carvacrol	Poultry antibiotic	Emulsion extrusion	Improved mechanical strength of microparticles, *in vivo* release faster than *in vitro,* fast release at high pH, slow at low pH	Zhang *et al.* (2014a)
Alginate, whey protein isolate	Insulin	Drug delivery	Cold gelation, adsorption	Good stability at pH 7.4 and 37 ℃ for 30-60 min, release limited at 30 min, then completed at 2 h at pH 4.5, depending on the whey protein isolate ratio	Déat-Lainé *et al.* (2013)
Amphiphilic triblock copolymer	Geraniol	Post-harvest decontamination of spinach	Flash nanoprecipitation	Sustained release at 25℃ in water, AM activity against *E. coli* and *S. enterica*	Yegin *et al.* (2016)
Baker's yeast, β-CD, modified starch	Curcumin	Protection against heat and light, improve solubility	Various	Improved storage stability in sunlight for 30 days, yeast cells offered best overall protection	Paramera *et al.* (2011)
Barley protein	Fish oil	Protect fish oil against oxidation, use in dairy	Micro-fluidization and spray drying	Good oxidative stability at pH 2 and 7 at 40℃ for 8 weeks	Wang *et al.* (2011b)
Cashew gum	Eucalyptus EO	Food preservation	Nanoencapsulation by emulsification and spray drying	Maintained stability for 365 days without light exposure at 25℃, good AM activity on Gram-positive bacteria	Herculano *et al.* (2015)
Chitosan	*Carum copticum* EO	Protection of oil	Emulsion-ionic gelation	Initial burst release at pH 3.5, slower at pH 7.4-10	Esmaeili and Asgari (2015)
Chitosan	Carvacrol	Controlled release of oil, AM activity	Emulsion-ionic gelation	Initial burst release, sustainable at pH 3, lower at higher pH 7-11, good AM activity	Keawchaoon and Yoksan (2011)
Chitosan	*Cinnamomum zeylanicum* EO	Cucumber cold storage	Emulsion-ionic gelation	Controlled sustained release for 40 days, extended shelf-life at 4, 10 and 21 degrees	Mohammadi *et al.* (2015a)

Wall	Guest	Application	Method	Comments	References
Chitosan	Clozapine	Drug delivery	Gel cross-linking, sieving	Initial burst release in acid, then slow sustainable pH 7.4 over 12 h	Agnihotri and Aminabhavi (2004)
Chitosan	Felodipine	Drug delivery	Emulsion-ionic gelation	Fast release at pH 7.4, sustained release with increased cross-linking	Ko *et al.* (2002)
Chitosan	Oregano EO	Controlled release of oil	Emulsion-ionic gelation	Initial burst release then slow at pH 7.4	Hosseini *et al.* (2013)
Chitosan	Strawberry polyphenols	Improve bioavailability and release	Ionic gelation	Initial burst release then sustained at pH 7.4	Pulicharla *et al.* (2016)
Chitosan	Summer savory oil	AM and AO food processing and packaging	Emulsion-ionic gelation	Strong AM and AO activity	Feyzioglu and Tornuk (2016)
Chitosan	*Zataria multiflora*	Control grey mold on strawberries	Emulsion-ionic gelation	Initial burst release, fast at pH 3 and 5, slower at pH 7	Mohammadi *et al.* (2015b)
Chitosan	α-lipoic acid	AO activity	Spray drying	Good retention of α-lipoic acid AO activity	Weerakody *et al.* (2008)
Chitosan, cashew gum	*Lippia sidoides* oil	Larvicide	Spray drying	Slow, sustained released with low gum content	Abreu *et al.* (2012)
Chitosan, collagen hydrolysate	Lavender oil	EO release, waste recovery	Coacervation	Initial burst release then sustainable release, recovery of tannery wastes feasible	Ocak (2012)
Ethylcellulose, methylcellulose	Thymol	Antibacterial cosmetic lotion	Solvent displacement	Good sustained AM activity compared to free thymol	Wattanasatcha *et al.* (2012)

Wall	Guest	Application	Method	Comments	References
Gelatin	Holy basil EO	Retention of volatile oil, AO activity	Coacervation	Maintained stability for 49 days at 60°C, equivalent to 18 months at 25°C	Sutaphanit and Chitprasert (2014)
Gelatin	Propolis extracts	Drug delivery, AM	Spray drying	Good AM activity, changed unpleasant flavour profile of extracts	Bruschi *et al.* (2003)
Gelatin, poly(γ-glutamic acid)	Lycopene extract	Protection of food ingredient	Emulsion freeze drying	Lycopene degraded with increasing temperature, fast release at pH 5.5-7, no release at pH 2-3.5	Chiu *et al.* (2007)
Guar gum, gum Arabic	Mint oil	Retention of oil	Spray drying	Good retention during storage at 27°C for 8 weeks, gum Arabic more effective	Sarkar *et al.* (2013)
Gum Arabic	Carvacrol, thymol	Packaging film AM coating	Oil/water emulsion	Good AM activity at very high concentrations, synergism when combined	Guarda *et al.* (2011)
Gum Arabic	Flaxseed oil	Food ingredient	Spray drying	Prevented oxidation, conditions optimized	Tonon *et al.* (2011)
Gum Arabic	Various esters	Retention of volatile aromas	Spray drying	Up to 90% retention, good storage stability at RH below 64%	Rosenberg *et al.* (1990)
Gum Arabic	Various monoterpenes	Protection from oxidation	Spray drying	Better stability for encapsulated β-pinene and citral than linalool and β-myrcene over 33 days	Bertolini *et al.* (2001)
Gum Arabic, carboxymethyl cellulose	Paprika oleoresin, various esters	Retention of volatile aromas	Solvent evaporation	Good retention at $a_w < 0.75$	Zilberboim *et al.* (1986)
Gum Arabic, maltodextrin	*Lippia sidoides* extracts	Antifungal agent for food, cosmetics	Spray drying	Good thymol retention with varying wall composition, good antifungal activity	Fernandes *et al.* (2012)
Gum Arabic, maltodextrin, methyl	Linseed oil	Functional foods, oxidation resistance	Spray drying	Good AO activity *in vitro*, reduced in baked bread	Gallardo *et al.* (2013)

Wall	Guest	Application	Method	Comments	References
cellulose, whey protein isolate					
Gum Arabic, maltodextrin, modified starch	Cardamom oleoresin	Protection against light, heat, oxygen	Spray drying	Combinations of wall components increased stability for 6 weeks storage	Krishnan *et al.* (2005)
Gum Arabic, maltodextrin, modified starch	Cumin oleoresin	Protection against light, heat, oxygen	Spray drying	Combinations of wall components increased stability for 6 weeks storage but not for all volatiles	Kanakdande *et al.* (2007)
Gum Arabic, maltodextrin, modified starch	Ubiquinone-10 (CoQ_{10})	Protection of agent	Spray drying	Enhanced storage and UV stability for at 30 degrees for six weeks, good UV light stability	Bule *et al.* (2010)
Gum Arabic, maltodextrin, modified starches	Green coffee oil	Retention of oxidative stability	Spray drying	Good oxidative stability maintained at a_w 0.70, RH <57.6% at 25 ℃	Silva *et al.* (2014b)
Gum Arabic, maltodextrin, soy lecithin, xanthan gum	Flaxseed oil	Retention of oxidative stability	Spray drying	Enhanced oxidative stability under storage at 28±2°C at 44 and 54% RH up to 49 days, physical structure maintained after 10 weeks storage	Khamis Ali *et al.* (2009)
Gum Arabic, soy protein isolate	Paprika oleoresin	Retention of carotenoids	Spray drying	Good retention at a_w below 0.274, 35°C, soy protein isolate more effective	Rascón *et al.* (2011)
Hydroxypropyl-β-CD	Carvacrol	AM food ingredient	Kneading, freeze drying	Good stability under storage, good AM activity but reduced AO activity	Kamimura *et al.* (2014)
Hydroxypropyl-β-CD	Chlorzoxazone	Drug delivery	Freeze drying	Improved bioavailability, reduce toxicity	Tang *et al.* (2015)

Wall	Guest	Application	Method	Comments	References
Hydroxypropyl-β-CD	Losartan potassium	Drug delivery	Freeze drying	Sustainable release at pH 7.4, improved bioavailability	de Paula *et al.* (2011)
Liposomes	Carvacrol, thymol	Preservation for food, cosmetics, medicine	Mechanical shaking	Enhanced AM activity of single agents and mixtures after encapsulation, improved AO stability	Liolios *et al.* (2009)
Lysozyme, carboxymethyl cellulose	Curcumin	Protection of AO activity	Coacervation	AO activity retained at pH 6 and 7 after heating	Li *et al.* (2015b)
Maltodextrin	Pomegranate peel phenolics	AO food additive, ice cream	Spray drying	Maintained phenolics under storage at 4°C for 90 days	Çam *et al.* (2014)
Maltodextrin	Star fruit polyphenols	AO food additive	Spray, freeze drying	Fast release at pH 1.2	Saikia *et al.* (2015)
Maltodextrin, Gum Arabic	Ginger extract	Food ingredient	Spray drying	Slight decline in AO activity and extract quality	Simon-Brown *et al.* (2016)
Maltodextrin, lentil protein	Flaxseed oil	Retention of oxidative stability	Emulsion, freeze drying	Oxidative stability maintained at room temperature for 30 days	Avramenko *et al.* (2016)
Maltodextrin, soy protein isolate, pea protein isolate, defatted milk powder, modified starch	Coenzyme Q_{10}	Functional foods	Spray drying	Good stability under UV light for 180 mins, heat at 80°C 3 h, improved bioavailability and release	Zhao and Tang (2016)
Maltodextrin, whey protein isolate	Avocado oil	Retention of oxidative stability	Spray drying	Maintained oxidative stability at ambient temperatures and below, decreased stability at 60°C but slightly improved with encapsulation	Bae and Lee (2008)

Wall	Guest	Application	Method	Comments	References
Maltodextrin, whey protein isolate	Thymol	Food additive	Emulsion evaporation	Good stability of hydrated capsules heated at 80°C for 15 mins	Shah *et al.* (2012)
Methylcellulose, hydroxypropyl methylcellulose phthalate	Thymol	Drug delivery	Spray drying	Good storage stability for 1 year, slow sustainable release at pH 1.2	Rassu *et al.* (2014)
Modified starch, skim milk	Cardamom oil	Retention of oxidative stability	Spray, freeze drying	Good storage stability after 6 weeks at 25 °C	Najaf Najafi *et al.* (2011)
Polycaprolactone, β-CD, 2-hydroxypropyl-β-CD	Eugenol	Flavour protection, AO activity	Emulsion-diffusion and molecular inclusion	Stable under light and dark at 25°C for 60 days, β-CD more effective for encapsulation	Choi *et al.* (2009)
Sodium caseinate, whey protein isolate, carboxymethyl cellulose	β-pinene	Protection of volatile agent	Coacervation	Addition of glycerol resulted in desorption of β-pinene at a lower temperature	Koupantsis *et al.* (2016)
Soy protein isolate, pectin	Propolis extracts	AM, AO food additive	Coacervation	Good stability, AO and AM activity, controlled release	Nori *et al.* (2011)
Whey protein concentrate, resistant starch	Folic acid	Nutraceuticals	Nanospray drying, electrospraying	Improved folic acid stability under dry conditions for both wall materials	Pérez-Masiá *et al.* (2015)
Whey protein isolate	Curcumin	Functional foods	Spray drying	Improved AO activity, increased solubility and bioavailability	Liu *et al.* (2016)

Wall	Guest	Application	Method	Comments	References
Whey protein isolate	Fish oil	Foods and pharmaceuticals	Spray drying	Good stability after 20-30 days at 40°C depend on guest to wall ratio, release also dependent on ratio	Wang *et al.* (2016)
Yeast, hydroxypropyl methyl cellulose	Fish oil	Retention of oxidative stability, odour control	Emulsion evaporation	Good storage stability at RH <70% for 30 days, better with hydroxypropyl methyl cellulose coating	Czerniak *et al.* (2015)
Zein	Carvacrol, thymol	Protection of volatile agents	Nanoprecipitation	Good storage and control of AM activity at 6 and 20°C for 90 days, sustained release at pH 7.4	da Rosa *et al.* (2015)
Zein	Carvacrol, thymol	AM, AO food additive	Liquid-liquid dispersion	Good AM and AO activity, improved solubility	Wu *et al.* (2012)
Zein	Fish oil	Retention of oxidative stability	Liquid-liquid dispersion	Storage stability improved during storage	Zhong *et al.* (2009)
Zein	Nisin, thymol	AM food additive	Spray drying	Good AM activity in 2% reduced fat milk at 25°C, burst then fast release at pH 2, lower at pH 6+, dependent on surfactant	Xiao *et al.* (2011)
Zein	Thymol, lysozyme	AM food additive	Spray drying	Sustained release of lysozyme at pH 6 over 49 days	Zhong and Jin (2009)
Zein, casein	Eugenol, thymol	AM food additive	Spray drying	Good AM activity in reduced fat milk, sustained release at pH 6-7	Chen *et al.* (2015b)
Zein, casein	Thymol	AM food additive	Antisolvent	Good stability after 8 months, good AM and AO activity	Li *et al.* (2013)
Zein, caseinate, chitosan	Thymol	AM additive for food, pharmaceutics, agriculture	Liquid-liquid dispersion	Enhanced AM activity of coated particles	Zhang *et al.* (2014b)

Wall	Guest	Application	Method	Comments	References
β-CD	2-nonanone	Protection of volatile agent	Co-precipitation	Good AM activity	Abarca *et al.* (2016)
β-CD	Carvacrol	AM, AO activity	Kneading, freeze drying	Improved stored stability and AM activity with encapsulation	Santos *et al.* (2015)
β-CD	Chlorzoxazone	Drug delivery	Freeze drying	Improved water solubility and bioavailability, release low at pH 1.2, high at pH 6.8	Tang *et al.* (2015)
β-CD	Curcumin	Food additive	Freeze drying, solvent evaporation	Improved stability in sunlight, pH variations and different temperatures, enhanced ice cream colour	Mangolim *et al.* (2014)
β-CD	Curcumin, bixin	Food additive, colourant	Kneading, co-precipitation, simple mixing	Co-precipitation most successful, colour intensification for both but better light stability with bixin	Marcolino *et al.* (2011)
β-CD	Eugenol	Protection of volatile agent	Molecular inclusion	Good stability at 20°C and 56% RH for 2 days, fast release at high temperature and RH	Eun-Ju *et al.* (2010)
β-CD	Garlic oil	Food additive	Co-precipitation	Good oxidative stability, release fast then sustainable at pH 1.5 for up to 12 h at 37°C	Wang *et al.* (2011a)
β-CD	*Lippia sidoides* oil	Protection of oil	Spray drying	Good stability at 40°C, 66% RH for 6 weeks	Fernandes *et al.* (2009)
β-CD	Oregano EO	Protection of volatile agent	Spray drying	Improved AM and AO activity, protection of main compounds	Arana-Sánchez *et al.* (2010)
β-CD	Propolis extracts	Drug delivery	Freeze drying	Release fast at pH 2, better with smaller particles	Kalogeropoulos *et al.* (2009)

Wall	Guest	Application	Method	Comments	References
β-CD	Red bell pepper pigment	Protection of colour for foods	Magnetic stirring, homogenization	Homogenization method offered better colour protection	Gomes *et al.* (2014)
β-CD	*Trans*-anethole	AM food additive	Co-precipitation	Good thermal stability, release slow at 40°C, fast at 60°C	Zhang *et al.* (2015)
β-CD	Vanillin	Protection against oxidation	Freeze drying	Improved oxidative stability and solubility of agent	Karathanos *et al.* (2007)
β-CD	Various EOEs	AM food additives	Freeze drying	Cinnamon bark, clove more effective AM agents than eugenol, *t*-cinnamaldehyde but all showed AM activity	Hill *et al.* (2013)
β-CD, alginate	Linalool	Controlled-release insecticide	Inverse gelation, oil-emulsion entrapment, interfacial coacervation, chemical precipitation	Highest yields for inverse gelation and oil-emulsion entrapment, controlled release best for oil-emulsion entrapment with use of glycerol/chitosan	Lopez *et al.* (2012)
β-CD, dimethyl aminoethyl methacrylate	Thymol	Drug delivery	Sealed-heating, co-precipitation	Good bioavailability *in vivo,* thymol rapidly absorbed and slowly eliminated, fewer higher doses required	Nieddu *et al.* (2014)
γ-CD, microcrystalline cellulose	Coenzyme Q_{10}	Drug delivery	Spray drying	Bioavailability enhanced by oral absorption,	Terao *et al.* (2006)

Gallardo *et al.* (2013) reported the oxidative stability of microencapsulated linseed oil in different wall compositions of GA alone, and its blends with MD and whey protein isolate (WPI) that were measured using the accelerated Rancimat test. The authors reported that the addition of MD to GA reduced the stability to 3.8 h compared to 8 h using GA alone. However, the incorporation of WPI in the composition increased the stability to 9 h. The low stability of the capsule containing MD and GA but without the protein material in these studies may have resulted from the use of MD with low DE (Carneiro *et al.*, 2013). Formulations with high DE are more stable (Runge, 2001) with capsules containing hydrolysed starch (i.e. MD with high DE), for example, providing better oxidative stability than MD with low DE in terms of oxygen permeation (Wagner and Warthesen, 1995). Hydrolysed starch of low DE provides lower stability because it contains a large proportion of long-chained saccharides that cause the wall to be vulnerable and more permeable to oxygen (Wagner and Warthesen, 1995).

The incorporation of amphiphilic biopolymers and polysaccharides within protein materials can also overcome the limitations of protein aggregation and poor physical stability. This is particularly important at the isoelectric point (IEP) of the protein during the capsule preparation or in food and pharmaceutical applications (Weinbreck *et al.*, 2004, Patel *et al.*, 2010, Zhang *et al.*, 2014b, Calderón-Oliver *et al.*, 2017). The effect of pH on protein materials varies widely and is dependent on the chemical structure of the protein and the pH range (Voilley and Etiévant, 2006). Shah *et al.* (2012) studied the effect of MD and its DE on the heat stability of WPI at pH 5 where the electrostatic interaction between WPI molecules is remarkably high. The authors reported the electrostatic interaction between the protein molecules decreased with increasing DE of MD resulting in transparent and heat-stable nano-dispersions containing thymol even at pH around the IEP of the WPI. Li *et al.* (2015b) suggested strong electrostatic interactions between lysozyme and carboxymethyl cellulose where higher degrees of substitution provided higher attraction and protection to curcumin which preserved its bioactivity at pH 6-7 before and after pasteurization at 60°C for 30 min. In another example, sodium caseinate was used to stabilize thymol-loaded zein nanoparticles where the nanoparticles were coated with chitosan. This resulted in shifting the IEP of zein from 6.18 to 5.05 thus improving its physical stability and desirable dispersibility in water at neutral pH (Zhang *et al.*, 2014b).

Modification of the wall chemical structure using enzymatic and/or heating-induced degradation can alter the guest molecular interactions within the wall. For example, heating proteins at a specific temperature is well known to result in the thermal denaturing of the protein. This can improve the interaction between the wall and the guest due to the alliteration of the protein conformation and subsequent structural changes thereby improving the binding sites and resulting in better efficiency and stability (Voilley and Etiévant, 2006). Some researchers have investigated the potential of protein thermal treatment in this context with the aim to improve the interaction of the guest and protein wall (Avramenko *et al.*, 2016, Wang *et al.*, 2016). Wang *et al.* (2016) evaluated WPI that was thermally treated to induce cross-linking to provide better protection to DHA-containing fish oil and improve stability under storage conditions at 40°C for 50 days. The authors suggested protein thermal treatment resulted in a particle with reduced oxygen permeability thereby protecting the guest oil against oxidation. In another study, Avramenko *et al.* (2016) investigated the effect of hydrolysed heated lentil proteins to improve the stability of flaxseed oil against oxidation. It was concluded that enzymatic and heat treatment of lentil proteins were not enough to alter the lentil proteins with no improvement in the encapsulation process when compared to their native form. Moreover, the treatment resulted in more surface oil with low EE confirming the inadequate treatment.

2.3.7 Effect of Storage Conditions

Storage plays a significant role in the protection of bioactive compounds under different conditions including exposure to light, temperature, moisture, acid or alkaline media and oxygen atmosphere. Once the wall structure is destroyed, as in the case of water uptake at high RH, the capsules will lose their contents (Rosenberg *et al.*, 1990, Soottitantawat *et al.*, 2005). The increase in the moisture intake can result in elevating the release and diffusion of encapsulated agents towards the surface causing a decrease in the retention of the guest (Soottitantawat *et al.*, 2005) and enhancing the oxidation process since the agent on the surface is more vulnerable to oxidation (Paramera *et al.*, 2011). Susceptibility of bioactive agents to oxidative degradation processes (Çam *et al.*, 2014) and dehydration reactions can also result in decreasing the retention of bioactive agents or can catalyse reactions that lead to the formation of derivatives (Bertolini *et al.*, 2001, Simon-Brown *et al.*, 2016). Surface oil is also an important factor influencing the storage

stability of microcapsules as it can easily be oxidized resulting in unacceptable off-flavours (Sarkar *et al.*, 2013).

For solid cosmetics, the moisture content is generally required to be 2% or less to maintain the product integrity and quality. For encapsulated products in cosmetic or microbiological applications, the capsule wall should have the ability to withstand a critical a_w value less than or equal to 0.6 (Kha *et al.*, 2010, Silva *et al.*, 2014b). Nonetheless, some studies have shown that encapsulated agents with some hydrophilic materials can be susceptible to oxidation as shown in Table 2.1. Others studies have revealed the use of proteins or the incorporation of proteins with hydrophilic materials results in better stability (Carneiro *et al.*, 2013, Gallardo *et al.*, 2013, Pérez-Masiá *et al.*, 2015). Hence, the choice of the wall composition plays a significant role in terms of protecting the guest materials, especially during long-term storage (Rosenberg *et al.*, 1990). Therefore, it is important to understand the behaviour of different materials under different storage conditions.

Water Activity

Water activity is determined by dividing the partial vapor pressure of water in a substance by the standard state partial vapor pressure of water and has long been considered to be one of the most significant quality factors particularly in the case of long-term storage (Rascón *et al.*, 2011). Carbohydrate wall materials are affected by low a_w values (Soottitantawat *et al.*, 2005) due to the presence of polar sites in their structure that adsorb water on the surface, and at high a_w values, dissolution occurs (Fernandes *et al.*, 2014). Starch wall materials such as MD and GA have been shown to be influenced by a_w, even at low levels, compared to other wall materials that showed better stability (see Table 2.1). In one example, the degradation rate of microcapsules prepared with GA was higher than the soy protein isolate matrix when the two capsules were compared at the same level of a_w (Rascón *et al.*, 2011). This study reported the maximum oxidative stability for carotenoid microcapsules occurs at a_w = 0.274 and 0.710 for GA and soy protein isolate respectively. In both walls, the microcapsules were unable to retain their structural integrity at a_w above 0.743.

Relative Humidity and Moisture

It has been frequently observed that environmental RH strongly affects capsule stability (Zilberboim *et al.*, 1986, Rosenberg *et al.*, 1990, Khamis Ali *et al.*, 2009, Eun-Ju *et al.*, 2010, Silva *et al.*, 2014b). With increasing RH, wall material water uptake also increases which lowers the glass transition temperature of the wall, altering its structure and affecting the stability of the encapsulated agents (Thies, 2001). Rosenberg *et al.* (1990) reported that at RH levels up to 64%, the microcapsule structure using GA caused some swelling and bridging of the capsules. Further increases in RH up to 75-92% resulted in the progressive dissolution of the wall until at 97% RH, a paste was formed. Czerniak *et al.* (2015) reported a similar result when they found the stability of encapsulated fish oil using a yeast matrix was improved so long as the RH was maintained low and no protection was noticed at 90% RH. The same authors suggested that coating the capsule further with a hydroxypropyl methyl cellulose layer improved the oxidative stability of the oil and promoted agglomeration. This decreased the external surface of the microcapsules resulting in a minimized oxygen diffusion rate. In contrast, Khamis Ali *et al.* (2009) reported that flaxseed oil encapsulated in a GA-MD-soy lecithin-xanthan gum matrix showed a lower peroxide value at 54% RH compared to storage at 44% RH which resulted in a higher peroxide value. The authors suggested at low RH the moisture uptake from the matrix to outside is higher resulting in cracking of the capsule structure at low RH which consequently accelerated the oxidation process.

Pérez-Masiá *et al.* (2015) encapsulated folic acid into WPC or resistant starch wall and found the guest protected the WPC against degradation during both aqueous solution and dry storage conditions. In comparison, the resistant starch capsules showed lower stability under aqueous solution storage conditions due to the dissolution of starch resulting in a faster release of folic acid. Nevertheless, the resistant starch capsule still provided protection under dry storage conditions. Wang *et al.* (2011b) reported fish oil encapsulated in barley protein showed high oxidative stability in aqueous solutions at pH 2 and pH 7 and at 40°C for 8 weeks. The results indicated the integrity of the capsule structure was stable at both pH values. The maintaining of the wall structure and strong stability of capsule structure in the study by Wang *et al.* (2011b) may be due to the hydration of barley protein powders with NaOH during the capsule preparation that plays the role of a hardening agent (Hsieh *et al.*, 2006).

Temperature

Storage conditions after encapsulation can result in loses of active ingredients with extended storage times at high temperatures (Chiu *et al.*, 2007). The stability of bioactive compounds with respect to temperature varies amongst the different studies depending on the wall matrix, chemical structure, and the affinity of the guest for the wall. For example, Nori *et al.* (2011) studied the stability of phenolic compounds and total flavonoids of propolis encapsulated in isolate soy protein and pectin wall materials under storage at temperatures of 10 and 25°C in the absence of light and oxygen for 180 days. In order to maintain the stability of the phenolic compounds and flavonoids for this extended time, the authors recommended the storage temperature be maintained at 10°C. In another study, the stability of nanoparticles loaded with thymol or carvacrol were measured at 6 and 20°C for up to 90 days and the results showed that the nanoparticles were stable during the storage period at each of the temperatures and with little variation (da Rosa *et al.*, 2015). Another example reported that hydrolysed starch with high DE improved the retention of encapsulated α- and β-carotene carrot powders during storage at a maintained, constant moisture level without removing oxygen at 37, 45°C and 65°C (Wagner and Warthesen, 1995).

2.4 Release Mechanisms

The range of sensitive bioactive compounds that can be encapsulated for controlled release include food additives (Siro *et al.*, 2006); nutraceutical compounds (Gleeson *et al.*, 2016), EOs (Zhang *et al.*, 2014a), phenolic compounds (Saikia *et al.*, 2015), drugs (Tang *et al.*, 2015), vitamins and AOs (Katouzian and Jafari, 2016) and proteins (Déat-Lainé *et al.*, 2013) among others. Recently, encapsulation for controlled release has received increasing interest for food and drug delivery applications (Esmaeili and Asgari, 2015). The controlled release of bioactive compounds offers a number of advantages over immediate release including: maximizing AM properties (Parris *et al.*, 2005, Pezo *et al.*, 2012), preventing release at an undesired stage (Arana-Sánchez *et al.*, 2010), improving solubility and reducing toxicity for clinical applications (Tang *et al.*, 2015), minimizing rapid gastrointestinal enzymatic degradation, increasing absorption rate for peptides and proteins *via* oral administration (Déat-Lainé *et al.*, 2013), improving the stability of bioactive constituents in the stomach and enhancing the release in the

intestine over a range of physiological pH values (Chiu *et al.*, 2007) and increasing the amount of compound being delivered to the lower gastrointestinal tract (Zhang *et al.*, 2014a). Hence, the encapsulated bioactive molecule must be released at a rate that allows a sufficient amount of it to maintain its bioavailability (Parris *et al.*, 2005). Nonetheless, the release rate must be adjusted to suit specific applications with consideration given to several key factors including: the physiochemical structure of the capsule and the chemical properties of the guest molecule (size, polarity, electrostatic charge and hydrophobic interactions within the release medium) and molecular interactions between the wall material and the guest material (Zhong and Jin, 2009); the type and composition of the matrix and its sensitivity and the degree of swelling to the dissolution medium (Terao *et al.*, 2006, Saikia *et al.*, 2015, Tang *et al.*, 2015); the wall-to-guest ratio (Li *et al.*, 2013); and size of the capsule (Weinbreck *et al.*, 2004). Other critical parameters include: environmental conditions such as temperature (Katouzian and Jafari, 2016), preparation processes (Ko *et al.*, 2002), crosslinking agents (Ko *et al.*, 2002, Ocak, 2012) and the addition of surfactants (Xiao *et al.*, 2011).

2.4.1 Effect of Capsule Morphology

The selectivity of the guest material plays an important role in minimizing the imbalance in release rates. This is because the vapour pressure and permeability of the guest materials depend on their physical and chemical properties and their structure which cannot be altered (Reineccius, 1995). For example, although thymol and carvacrol are isomers, encapsulating them in GA (Guarda *et al.*, 2011) and zein (da Rosa *et al.*, 2015) results in a faster release of carvacrol than thymol as observed in both studies. This may be due to their physical states where carvacrol is a non-crystallisable liquid phenol whereas thymol is a crystalline solid at room temperature, thus carvacrol can be more easily disseminated through the wall (Guarda *et al.*, 2011). In addition, the stronger the hydrophobic attraction between the guest and wall, the slower the release rate which may be the case with thymol which has lower solubility in water (Chen *et al.*, 2015b).

In another example, the release of carvacrol from chitosan nanoparticles reached a plateau on day 30 with 53%, 23% and 33% when placed in buffer solutions at pH 3, 7 and 11, respectively (Keawchaoon and Yoksan, 2011). In a similar study, carum copticum oil released from chitosan nanoparticles reached a plateau in 72 h with 90%, 82%, 65% and 74% released into buffer solutions at pH 3, 5, 7.4 and 10, respectively (Esmaeili and

Asgari, 2015). When comparing these two studies, the release of carvacrol was slower and the time to reach the plateau was longer than in the case of the carum copticum oil which is comprised of a mixture of different molecules such as thymol, γ-terpinene and, p-cymene. A faster release within a shorter time may therefore be expected for a mixture of compounds although the reasons for this are unclear.

The concentration of the guest material and the thickness of the wall material are also important factors in the release mechanism and subsequent release rate and both can be utilised to design controlled release microcapsules (Ocak, 2012, Hosseini *et al.*, 2013). For example, increasing the thickness and compactness of the wall matrix increases the barriers for oils to be released from microcapsules (Ocak, 2012). However, with a concurrent increased oil loading, the release of the oil from microcapsules will also increase (Ocak, 2012, Li *et al.*, 2013). A decrease in the wall thickness of the microcapsules becomes less effective at higher oil loadings resulting in the encapsulation of larger oil vesicles (Ocak, 2012). If the guest material is only encapsulated by physical embedding, once the outer layer of the capsule is dissolved the protection of the inner oil is compromised and a fast release will be expected (Wang *et al.*, 2016). Other methods of controlling release include examples such as the thermal treatment of WPI emulsions prior to spray drying which have been shown to assist in the slow release of oil in simulated gastric fluids at pH 1.2 (Wang *et al.*, 2016). This study also showed that holding the lipid inside the capsule decreased the wettability of the final particle, most likely by forming a crosslinked protein network and consequently reducing leakage of the oil into the solution.

Li *et al.* (2013) reported that the release of thymol from zein-casein was dependent on the ratio of thymol to zein in the complex. It was found that increasing the thymol-to-zein ratio resulted in a 30-40% increase in the burst effect and the cumulative release after 6.5 h. However, contrary results have been reported in a similar study by Hosseini *et al.* (2013) who suggested the cumulative release after 3 h was reduced from 82% to 12% as the concentration of oregano oil in a chitosan matrix was increased from 0.1 to 0.8 g g^{-1} oregano in chitosan. The differences between the studies of Li *et al.* (2013) and Hosseini *et al.* (2013) could be related to the resulting particle surface morphology. Un-encapsulated oils can leak at the capsule surface due to the use of high oil concentrations which can enhance oxidation processes in addition to contributing to the dissolution

behaviour (Zhao and Tang, 2016). Capsules with low EE and high surface oils have low dissolution properties which can consequently affect the bioavailability of the oil (Zhao and Tang, 2016) since the hydrophobic components are exposed to the outer aqueous environment. The particles tend to have lower density which can affect the wetting behaviour and as a consequence, these particles would be prevented from being fully immersed into the solution resulting in slow release (Wang *et al.*, 2016).

2.4.2 Effect of Medium

The behaviour of encapsulated bioactive compounds in a simulated gastric fluid medium is always dependent on the surrounding matrix composition and their resistance or susceptibility to digestive enzymes as well as on the simulated gastro-intestinal conditions like pH range (Saikia *et al.*, 2015). A number of studies have evaluated the release mechanisms and kinetics of bioactive compounds at different pH values (acidic, basic and neutral) to determine the optimum conditions for releasing these compounds (Chiu *et al.*, 2007). Molecular interactions between the wall material and guest material at different pH values are important to the release characteristics where strong repulsive forces between the guest and the wall can result in quicker and relatively more complete release, with moderate attractive forces resulting in possible sustained release (Zhong and Jin, 2009).

Furthermore, electrostatic or hydrophilic attractive forces between polymers and bioactive compounds drive the release rate of active compounds (Chiu *et al.*, 2007). The slow release observed at specific pH values is due to the formation of a tight structure through an electrostatic interaction between the bioactive compounds and the selective wall material. Xiao *et al.* (2011) reported the release of nisin from zein was fast under acidic conditions (i.e. pH 2), then more sustainable as the pH increased, and limited at pH 8. Nisin is generally more positively charged at a lower pH and the high release at pH 2 is due to the repulsive forces between the positively charged zein and nisin. The more sustainable release with increasing pH is due to an increase in attraction forces between the positively charged wall and the negatively charged guest. With the change in pH, the more limited release at pH 8 is due to the higher attraction between the negatively charged zein and slightly positively charged nisin. In addition, the hydrophobic attraction and electrostatic interactions are stronger between nisin and zein with increasing pH the release rate decreased (Xiao *et al.*, 2011).

In another study, Zhong and Jin (2009) found the release of lysozyme at different pH values is also affected by the hydrophobicity and interactions between lysozyme and the zein matrix. The stronger repulsive forces between zein and lysozyme resulted in quicker and relatively complete release at pH 2 with slower, incomplete release at pH 6 and pH 8. However, Xiao *et al.* (2011) reported that the kinetic release of thymol from the zein matrix is pH-independent, and therefore sustained the controlled release of thymol over 144 h. In another study, da Rosa *et al.* (2015) reported the controlled release of thymol and carvacrol from a zein matrix in a pH 7.4 buffer solution over time was also achieved without burst effects due to a strong interaction between the agents and the zein wall.

Some wall materials do not possess the desired optimal release properties for a specific application or requirement. For example, the general release of bioactive ingredients from chitosan-based capsules is high under acidic pH conditions which may be undesirable. This is due to the swelling and partial dissolution of the capsule caused by ionic repulsion of protonated free amino groups on one chitosan chain with its neighbouring chains which consequently enhances the release of bioactive ingredients (Esmaeili and Asgari, 2015). The slower release under basic pH conditions might be due to the aggregation and precipitation of the particles that occurs in buffered solutions at high pH and this may cause a reduction of particle surface area exposed to the media (Keawchaoon and Yoksan, 2011). An “initial burst” effect followed by the rapid release of bioactive compounds from the matrix of chitosan-based delivery systems is a commonly observed phenomenon (Agnihotri and Aminabhavi, 2004) see (Table 2.1). Conversely, at acidic pH, Zhang *et al.* (2014a) suggested the thickened calcium-alginate outer layer shrinks at low pH and thus could minimize the penetration of enzymes resulting in better gastric resistance when fed to chickens. The microparticles could therefore more effectively prevent a reduced tendency of the EOs to interact with food or feed components, leading to reduced AM activity before the EOs reach the gastrointestinal tract.

In order to attain a fast, slow and/or sustainable release of an encapsulated bioactive compound, a common practice involves mixing two or more materials (da Silva *et al.*, 2014). Several studies have explored and have established the potential of using a combination of different polymers to encapsulate sensitive bioactive compounds where

the selective polymers exhibit opposing mechanistic behaviour at the same pH (Deladino *et al.*, 2008). Some studies have investigated the formation of alginate microparticles through electrostatic attraction as carriers for the specific delivery and controlled-release of bioactive compounds (Lacerda *et al.*, 2014). At acidic pH, the release of bioactive molecules from alginate matrices is slow due to the protonation of alginate to give the insoluble form of alginic acid where the electrostatic attraction among these groups are high (Déat-Lainé *et al.*, 2013, Lacerda *et al.*, 2014, Zhang *et al.*, 2014a). In order to adjust the release at acidic pH where the alginate matrix shrinks, the addition of WPI, which behaves differently to alginate at the same pH (i.e. swelling and relaxation), can result in a more sustainable and controlled release of insulin (Déat-Lainé *et al.*, 2013).

In another study, the controlled release of the antibiotic rifampicin was achieved using sodium alginate-chitosan microparticles (Lacerda *et al.*, 2014). At acidic pH, the release of the high molecular weight molecule was slow due to its interaction with chitosan which has positively charged groups that interact strongly with alginate resulting in reduced swelling and a slower release rate. A complete release over 250 min was found at pH 6.8 due to a weakening of the extent of the internal interactions between chitosan and sodium alginate and the ionization of the sodium alginate carboxyl groups. Zhang *et al.* (2014a) reported different observations, where despite the mixing of alginate with WPI to encapsulate carvacrol, the release in acidic pH was low and therefore WPI did not contribute to the release but rather it acted effectively as an emulsifier that coated and stabilized the carvacrol droplets. The release was affected more by the physical structure where the capsule outer layer was mainly covered by Ca-alginate, and the WPI layer was covered with the carvacrol layer at the immediately adjacent layer. However, the release in simulated intestinal fluid was more dominated by the WPI concentration rather than alginate, and hence optimizing the concentration of WPI could alter the release rate depending on the release requirements.

Some studies have investigated the effect of incorporating a hydrophilic matrix such as cashew gum or an amphiphilic biopolymer such κ-casein to control the release rate of bioactive agents (Abreu *et al.*, 2012). In this study, Abreu *et al.* (2012) found that increasing the quantity of cashew gum in chitosan-cashew and alginate-cashew gum matrices increased the hydrophilicity and consequently increased the release of the guest material. In another study, Chen *et al.* (2015b) found a faster release rate of thymol from

zein-κ-casein capsules was controlled by the presence of κ-casein in the zein nanoparticles.

2.4.3 Effect of Capsule Swelling Capacity

The degree of swelling by the wall due to its plasticization with water or by water uptake from the environment can result in increases in both the intramolecular free volume and extra-molecular pore space (Reineccius, 1995). In most studies investigating this effect, relaxation due to the swelling and conversion of glassy polymers into a rubbery matrix or the dissociation of the matrix was found to affect the ability of the bioactive agent to diffuse. A number of studies have investigated the effect of incorporating crosslinking agents on the swelling capacity and release rate of bioactive compounds. In several studies, it was found that crosslinking agents decrease the swelling capacity of microparticles and therefore decrease the release rate of these compounds into the dissolution medium. For example, microcapsules containing higher amounts of crosslinked tripolyphosphate (Ko *et al.*, 2002) or glutaraldehyde (Agnihotri and Aminabhavi, 2004, Ocak, 2012) exhibited more prolonged release than those containing a lower amount of the crosslinked agents. Manca *et al.* (2008) found the addition of glutaraldehyde at pH 7.4 decreased the release of rifampicin from chitosan microparticles in contrast to the case where no crosslinking agent was added. However, under acidic conditions (i.e. pH 4.4), the release of the drug was considerably faster compared to the release observed at pH 7.4. The authors suggested the drug release rate under acidic conditions is attributed to the higher solubility of chitosan at lower pH and therefore the crosslinking reactions cannot substantially modulate the release rate. In this case, the release of the drug under the acidic conditions is predominantly determined by the solubilization of chitosan, and the diffusion of the drug molecule through the polymeric matrix (Manca *et al.*, 2008).

However, Pulicharla *et al.* (2016) reported the release profiles of polyphenols from chitosan crosslinked with tri-polyphosphate is influenced by the polyphenols rather than the matrix. It was found that the release was higher at pH 7.4 than at pH 1.4 and was characterized by an initial rapid release followed by a more sustained release. The authors suggested the high solubility of polyphenols due to the presence of anionic groups at pH 7.4 resulted in the higher release rate compared with that at the lower pH. In another study, Harris *et al.* (2011) measured the release of yerba mate extract (polyphenols) in

chitosan hydrochloride crosslinked with sodium tri-polyphosphate and reported the release profiles of the polyphenols was higher at pH 5.7 than at pH 6.5 due to the solubility of chitosan hydrochloride at low acidic pH. A similar example reported the release of encapsulated polyphenols from an MD wall was higher in simulated gastric fluids at pH 1.2 than in simulated intestinal fluid at pH 6.8 (Saikia *et al.*, 2015).

Other studies have investigated the effect of non-ionic surfactant Tween 20 and glycerol, the most commonly-used plasticizer, on the release kinetics of nisin and thymol from zein capsules (Xiao *et al.*, 2011). It was found the addition of the Tween 20 weakens the hydrophobic attraction between nisin and zein and enables the release of nisin from the capsules at pH 6.0 and complete release at pH 8.0. It was observed that the kinetic releases of thymol and nisin from a zein matrix were increased with increasing levels of Tween 20 and glycerol in the capsule.

2.4.4 Stabilizing Agents

Reticulating agents, also known as crosslinking agents, include formaldehyde (Dong *et al.*, 2008), glutaraldehyde (Agnihotri and Aminabhavi, 2004, Ocak, 2012), transglutaminase enzyme (Dong *et al.*, 2008), calcium chloride (Soliman *et al.*, 2013), and surfactants such as sodium dodecyl sulfate (SDS), Tween 20 and sodium lauryl sulfate (Li *et al.*, 2009). These agents are commonly used to harden microcapsule walls, consequently stabilizing and strengthening the wall structure by facilitating a better interaction with the guest. Dong *et al.* (2008), studied the optimum cross-linking parameters to encapsulate peppermint oil in gelatin–GA microcapsules crosslinked with transglutaminase. It was found that a crosslinking time of 6 h at 15°C and pH 6 resulted in improved structural stability during incubation at 80°C.

Some studies have investigated the effect of surfactants and reticulating agents on the stability of microcapsules (Paramera *et al.*, 2011) with the concentration of surfactant an important factor affecting the capsule thermal stability. Li *et al.* (2009) reported the use of SDS in preparing microcapsules containing tetrachloroethylene as the guest in gelatin-sodium/carboxymethyl cellulose wall materials. They reported an enhanced process yield up to a concentration of 1 mM of the surfactant, however, increasing the concentration to 3 mM dramatically decreased the yield to about 8%. Thermogravimetric (TG) analysis showed the thermal stability of the microcapsules decreased as the

concentration of SDS in the formulation was increased. When the SDS concentration was 2.5 mM or more, a weight loss was observed suggesting the capsule wall was less compact where gelatin desorbed from the oil/water surface at high SDS concentrations and no stable microcapsules could be formed. In another study, Paramera *et al.* (2011) reported that DSC scans of curcumin/MS encapsulation powders with or without Tween 80 did not exhibit the characteristic melting peak of curcumin and protected the guest material up to 240°C. Above this temperature, decomposition of the powder occurred indicating that the presence of the emulsifier did not further enhance the protection of curcumin.

The potential for adverse health side-effects of using reticulating agents and the high price of certain enzymes have driven researchers to investigate the effectiveness of more natural, and economically sustainable agents such as glycerol and tannic acid. Koupantsis *et al.* (2016), for example, studied the addition of glycerol as a reticulating agent to encapsulate *β*-pinene in sodium caseinate or WPI/carboxymethyl cellulose wall materials in order to improve the quality of the capsule. It was found the addition of glycerol led to more than a two-fold increase in the EE, however, TG analysis showed the addition of glycerol resulted in desorption of the encapsulated *β*-pinene at a lower temperature. The authors suggested that heat increased the glycerol mobility resulting in faster desorption of *β*-pinene thereby accelerating its loss. In another study, Agnihotri and Aminabhavi (2004) encapsulated clozapine in a chitosan wall and the active agent was crosslinked with glutaraldehyde. It was found that significant weight loss of clozapine occurred above its melting point at 182°C and the microparticles started to decompose at 260°C. The TG results indicated that the encapsulation process did not modify the melting point of clozapine, and hence the clozapine was not chemically changed in the encapsulation process. However, from the heat stability point of view, this result may support the findings of the study by Koupantsis *et al.* (2016) who suggested that surfactant or crosslinked agents could improve EE but consequently could interfere with guest stability.

2.5 Activity of Encapsulated Bioactive Agents

The demand for the use of natural AM additives to inhibit the growth of microorganisms, improve safety and extend the shelf-life of foods whilst maintaining its quality, as well as preventing lipid oxidation has resulted in a large number of studies that

examined the effectiveness of natural AM agents in different applications. Lipid oxidation can result in food quality losses (Fukumoto and Mazza, 2000, Velasco *et al.*, 2004) and therefore the use of AO agents can: (i) delay or prevent lipid oxidation and minimize rancidity in foods; (ii) prevent toxicity resulting from the oxidation process, (iii) maintain nutritional quality; and (iv) increase product shelf-life (Fukumoto and Mazza, 2000). Consequently, maintaining AM and AO activities of bioactive agents after the encapsulation process is one of the more significant considerations.

2.5.1 Antioxidant Activity

The AO activity after encapsulation has been studied using different techniques including Trolox equivalent antioxidant capacity (Santos *et al.*, 2015); 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay (Weerakody *et al.*, 2008); ferric ion spectrophotometric assay (Wu *et al.*, 2012); and thermal analysis using DSC (Liolios *et al.*, 2009). Some studies have reported the AO activity of encapsulated agents were reduced in comparison with their free form (Kamimura *et al.*, 2014, Santos *et al.*, 2015, Simon-Brown *et al.*, 2016), whereas others have reported that encapsulated agents have better AO properties (Liolios *et al.*, 2009). The reduction of AO activity after the encapsulation process can be related to a number of factors including solubility, chemical structure and the composition of the guest and wall, experimental parameters, and the method of evaluation (Esmaeili and Asgari, 2015).

In general, without encapsulation the solubility of bioactive agents in solutions and the subsequent steric hindrance of compounds can contribute to the observed differences in the AO activities (Fukumoto and Mazza, 2000). However, in the case of encapsulated agents, the solubility of the wall in the extracting solvent plays a role in determining the capacity of the agent to quench free-radicals (Esmaeili and Asgari, 2015). This can occur when the capsule is not completely destroyed or the diffusion of the encapsulated agent during the analysis does not allow it to reach its maximum concentration due to structural interactions between the wall and the AO agent. As a result, this can contribute to the lower AO activity when compared to the free form and can subsequently result in a reported reduction of the AO activity (Esmaeili and Asgari, 2015).

Weerakody *et al.* (2008) found the AO activity of encapsulated lipoic acid in a chitosan matrix using the DPPH assay exhibited a reduced AO activity when extracted with ethanol. However, when the capsule was extracted with a 1:1 mixture of ethanol and 2% (v/v) acetic acid, the AO activity was higher. Chitosan is soluble in acidic media and hence bioactive agents can diffuse more easily outside the chitosan wall resulting in a higher AO activity. Nevertheless, organic solvents such as ethanol and methanol are necessary in analyses using the DPPH assay due to the hydrophobic properties of DPPH that limits its solubility in solutions containing less than 50% organic solvent (Ferreira *et al.*, 2013).

Feyzioglu and Tornuk (2016) reported the AO activity of summer savory (*Satureja hortensis* L.) EO encapsulated in chitosan nanoparticles correlated with increasing concentration of the EO. The zeta potential (ZP) of the nanoparticles varied from -7.54 mV to -21.12 mV as the concentration of the active agent was increased and was attributed to the highly negatively-charged active agent counteracting the positively charged chitosan. The reduction of the ZP value is a clear indication of the encapsulated agent being adsorbed on the surface of the wall (Keawchaoon and Yoksan, 2011, Li *et al.*, 2013, Gomes *et al.*, 2014). Therefore, the observed increasing AO capacity with increasing EO concentration may be the result of the EO coated on the particle surfaces having a high solubility in the ethanol mixture used during the quenching experiment.

In another study, the AO activity was found to depend on experimental parameters used to produce encapsulated agents where high concentrations of wall compounds and the inlet air temperatures used for spray drying were reported to affect the physicochemical properties and consequently reduce AO activity (Kha *et al.*, 2010). Increasing the concentraiton of wall material resulted in viscous emulsions that required longer exposure to atomization during spray drying which favors lipid oxidation and can reduce AO activity (Tonon *et al.*, 2011). Similarly, Wu *et al.* (2012) reported that the AO activity of encapsulated thymol and carvacrol in zein depends on the conditions used during the formulation such as the pH used to prepare the capsule and the incubation time with free radicals during the AO test. It was found that the longer the incubation time, the greater the number of free radicals that are quenched. Despite the fact that thymol and carvacrol are isomers, the authors found that thymol showed higher AO activity than carvacrol suggesting that steric hindrance of the thymol phenolic group may be

responsible for this observation. The observed higher AO activity of thymol compared to carvacrol is also in agreement with the studies (Liolios *et al.*, 2009). Harris *et al.* (2011) also found the effectiveness of AO in the medium increased when more polyphenol was released. A study by Belščak-Cvitanović *et al.* (2011) investigated the possibility of using ascorbic acid to dissolve chitosan instead of using acetic acid to improve the AO stability of six encapsulated herbal polyphenolic extracts in alginate–chitosan microbeads. The authors concluded the presence of ascorbic acid neither improved nor provided AO stability to the microencapsulated polyphenolic. Moreover, the presence of ascorbic acid elevated the degradation process of the encapsulated compounds. The authors suggested the high content of transition metal ions of these extracts contributed to the rapid degradation of ascorbic acid and consequently this initiated and affected the degradation of polyphenolic compounds (Belščak-Cvitanović *et al.*, 2011).

Arana-Sánchez *et al.* (2010) evaluated the AO activity of three Mexican oregano EOs encapsulated in β-CD using the DPPH assay and found that the AO activity of the encapsulated oils improved by four- to eight-fold when compared with the pure forms. In contrast, Kamimura *et al.* (2014) and Santos *et al.* (2015) reported carvacrol encapsulated in HB-β-CD and β-CD was stable during storage for 3 months at room temperature when exposed to light and further reported that light exposure did not affect the AO activity of the encapsulated carvacrol throughout the storage period. However, the AO activity in both studies was lower than that of free carvacrol. The differences in the AO activities using β-CD might be due to the experimental parameters whereby the Arana-Sánchez *et al.* (2010) study used a longer incubation time in the test assay with a high concentration of the organic solvent which may have resulted in the observed increase in the AO activity.

In contrast, other studies measuring AO activity by thermal techniques such as DSC have shown that the AO activity was enhanced when the active agent was encapsulated compared to when it is present in its free form (Liolios *et al.*, 2009). Therefore, it is important to assess the AO stability and activity using a combination of methods to give a more complete and comprehensive understanding of the effect of encapsulation on the AO activity of a given agent (Viuda-Martos *et al.*, 2010).

2.5.2 Antimicrobial Activity

The mode of action of naturally occuring AM agent primarily depends on the polarity and hydrophobicity of the agent (Kuorwel *et al.*, 2011b). There is an agreement amongst different studies regarding the sensitivity of Gram-negative and Gram-positive bacteria to the action of AM agents that is due to the differences in the structure of the outer cell membrane (da Rosa *et al.*, 2015). The preservation and enhancement of the activity of AM agents in a controlled manner to inhibit a wide range of pathogenic microorganisms using encapsulation techniques has been extensively studied and these finding are summarised in Table 2.1. Most of these studies have investigated the effect of the encapsulation process on the activity of the AM agents, for example, if encapsulation offers better, the same, or hindered activity. These studies have hypothesised a range of suggestions as to the reasons for better action of encapsulated AM agents when examined in specific applications using a different range of wall materials and techniques.

The degree of swelling of the wall affects the ability of the bioactive agent to diffuse. This is the common phenomenon that is anticipated to be the mechanisms responsible for the controlled release of the encapsulated agent to the outside of the matrix, and therefore the activity of the AM or AO agent in a specific application (Agnihotri and Aminabhavi, 2004, Deladino *et al.*, 2008, Bayarri *et al.*, 2014). Some wall materials are pH sensitive, and therefore, the degree of swelling and the relaxation of the wall to diffuse active agents from the capsule can be affected by the pH due to the electrostatic attraction or repulsions at a given pH (Déat-Lainé *et al.*, 2013). Others studies have suggested the improvement in AM actions is due to factors including: the enhanced solubility of the agents (Arana-Sánchez *et al.*, 2010, Hill *et al.*, 2013, Santos *et al.*, 2015); the physicochemical structure of the capsule (Liolios *et al.*, 2009); and the digestion of the encapsulated wall by the pathogenic organisms (Wu *et al.*, 2012).

The encapsulated AM agent activity is significantly influenced by the physicochemical characteristics of the wall that include the wall composition, size and charge, as well as by the composition of the bacterial membrane (Liolios *et al.*, 2009). Most research studies performed *in vitro* have found that the encapsulation process enhances the solubility of the AM agent which is the dominant factor contributing to the higher activities compared to the free AM agents. This phenomenon is more dominant if the AM agents are examined in aqueous environments that bring the AM agent to the cell

membrane surface, thus improving its solubility and accessibility to microbial cells (Arana-Sánchez *et al.*, 2010, Hill *et al.*, 2013, Santos *et al.*, 2015). Liolios *et al.* (2009) found the addition of cholesterol liposomes changed the degree of head group dissociation and provided better interaction with lipophilic compounds. These actions could enhance the solubility of AM agent in the water phase, subsequently improving the cellular transport and release of the AM agent at the membrane and inside the cytoplasm of the bacteria. This action can alter the pH and equilibrium of inorganic ions inside the cytoplasm, and cause damage to the proteins and lipids resulting in dramatically increased AM activity (Mohammadi *et al.*, 2015b). This study also revealed that capsule size plays a role in elevating the encapsulated AM activity outcome (i.e. nano-size capsules improve the activity compared to micro-size capsules).

Furthermore, controlling the surface charge or ZP of the capsule has also been suggested to improve the AM activity of encapsulated agents (Kurincic *et al.*, 2016). A negatively-charged microbial cell wall responds better to positively-charged surface materials due to the electrostatic interaction (Donsì and Ferrari, 2016). This can increase the availability of the AM agent at the active site of the cell membrane and consequently inhibit cell growth. Chitosan is one material that has such characteristics with positively-charged surfaces and has been shown to work effectively against different pathogenic organisms (Mohammadi *et al.*, 2015b). The hypothesis that electrostatic interactions between the capsule wall and pathogenic cell membrane facilitates the activity of the AM agent was confirmed by the results of Esmaeili and Asgari (2015). The authors reported the activity of carum copticum EO encapsulated in chitosan showed better AM activity than the pure form of the oil against *S. aureus, S. epidermidis, B. cereus, E. coli, S. typhimurium and P. vulgaris*.

This hypothesis may also be supported by the results of Zhang *et al.* (2014b) who found that encapsulated thymol loaded in zein nanoparticles stabilized by sodium caseinate–chitosan hydrochloride double layers also effectively inhibited the growth of *S. aureus* for 16 h. In addition, a formulation containing a high mass ratio of sodium caseinate to chitosan hydrochloride of 1:4 significantly inhibited *S. aureus* for the whole test period of 24 h in contrast to formulations containing 1:1 or 1:2 mass ratios where inhibition lasted for 16 h or less. The analysis of the ZP of sodium caseinate-stabilized nanoparticles showed that the charge changed from negative to positive after it was coated

with the chitosan layer (Zhang *et al.*, 2014b). In another study, Keawchaoon and Yoksan (2011) found that positively-charged carvacrol-loaded chitosan nanoparticles displayed AM activity against *S. aureus, B. cereus and E. coli*. However, Abreu *et al.* (2012) found the relationship between the mortality rate of St. *aegypti* larvae using cashew gum-chitosan loaded with *Lippia sidoides* oil was related to the concentration of the oil in the formulation. The highest ZP value found in this study was 49 mV which corresponded to the 1:10 gum:chitosan formulation, although the levels of mortality were low. Nevertheless, some studies have reported that encapsulated materials with a negative ZP surface charge also improves the AM activity and inhibits the growth of microorganisms (Li *et al.*, 2013, Herculano *et al.*, 2015, Feyzioglu and Tornuk, 2016).

The digestion of the encapsulating wall by the pathogenic organism has also been reported to be responsible for AM action (Wu *et al.*, 2012). For example, the AM activity of thymol/carvacrol loaded in zein against *E. coli* evaluated using lysogeny broth medium showed a reduction in the concentration of *E. coli* by 0.8-1.8 $\log_{10}$ CFU mL^{-1} after the 48 h incubation at 35°C compared to the control. Wu *et al.* (2012) observed that zein nanoparticles quickly released all encapsulated carvacrol and thymol into the test medium, and also reported the solubility of the encapsulated agents increased by 14-fold by incorporating it in a zein wall. They suggested that the fast release of thymol and carvacrol could be the result of the digestion of the zein nanoparticles by *E. coli* so as a consequence the nanoparticles work effectively in the reduction of the bacteria (Wu *et al.*, 2012).

In another study using a diffusion agar plate, Benavides *et al.* (2016) inoculated the plate with bacterial cultures including *E. coli* (ATCC 25922), *S. aureus* (ATCC 6538), and *L. monocytogenes* (ATCC 7644) with the wells loaded with 50 mg of alginate/thymol microspheres. The authors reported that the microcapsules were still able to maintain their AM properties despite the slow swelling of the microspheres which were tested in a relatively dry, solid phase and as such there was no affinity with the agar medium. It was suggested the microspheres were affected by pH fluctuations, peristalsis and other metabolic factors that favour swelling and subsequent release which, in turn, inhibits the growth of microorganisms.

The physical and chemical properties of EOs are the main aspects that determine their AM action against pathogenic organisms. The physical and chemical properties of the guest materials depend on their chemical structure which cannot be altered, whereas the extent of activity of the EOs depends on the extent of their volatilization in the case of volatile agents (Reineccius, 1995). In an example of a non-volatile agent, Carrizo *et al.* (2016) studied the AO activity of green tea in a multilayer packaging application for dark chocolate peanuts and milk chocolate cereals. Sampling over a period of 16 months showed AO activity although the complete absence of migration indicated that catechins in the green tea do not diffuse through the polymer. It was concluded that free radicals cross the polymeric layer and diffuse through the polymer where they are trapped and consumed by the catechins.

In order to prolong the shelf-life of food products and inhibit the growth of microorganisms, the mass transfer of the AM additive must be slow, so that the rate of its release remains greater than the growth rate of the target microorganism (Appendini and Hotchkiss, 2002). If not, then the AM additive will consequently lose its activity before the end of the storage period. For EOs and their extracts, the encapsulation process can minimize the evaporation rate of the oil, thus increasing its AM activity by protecting it from evaporation losses (Soliman *et al.*, 2013) or dilution with the food components.

The polarity of an AM agent is related to the presence of hydrophilic groups (e.g. hydroxyl functional group) and is the vital characteristic in determining its physical properties (Davidson and Branen, 2005). The solubility of the hydrophilic part of the AM agent also plays a role in dissolving it in the water phase, where the growth of microorganisms primarily occurs. Furthermore, one of the main characteristics of EOs that is responsible for their AM activity is their hydrophobicity. This enables them to act on the hydrophobic membrane of microorganisms and break down the lipids in the bacterial cell membrane walls causing leakage of ions and other cell compounds and thereby resulting in the eventual death of the cells that constitute the microorganisms (Becerril *et al.*, 2013, Feyzioglu and Tornuk, 2016, Nerin *et al.*, 2016a). In addition, the AM agent is able to cross the bacterial membrane and exert a bactericidal effect by acting on several cellular targets without damaging the membrane. This results in changes in essential cell components such as saccharides, amino acids, proteins, lipids, or enzymes,

thus impacting several cellular functions such as respiration, metabolism, or cell cycle (Clemente *et al.*, 2016a, Clemente *et al.*, 2017).

2.6 Practical Applications

In practice, the advantageous outcomes of encapsulated bioactive agents include increased stability and activity, as well as controlled, and often sustained release (Silva *et al.*, 2014a). To achieve these outcomes, it is important to consider the particular applications or industries where these compounds are used and test the encapsulated agents accordingly. The vast majority of studies of encapsulated agents have investigated or established the benefit of encapsulation techniques primarily using *in vitro* studies. Although the outcomes of these *in vitro* studies offer crucial information for the development of encapsulation formulations, it is becoming more important to assess the outcomes *in vivo* to more fully assess the efficacy for the desired application. Clearly the stability and release of encapsulated agents in applications may be different to those found under controlled laboratory experimental conditions so it may be difficult to correlate the *in vivo* and *in vitro* tests.

2.6.1 Foods and Pharmaceuticals

Studies that have focused on practical applications of encapsulated bioactive agents include food applications (Marcolino *et al.*, 2011, Gallardo *et al.*, 2013, Çam *et al.*, 2014, Gomes *et al.*, 2014, Mangolim *et al.*, 2014, Mohammadi *et al.*, 2015a, Mohammadi *et al.*, 2015b) and drug and oral applications (Agnihotri and Aminabhavi, 2004, Terao *et al.*, 2006, de Paula *et al.*, 2011). For example, Gallardo *et al.* (2013) reported unexpected findings in the field compared to laboratory experimental results when linseed oil GA microcapsules were incorporated in bread fortification. The GA microcapsule was selected based on the outcomes of the experimental investigations that showed GA microcapsules to have the highest EE and excellent stability against oxidation using the Rancimat test when compared to GA-MD-WPI microcapsules that had a lower EE. When the capsule prepared with GA wall alone was used, they found a reduction in the α-linolenic acid content which is the major component of linseed oil. It was suggested other factors related to the bread manufacturing may also be associated with the decrease in the α-linolenic acid content, such as water content, dough kneading and incubation at 80% RH, as well as baking at 220°C. In another example, encapsulated fish oil in barley protein showed high oxidative stability in milk and yogurt when both products were

pasteurized at 80°C for 30 min before storage for 4 and 5 weeks, respectively, corresponding to their average shelf-life (Wang *et al.*, 2011b). The results suggested the oxidative product stability was significant in yogurt even after the prospective shelf-life. Çam *et al.* (2014) found the addition of pomegranate peel phenolics microencapsulated in MD by a spray drying method improved the AO activities of ice cream products.

Marcolino *et al.* (2011) suggested the complexation of bixin and curcumin in β-CD favours their use in low-fat foods. It was found that the addition of complexed curcumin in cheese and yogurt, and bixin in the curd, did not alter the initial characteristics of the products and were well accepted based on their sensory properties. In another study, Mangolim *et al.* (2014) found the use of the β-CD/curcumin complex in vanilla ice creams increased the colour intensity and provided better dispersion without altering the products attributes such as texture, taste and flavour. Furthermore, the use of the complex provided better commercial advantage since the colourant quality of the complex was 83% less in the prepared product compared with the pure colourant.

In order to attain the maximum antibacterial activity of EOs for oral delivery to the small intestine of animals, it is desirable to minimize the release of agents in the upper gastric track and increase the amount of EOs being delivered to the intestine (Wang *et al.*, 2009). Typically, the gastric emptying time for humans and some monogastric animals higher up the food chain can range between 1 and 4 h, and therefore encapsulated bioactive compounds should have little interaction with other components in the food, have limited digestibility in the stomach, slow release in the small intestine, and more rapid release in the large intestine (Parris *et al.*, 2005).

In a pharmaceutical application, losartan potassium, a highly soluble, orally active, non-peptide drug used in the treatment of hypertension, was complexed with HP-β-CD (de Paula *et al.*, 2011). The encapsulation process enhanced the extent and duration of the medication antagonist action and increased its bioavailability by improving its sustained release. Hence the encapsulation process increased the drug efficacy and reduced the dose or spacing time required with each dose intake . In another example, Agnihotri and Aminabhavi (2004) reported the release of clozapine from a chitosan matrix *via in vitro* studies was affected by the concentration of the drug in the formulations as well as the crosslinking agents used in the formulation. In contrast, *in vivo* tests in the same study showed delayed drug absorption indicating that the absorption

under more controlled conditions. The results showed the encapsulation process increased the amount of drug reaching the systemic circulation unchanged as well as enhanced the drug on the pass of the first metabolism stage as well as improved its duration.

In some clinical applications, a fast dissolution rate can significantly increase the solubility and decrease the toxicity of certain bioactive agents. For example, Tang *et al.* (2015) studied the dissolution of chlorzoxazone complexed with HP-β-CD and β-CD and reported that when complexed with the former, the agent exhibited better dissolution properties than the one complexed with the latter. It was reported that up to 100% and 70% of the drug is released into the three media after 10 min and 90 min for HP-β-CD and β-CD respectively. Terao *et al.* (2006) conducted a clinical trial involving single-dose administration of CoQ10-*γ*-CD to healthy adult males and females. The results showed the complexation of the lipophilic drug capsule significantly enhanced the oral absorption and bioavailability of the enzyme in the subjects after 4 h with a slow gradual sustained increase in blood plasma levels for up to 24 h.

In another study, Zhang *et al.* (2014a) reported the results of *in vitro* tests of the release of carvacrol from alginate-WPI microparticles in simulated gastric fluids from different formulations were in the range of 8 to 11%. In addition, the release into simulated intestinal fluid was relatively high with more than 80% of encapsulated carvacrol released within 2 h. In this case, the *in vivo* application resulted in an observed faster release of carvacrol in the lower gastrointestinal tract of chickens than that measured *via in vitro* simulation (Zhang *et al.*, 2014a).

2.6.2 Food Packaging Applications

Active packaging (AP) technologies are among the key growth areas where the incorporation of natural agents such as EOs in food packaging materials are promoted as an effective method of food preservation. Rather than the addition of traditional preservatives into food products, AP relies on the addition of active agents into packaging materials to impart AO and/or AM activity with the aim to protect food quality and potentially extend the shelf-life of the food products. The shift in the use of synthetic to natural AM additives has resulted in many researchers investigating the effectiveness of such additives in inhibiting the growth of food-borne microorganisms such as bacteria, fungi, yeast, and mould *in vitro* and *in vivo* where the growth of these microorganisms can result in serious food-borne diseases (Smith-Palmer *et al.*, 1998, Suppakul *et al.*,

2006, Chalier *et al.*, 2007, López *et al.*, 2007, Gutierrez *et al.*, 2008, Suppakul *et al.*, 2008, Kuorwel *et al.*, 2011a, Muriel-Galet *et al.*, 2012a, Ramos *et al.*, 2012). The effectiveness of these natural AM additives is due to their chemical structure, low molecular weight, and high lipid solubility which enables them to target their action directly in the cell membrane of the microorganism. Examples of natural AM agents that possess strong AM activity against spoilage and pathogenic microorganisms include extracts from clove, cinnamon, oregano, thyme, sage, rosemary, basil and vanillin (Aurelio *et al.*, 2005). However, the volatility, poor water solubility, and low physiochemical stability limits the use of these agents in some of the abovementioned applications (Suppakul *et al.*, 2008). Recently, there has been an increase in the use of encapsulation techniques in food packaging applications with a particular interest in natural EOs and their extracts. The use of encapsulation techniques is desired to overcome the limitations of using natural AM agents directly without encapsulation. These limitations include their relative instability at high temperatures; high volatility, even at room temperature (Levi *et al.*, 2011); and their potential to change the organoleptic qualities of the food at high concentrations (Gutiérrez *et al.*, 2009a).

Makwana *et al.* (2014) studied nano-encapsulated cinnamaldehyde by lipid bilayers of polydiacetylene-N-hydroxysuccinimide nanoliposomes which were immobilized on glass slides and poly(lactic acid) (PLA) films. After 48 h, the glass surfaces coated with nanoparticles and PLA films coated with cinnamaldehyde showed high AM activity against *E. coli* W1485 and *Bacillus cereus* ATCC 14579. Nonetheless, no inhibition was detected when the nanoencapsulated cinnamaldehyde/liposomal coated on the surface of PLA film. Bayarri *et al.* (2014) studied the formation of a complex between lysozyme and low methoxyl pectin in order to develop an edible AM film. At a level of 0.2 g L^{-1} of low methoxyl pectin the complex formation was shown to significantly decrease the lysozyme AM activity probably due to the limited diffusion of the lysozyme substrate and/or a decrease of the enzyme mobility. The lysozyme activity increased and remained stable at a concentration of 2 g L^{-1}. The study showed the presence of pectinases in the release medium enhanced the lysozyme release confirming that the developed edible AM film can be used to protect foods against lysozyme-sensitive microorganisms and particularly those producing pectinolytic enzymes

In another study, Guarda *et al.* (2011), investigated the AM activity of thymol and carvacrol encapsulated in GA using the agar plate test. A bi-axially oriented

polypropylene film was coated with the encapsulated agent emulsion, was dried at room temperature, and was tested against *L. innocua*, *S. aureus* (Gram-positive bacteria), *E. coli* (Gram-negative bacterium), *S. cerevisiae* (yeast) and *A. niger* (mold filament). No zone of inhibition could be detected for encapsulated thymol and carvacrol at any of the concentrations tested (1%, 2%, 5% and 10% each) even though the higher two concentrations were greater than the minimum inhibitory concentration for the most resistant microorganisms such as *E. coli*. The authors reported that growth inhibition was detected for *S. aureus, L. innocua, E. coli* and *S. cerevisiae* only when both the encapsulated thymol and carvacrol were mixed together at 10% each, with of inhibition zones of 11.3 ± 1.3 mm, 8.8 ± 0.9 mm, 9.0 ± 0.8 mm and 4.3 ± 1.3 mm respectively. In addition, the film did not show a zone of inhibition for *A. niger* but an inhibition of the growth of the mycelium was detected. The authors suggested this is due to the relatively low release of the AM agents that resulted from high attraction between the AM agent and GA as it contains a fraction of glucuronic acid that develops negative charges around the particles in an oil emulsion. In addition, the release of the AM agent slows down as the fraction of arabinogalactan-protein in the GA has a high viscosity which slows down the movement of the oil particles.

Considering the previously mentioned limitations, there is currently a growing interest in using encapsulated natural AM agents in food packaging applications. Further studies are also required to provide a better understanding of the encapsulated AM agents' behaviour in prolonging the shelf-life of packaged food products. Most studies that have examined encapsulated AM agents indicated that in liquid media the encapsulated AM agents generally work effectively in inhibiting the growth of microorganisms. Nevertheless, the incorporation of these within films often affects their activity. Sometimes, the encapsulation process can lead to a decrease in the activity of the active agent (Donsì and Ferrari, 2016) due to several factors including: (i) a reduction in the mobility of the active agents; (ii) some of the active sites could be hidden and become inaccessible to the microbial cells, and/or (iii) the process results in random orientations of the active agents that are initiated by weak electrostatic interactions resulting in a decrease of the active agent's interactions with the anionic sites of the microbial cell wall (Bayarri *et al.*, 2014). Though, in food packaging applications the active ingredients must undergo controlled release where the active agent transfers from the polymeric carrier to the surface of the food. This release profile is necessary in order to maintain a

predetermined concentration and thus to prolong the shelf-life (Siro *et al.*, 2006) where the release could take place for a few weeks or even months. Nevertheless, the burst effect high release rate of active agents at the initial stage could be desirable to strengthen the performance of the active agent followed by a more sustainable release rate (Agnihotri and Aminabhavi, 2004).

It is therefore important to investigate the reason behind the hindrance of the activity of encapsulated AM agents when these are incorporated in food packaging materials. Furthermore, it is also necessary to determine if the activity of encapsulated AM agents will be different when these are applied to real systems. In real food applications, other factors that may affect the release of encapsulated AM agents, and hence improve or hinder their activity, include: food components such as fats, proteins and carbohydrates; the pH, water activity and humidity of the packaging as well as the temperature and temperature fluctuations during storage and transport. Many of the published reports about the benefits of encapsulating active agents have been reported on the basis of results from laboratory experiments and there are few studies have been reported based on in-service applications.

2.7 Summary

Encapsulation processes are designed to provide stability to bioactive compounds against degradation reactions when these compounds are exposed to one or more potentially detrimental environmental factors such as light, temperature, moisture, pH and oxygen. Carbohydrates and proteins are the most common wall materials used for the encapsulation of bioactive agents due to their well-established properties and their ability to stabilise a wide range of guest agents. Studies of encapsulation for enhanced stability, controlled release, improved bioavailability and bioactivity have therefore received increasing interest over recent years for a range of applications including drug delivery, nutraceutical cosmetics, agricultural, food and food packaging applications.

The effectiveness of the encapsulation process depends on numerous factors including the physiochemical structure of the wall material; the overall composition of the wall material; the ratio and the affinity of the bioactive agents to the wall; the storage conditions (including a_w, RH, temperature); the sensitivity and degree of swelling of the wall to the dissolution medium; the addition of surfactants and stabilising agents; and the

size and surface charge of the capsule. In addition, the mobility of the active agents from the capsule is a significant property that affects the bioavailability and/or activity of the agents. The use of different classes of materials and in different combinations, as well as the use of cross-linking and surface-active agents are among the several approaches that may enhance encapsulation efficiency. These approaches are used to optimize the capsule quality, stability, and release of the active agents to ensure the highest possible availability and bioactivity of the incorporated bioactive compounds.

Chapter 3 Optimization of β-CD Complexes

3.1 Overview

In this chapter, a method is developed to optimize the synthesis of β-CD complexes with thymol, carvacrol and linalool. This includes the materials used for this research including standards and reagents, reference materials, sample materials (preparation, extraction protocols, etc.) and the preparation methods through to the analytical instrumentation. This chapter has been published in the *Journal of Microencapsulation*, and the publication is presented in Appendix A.

3.2 Introduction

Microencapsulation techniques are used in various industries to entrap a wide range of substances such as EOs, oleoresins, aroma, and flavour mixtures (Balasubramani *et al.*, 2015, Michalska *et al.*, 2017). The encapsulation of these substances in an external matrix offers many advantages including: providing stabilization and protection against oxidation and thermal or light-induced decomposition (Wang *et al.*, 2014); reducing evaporation losses of volatile substances; converting liquid substances to solids to facilitate handling or separation (Reineccius, 1995, Desai and Hyun Jin, 2005); preventing discolouration; providing thickening; improving bioavailability (Xiao *et al.*, 2014a, Lima *et al.*, 2016); and masking unpleasant aromas or flavours in food applications (Risch, 1995, Astray *et al.*, 2009). The most common encapsulation materials are the bio-derived cyclodextrins (CDs) such as α-, β- and γ-CDs, with 80–90% of all CDs primarily used in pharmaceutical and food-related applications (Astray *et al.*, 2009, Nieddu *et al.*, 2014, Pinho *et al.*, 2014). Given the high costs required to purify α- and γ-CDs, more than 97% of the CDs used are β-CDs.

The typical preparation of β-CD complexes involves the use of co-solvents such as ethanol, methanol, acetone, or dichloromethane to aid in the dissolution of non-polar active agents and/or to dissolve the β-CD (Szente and Szejtli, 1986, Bhandari *et al.*, 1998, Chen *et al.*, 2007, Nunes and Mercadante, 2007, López and Pascual-Villalobos, 2010, Lopez *et al.*, 2012, Gomes *et al.*, 2014, Mangolim *et al.*, 2014, Tang *et al.*, 2015). Although β-CD is not soluble in 100% ethanol (Coleman *et al.*, 1993), mixtures of ethanol

with water are commonly used during β-CD complex preparation. However, many studies using ethanol report low inclusion efficiencies (IE's) and examples of relatively low or varied IE's in β-CD include: 31% for linalool (López and Pascual-Villalobos, 2010); 6% (Paramera *et al.*, 2011) and 74% (Mangolim *et al.*, 2014) for curcumin; 53% and 62% for red bell pepper extract prepared by magnetic stirring and ultrasonic mixing respectively (Gomes *et al.*, 2014); and 35% for 2-nonanone (Abarca *et al.*, 2016). The encapsulation of curcumin has also been studied using other co-solvents including methanol (Paramera *et al.*, 2011) and acetone (Mangolim *et al.*, 2014) resulting in maximum IEs of 17% and 14% respectively. Dichloromethane has also been used to dissolve lycopene with a resulting IE of 50% (Nunes and Mercadante, 2007) and a mixture of dichloromethane/acetone has been used to dissolve astaxanthin with an IE of 49% (Chen *et al.*, 2007). In each of these studies, there were no systematic investigations on the effect of optimising the level of co-solvent on the IE, the yield, or the quantification analysis.

The use of AM additives in food packaging films is a popular alternative to the direct addition of conventional preservatives to food products (Suppakul *et al.*, 2003, Muriel-Galet *et al.*, 2012b, Makwana *et al.*, 2014, Sung *et al.*, 2014, Nerin *et al.*, 2016b). Of the wide range of natural AM agents utilized in food packaging, EOEs are prevalent due to their availability and, in many cases, broad-spectrum AM activity (Gutierrez *et al.*, 2008, Rodríguez *et al.*, 2014). Examples of EOEs include thymol, carvacrol and linalool, which are hydrophobic AM agents extracted from basil and oregano plants. These active components have shown significant promise in AM packaging applications (Kuorwel *et al.*, 2011b), and thymol, for example, has been shown to exhibit strong antibacterial, AM and some AO activity (Suhr and Nielsen, 2003, Mourtzinos *et al.*, 2008). Carvacrol and linalool have also been shown to possess antifungal, insecticidal, AO, and antiparasitic properties (Lopez *et al.*, 2012, Ramos *et al.*, 2012).

The addition of the abovementioned and other AM agents directly into packaging films is limited however, primarily due to the volatile nature of EOEs which can result in unacceptable losses during the production of AM films (Suppakul *et al.*, 2011a, Ramos *et al.*, 2012). Microencapsulation of these AM agents using β-CD offers a promising technique that can protect the AM agents during processing to significantly reduce these losses (Guarda *et al.*, 2011, Tao *et al.*, 2014). Several methods have been developed to prepare β-CD inclusion complexes including: co-precipitation, slurry, paste, and dry

mixing (Hedges, 1998, Wang *et al.*, 2011a); kneading, freeze-drying and spray-drying (Marques, 2010); and neutralization (Choi *et al.*, 2001). The method of co-precipitation is one of the simplest techniques to prepare inclusion complexes, however, large-scale production is limited due to the size of the reaction tanks required and wastewater disposal considerations (Hedges, 1998). Although the encapsulation of thymol, carvacrol, and linalool using various methods have been previously reported (Ponce Cevallos *et al.*, 2010, Bonetti *et al.*, 2015, Guimarães *et al.*, 2015), these studies have not quantified the IE's of the processes.

Improving the IE, preventing rapid losses of guest materials during storage, and minimizing the amount of un-encapsulated oil at the surface of wall materials are essential to producing high-quality inclusion complexes with maximum yields (Choi *et al.*, 2001, Jafari *et al.*, 2008). Volatile liquids, however, may also be susceptible to evaporation losses during the preparation of their inclusion complexes (Guarda *et al.*, 2011). It is therefore important to investigate factors influencing the efficacy of any technique in order to optimize the process of encapsulation (Serafini *et al.*, 2012). In view of the wide range of existing applications of inclusion complexes, the potential use of these to overcome the volatility problems associated with incorporating natural AM agents in food packaging materials, and the need for improved and efficient methods of complex preparation that deliver higher yields with greater IE, the present study was undertaken. The primary aims were to: (i) investigate the experimental parameters that affect the IE and the yield of β-CD inclusion complexes with the AM agents: thymol, carvacrol, and linalool, and (ii) hence produce the target complexes that are suitable for food packaging applications under a co-precipitation method that is more efficient than existing methods.

3.3 Materials and Methods

3.3.1 Materials

Ethanol (absolute) was purchased from Merck Australia and the following chemicals were purchased from Sigma-Aldrich Australia: isooctane (anhydrous, 99.8%), beta-cyclodextrin (β-CD, 98.5% purity, 1.5% (w/w) water content), the AM agents: thymol (99.5% purity), carvacrol (98% purity) and linalool (97% purity). Milli-Q water was used in all preparations.

3.3.2 Pre-Optimized Thymol/β-CD Complex Preparation

The pre-optimized preparation of a thymol/β-CD complex was performed according to a precipitation method reported previously (Szente and Szejtli, 1986, Bhandari *et al.*, 1998, Bhandari *et al.*, 1999) with minor changes to the drying method and the ratio of the components. A quantity of β-CD (3.8352 g, 3.33 mmol) was dissolved in 38 mL of a 50% (v/v) ethanol/water mixture at 55°C. Thymol (0.5000 g, 3.33 mmol) was dissolved in absolute ethanol and added with continuous stirring to the warm β-CD solution at a 10% (w/v) ratio and the solution was covered. After the addition of thymol, the heating was discontinued and the mixture was cooled to room temperature (RT) with stirring for 4 h. The solution was then further cooled to 4°C and maintained at this temperature for 24 h. The complex was collected by vacuum filtration and was dried for 5-9 days at room temperature.

3.3.3 Electrospray Ionization Mass Spectrometry Analysis

A mass of 0.1 g of thymol/β-CD complex was dissolved in 25 mL of water followed by the addition of 25 mL of ethanol with stirring. A Thermo Electron Corporation LCQ-DECA-XP-MAX electrospray ionization mass spectrometry (ES/MS) instrument was used to record the mass spectrum by the direct diffusion method. The ESI probe voltage was 3 kV and samples were delivered at a flow rate of 30 μL min^{-1} *via* a syringe pump. The capillary temperature was maintained at 260°C and no sheath or desolvation gas was used.

3.3.4 Gas Chromatographic Analysis

Gas chromatographic analyses were performed using a Varian Star 3400-CX gas chromatograph (GC) equipped with a fused silica capillary column DB-5 (30 m × 0.32 mm i.d.; film thickness: 0.25 μm; J & W Scientific, USA). The GC was operated using the following conditions: injection volume: 1.0 μL; initial column temperature: 80°C; heating rate: 5°C min^{-1} up to 180°C; kept at this temperature for an additional 1 min; injector temperature, 250°C; split ratio: 1:60; FID detector temperature: 300°C; and carrier gas: nitrogen.

3.3.5 Yield, Inclusion Efficiency and Un-Complexed AM Agent

Yield of Complex

The yield of the complex (expressed as a mass percentage) was calculated as the ratio of the recovered mass of the dried complex to the theoretically expected mass for 100% conversion, based on the mass of the materials used (i.e. AM agent plus β-CD) in the synthesis.

Inclusion Efficiency

The total mass of AM agent associated with the β-CD complex comprises the encapsulated AM agent and any AM agent that may be adsorbed on the exterior surface of the β-CD wall. The total mass of AM agent was determined by extraction in ethanol that is a solvent capable of fully extracting the complexed AM agent from the β-CD as well as any AM agent adsorbed to the surface of the wall.

To determine the IE, a mass of *ca.* 40 mg of the complex was placed in a round-bottom flask and 25 mL of ethanol was added. The flask was sealed and its contents stirred for 150 min. A volume of 5 mL of the extract was filtered using a Phenex RC 0.45 µL syringe filter and an aliquot of the filtrate was directly analysed by GC under the conditions described previously. The content of AM agent in the extract was determined using a standard calibration curve and the results are expressed as an average of six extractions along with the corresponding standard deviation. The IE was calculated using the following equation:

$$\text{IE}\ (\%) = 100 \times (m_{\text{complex}} / m_{\text{theoretical}}) \qquad (3.1)$$

where m_{complex} is the mass of AM agent extracted from the inclusion complex and $m_{\text{theoretical}}$ is the theoretical mass of AM agent for the given mass of complex assuming a 1:1 guest/wall ratio and complete complexation. The value of $m_{\text{theoretical}}$, assuming a 1:1 guest/wall mole ratio, is determined using:

$$m_{\text{theoretical}} = m_{\text{complex}} \times \text{MW}_{\text{thymol}}/(\text{MW}_{\beta\text{-CD}} + \text{MW}_{\text{thymol}}) \qquad (3.2)$$

where $\text{MW}_{\text{thymol}}$ and $\text{MW}_{\beta\text{-CD}}$ are the molecular weights of thymol and β-CD respectively. The assumption of complete complexation excludes guest molecules being entrapped

among adjacent β-CD monomers, dimers or polymers, and guest molecules being adsorbed on the surface (Loftsson *et al.*, 2002, Loftsson *et al.*, 2004).

To quantify the mass of AM agent that was adsorbed to the surface of the β-CD, a mass of 0.1711 g of the complex was firstly mixed with 3 mL of isooctane in a sample vial. Isooctane is sufficiently non-polar so as not to dissolve the β-CD and thereby extract the AM agent that is complexed. The vial was capped and thoroughly agitated for 5 min using a vortex mixer set at high speed. The complex was then filtered under vacuum and an aliquot of the filtrate was directly analysed using GC under the conditions described previously. Extractions were performed in triplicate and the amount of adsorbed AM agent was calculated using a standard calibration curve. The amount of AM agent (AO), assumed to be present as an adsorbed oil, was calculated using the following equation:

$$\text{AO}\ (\%) = 100 \times (m_{\text{isooctane}} / m_{\text{theoretical}}) \qquad (3.3)$$

where $m_{\text{isooctane}}$ is the mass of AM agent detected in the isooctane solvent and $m_{\text{theoretical}}$ is the theoretical mass of AM agent assuming a 1:1 guest/wall ratio (see Equation (3.2)).

Quantification of Un-Complexed Thymol

In order to determine the amount of AM agent remaining in the filtrate following the initial synthesis step, a volume of 2 mL of filtrate was placed in a 15mL vial. Isooctane (5 mL) was added and the vial capped and gently shaken to facilitate mixing before the organic phase was separated and placed in a volumetric flask. The filtrate was washed further with isooctane and the organic phase (total of 10 mL) collected. An aliquot of the collected organic phase was directly analysed using GC in accordance with the method described previously.

The amount of un-complexed agent (UA) was calculated as the ratio of recovered AM agent in the total volume of the filtrate to the amount of AM agent originally added to the reaction mixture:

$$\text{UA}\ (\%) = 100 \times (m_{\text{organic}} / m_{\text{added}}) \qquad (3.4)$$

where m_{organic} is the mass of AM agent detected in the organic phase and m_{added} is

the mass of AM agent originally added to the reaction mixture. The value of m_{added} varies from $m_{theoretical}$ in that m_{added} is the experimental mass of the agent added to the mixture. The analysis was performed in triplicate and the amount of AM agent was calculated using a standard calibration curve.

3.3.6 Synthesis Optimization Experiments

A series of optimization experiments was performed in order to maximize the yield of the complex and IE of thymol. A summary of the experimental conditions is presented in Table 3.1.

Table 3.1: Experimental parameters for optimizing the β-CD complexes

Solvent composition: ethanol/water/ % (v/v)	Initial reaction temperature /°C	Total solvent volume/mL	Other experimental conditions
50/50	55	204	Mix reactants at 55°C, cool to RT with stirring over 4 h period, cool to 4°C and maintain at this temperature for 24 h
60/40			
70/30			
80/20			
90/10			
92.5/7.5			
95/5			
100/0			
97.5/2.5	55	204	
	60		
	80		
99.5/0.5	55	204	Mix reactants at 55°C, maintain at 55°C with stirring for 4 h, cool to 4°C and maintain at this temperature for 24 h
99.5/0.5	55	204	Mix reactants at 55°C, cool to RT with stirring over 4 h period, cool to 4°C and maintain at this temperature for 24 h
		122	
		102	
		60	
100/0	55	60	Mix reactants at 55°C, maintain at 55°C with stirring for 4 h, cool to 26 ± 1°C with stirring over 4 h, cool to 4°C and maintain for 24 h

In these experiments, the effect of the concentration of ethanol co-solvent on the formation of the complex was investigated using a constant total solvent volume of 204 mL. The preparation of the complex was achieved by firstly dissolving β-CD (3.8352 g, 3.33 mmol) in water at 55°C. In each case, thymol (0.5000 g, 3.33 mmol) was dissolved in different volumes of ethanol and added to the β-CD solution. In this series of

experiments, the final ethanol/water ratio was varied from 0-50% (v/v) and the remaining preparation conditions were as described previously.

To optimize the reaction conditions with respect to the heating and cooling regimes, the effect of using higher temperatures during the synthesis of the complex was also investigated with complexes prepared at 55, 60 and 80°C. In each of these experiments, the concentration of ethanol and the total volume of the reaction mixture were held constant at 2.5% (v/v) and 204 mL respectively. The remaining preparation conditions were as described above. In addition, the effect of maintaining the temperature at 55°C for 4 h under continuous stirring rather than removing the heat source once the components were mixed was explored. The remaining preparation conditions for these experiments were also as described previously.

The effect of the total volume of the ethanol/water solvent system was investigated by successively reducing the total solvent volume from 204 mL to 60 mL in separate experiments. The ethanol concentration was held constant at 0.5% (v/v) and all other conditions were the same as those described previously.

The effect of extending the time allowed for the reaction to be completed under continuous stirring on the yield of complex and the IE was also investigated. In these experiments (not summarized in Table 1), the stirring was continued for a further 48 h after the heating was discontinued and the temperature of the reaction mixture reached 26 ± 1°C. The ethanol concentration was held constant at 0.5% (v/v) with all other conditions being the same as those described above.

3.3.7 Synthesis of Complexes Under Optimized Conditions

Beta-cyclodextrin complexes encapsulating thymol as well as carvacrol and linalool were each prepared under the optimized conditions as determined from the previous experiments. In these cases, β-CD (3.8352 g, 3.33 mmol) was reacted with the AM agents: thymol, carvacrol, and linalool in a 1:1 mole ratio in each case. The β-CD was dissolved in 60 mL of water at 55°C to which the appropriate mass of AM agent was added and the reaction mixture vigorously stirred. The solutions were covered and maintained at 55°C for 4 h with continuous stirring after which the heating was discontinued and the mixture was continuously stirred for a further 4-5 h until the

temperature decreased to 26 ± 1°C. The mixture was then cooled to 4°C, maintained at this temperature for 24 h and the precipitated complexes were recovered by vacuum filtration. The complexes were dried in a vacuum desiccator for 1 h after which the vacuum was removed. The complexes remained in the desiccator for a further 12 h to remove any residual water and until constant masses were attained. The yield, IE, absorbed AM agent on the surface of the β-CD, and amount of un-complexed agent were determined in accordance with the methods described above.

3.3.8 Data Analysis

All experiments were performed in triplicate with the exception of the yield which was performed in duplicate. Error bars and errors presented in the tables and text are based on the standard deviation from the mean.

3.4 Results and Discussion

3.4.1 Pre-Optimized Complex Yield and Inclusion Efficiency

The preparation of the thymol/β-CD complex in accordance with a previously reported precipitation method (Szente and Szejtli, 1986, Bhandari *et al.*, 1998, Bhandari *et al.*, 1999) resulted in a final product yield of 77.0%. In the current study, it was observed that under the same synthesis conditions, no precipitate formed after the addition of thymol at 55°C despite having used the minimum amount of solvent during the preparation. The precipitate started to form only at temperatures below 40°C and the precipitate that was eventually recovered was analysed using ES/MS revealing a low-intensity peak corresponding to the 1:1 mole ratio β-CD complex (m/z = 1284.2) as shown in Figure 3.1. Peaks corresponding to β-CD and free thymol were also detected in the spectrum. The 1:1 mole ratio observed for the thymol/β-CD in the current study is consistent with the work of Bethanis *et al.* (2013) who determined a 1:1 mole ratio of thymol/β-CD in the solid state using x-ray crystallography.

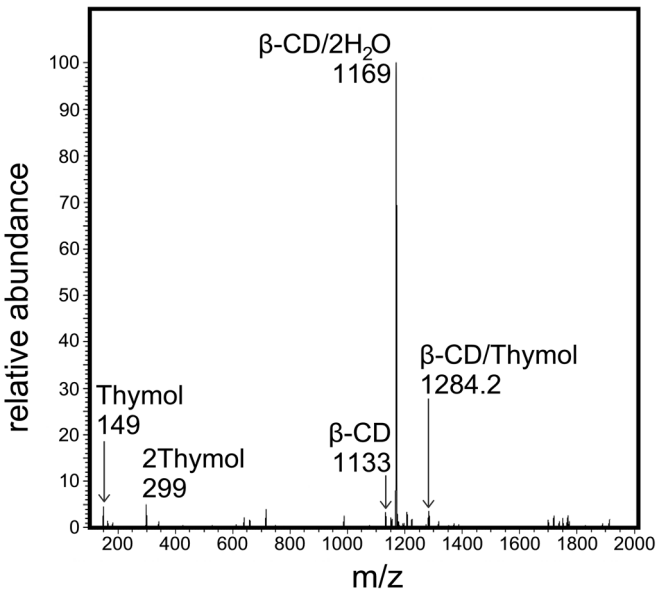


Figure 3.1: Example of thymol/β-CD complex ES mass spectrum prepared without ethanol confirming the presence of the inclusion complex at m/z = 1284.2

Although the qualitative analysis using ES/MS indicates that thymol was complexed with β-CD, the results do not enable the efficiency of the process to be determined and so a quantitative analysis using GC was used to assess the IE. In this study the IE as defined by Gomes *et al.* (2014) was used as this provides the most accurate calculation of the percentage of the guest molecules that are complexed within the β-CD cavity.

Other authors have defined and/or used terms such as "complexation efficiency" (Mangolim *et al.*, 2014), and "entrapment efficiency" (Hill *et al.*, 2013) to characterize the efficiency with which complexation has been achieved and it is important to note that these quantities are not directly comparable to each other or the "inclusion efficiency" as calculated in the current study. In this study, thymol was extracted from the thymol/β-CD complex using ethanol and the pre-optimized IE was 79.6 ± 0.5% (w/w) of the maximum that would be expected if each β-CD molecule formed a complex with one molecule of the AM agent.

3.4.2 Optimized Preparation of Complex

Effect of Ethanol Co-Solvent

The effect of varying the concentration of the co-solvent ethanol on the yield and IE was investigated over the co-solvent range of 0-50% (v/v) ethanol/water. It was observed that for syntheses using concentrations of ethanol greater than 20% (v/v), no precipitate was formed unless the reaction mixture was cooled overnight at 4°C. Syntheses using lower amounts of ethanol resulted in a precipitate even at room temperature. As shown in Figure 3.2, the yield of complex decreased as the amount of ethanol was increased in the reaction mixture. This suggests that the ethanol content of the reaction mixture significantly influences the yield of complex. This is most likely due to the complex being more soluble in the reaction mixture containing higher levels of ethanol; it therefore remains in solution and is not recovered, leading to lower isolated yields.

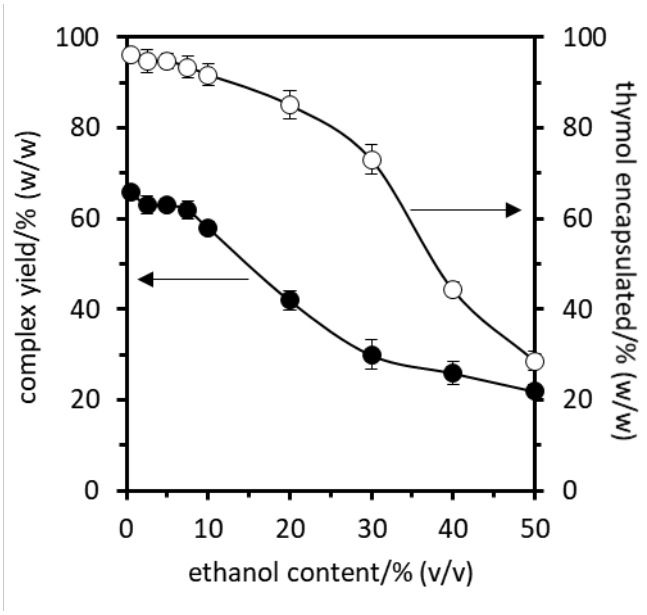


Figure 3.2: Effect of co-solvent composition on yield and IE (a) yield of thymol/β-CD complex (filled circles) (n=2) and (b) IE of thymol in the complex (open circles) (n=3).

Figure 3.2 also shows that the lower the amount of ethanol used in the synthesis, the higher the thymol/β-CD IE where the product prepared with 0.5% (v/v) ethanol has the greatest thymol IE of 96.2 ± 1.0% (w/w). These findings are consistent with the notion

that ethanol is capable of disrupting the non-covalent bonding of thymol to the β-CD structure (Kalathenos and Russell, 2003). Hydrophobicity is the main driving force for guest/β-CD inclusion complexation and so the hydrophobic interactions necessary for encapsulation are stronger in polar aqueous environments compared with less polar solvent media such as ethanol/water mixtures. In the latter case, the solubilization of thymol by ethanol is more favourable than the interaction of thymol with β-CD. In a similar study, the maximum complexation efficiency of curcumin in β-CD prepared by co-precipitation in ethanol/water using a molar ratio of 1:2 was found to be 74% (Mangolim *et al.*, 2014). This low efficiency may be attributed to the combined use of ethanol and rotary evaporation to remove the co-solvent before cooling the mixture. However, it is important to note that in the latter study the authors calculated the efficiency based on the mass of the complexed curcumin to the total mass of curcumin added initially.

The filtrate from the syntheses in the present study was also analysed to investigate the amount of thymol that remained un-complexed with and unbound to the surface of the β-CD. Figure 3.3 shows plots of: (i) the percentage of the total thymol used in the synthesis that is adsorbed on or complexed with the β-CD and (ii) that which is un-complexed and remains in the filtrate, as a function of the percentage of ethanol in the solvent system that was used in the synthesis of the complex.

The percentage of thymol that was adsorbed/complexed (see Figure 3.3) was determined by quantitative GC analysis thymol in an ethanol extract of the prepared complex and the percentage of thymol in the filtrate was also obtained by GC analysis. Figure 3.3 also shows a mass balance plot in the form of the sum of the two other percentages. The results are consistent with the previous observation where reaction mixtures with lower ethanol content resulted in higher IE's of thymol in the β-CD wall. Interestingly, the mass balance of the total thymol in the filtrate and that recovered from the β-CD complex suggests that some loss of thymol from the system occurred (see Figure 3.3). The loss also increased with increasing ethanol content in the synthesis solvent and this may be attributed to evaporation of thymol during the heating stage, or some of the thymol having remained in the aqueous phase in the form of the complex following the extraction process. In the case of the latter, the addition of isooctane was observed to result in the formation of a gel layer that may have trapped some of the thymol/β-CD

complex. Some of the thymol adsorbed on the surface of the β-CD is also expected to have been lost by evaporation during the drying step (Bhandari *et al.*, 1998).

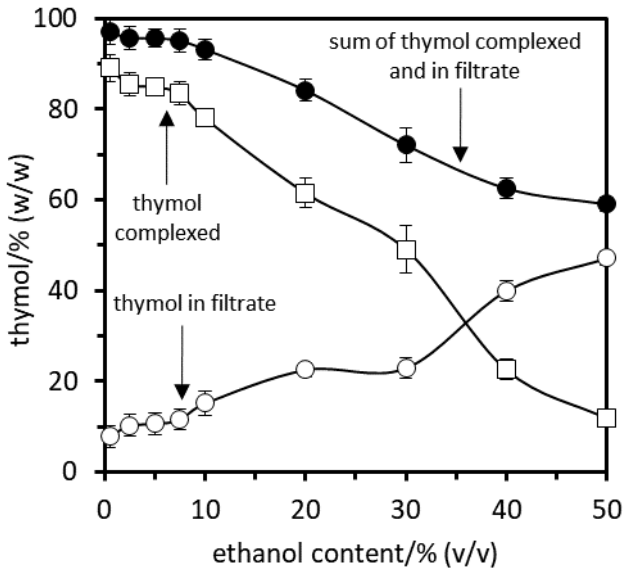


Figure 3.3: Effect of co-solvent composition on complex formation (a) percentage of the total thymol adsorbed/complexed with the β-CD (□), (b) percentage of un-complexed thymol in filtrate (○) and (c) sum of (a) and (b) (●) (n=3)

The thymol/β-CD complex was also prepared in the absence of ethanol to investigate the possibility of preparing the complex without using a co-solvent. It was found that without ethanol, the IE and yield were 99.8 ± 1.0% (w/w) and 87% (w/w) respectively. This suggests that in some cases the aqueous solubility of hydrophobic agents such as thymol may preclude the need for a co-solvent to aid dissolution (Chen *et al.*, 2007). Nonetheless, it is expected that this will not be the case in general and so it will usually be necessary to use small amounts of co-solvent to aid in the formation of the inclusion complex.

The amount of thymol absorbed on the surface of β-CD for samples prepared using only water as the solvent was determined by washing the complex with isooctane. Analysis of thymol in the washing solutions showed that only 0.26 ± 0.01% (w/w) thymol was adsorbed to the surface of the β-CD. This also confirms that isooctane did not extract the thymol from the β-CD complex and that isooctane is a good choice of solvent for washing the complex during the clean-up process.

Effects of Temperature, Reaction Time and Total Solvent Volume

The yield of the complex and IE of thymol at 55, 60 and 80°C using 2.5% (v/v) ethanol in the reaction mixture were also investigated. It is well known that the solubility limit of β-CD is up to 10 mM under ambient conditions (Marcolino *et al.*, 2011). It is expected that the thymol will be more soluble in aqueous solutions at the higher temperatures and this increased solubility may lead to greater IE's due to an increased concentration of thymol in solution (Mangolim *et al.*, 2014). The yields and IE's obtained at 60 and 80°C were found to be close to those obtained at 55°C which were 63% (w/w) and 94.7 ± 0.9% (w/w) respectively. This suggests that any difference in the solubility of the thymol at the increased temperatures does not affect the IE and therefore conducting the synthesis at higher reaction temperatures has no significant effect on the formation of the complex. Temperatures below 55°C were not explored as it was observed that β-CD is not completely soluble in the solvent system at these temperatures.

Under the pre-optimized conditions described above, the reaction mixture was stirred for 4-5 h after the heating was discontinued. To determine if an extended length of time may improve the yield and/or IE, a sample prepared using 0.5% (v/v) ethanol was reacted for a total of 48 h with continuous stirring. The extended time that allowed for the dissolution of thymol in the system resulted in no significant difference in the IE or the yield. In addition, the total volume of the ethanol/water co-solvent was varied to assess its effect on the yield. Several complexes of β-CD with thymol were prepared using smaller total solvent volumes but maintaining the level of ethanol at 0.5% (v/v). Decreasing the total solvent volume from 204 mL to 60 mL improved the yield from 66±1 to 87±3% (w/w) and it was found that this increase was achieved without a compromise in the IE.

The minimum total volume of solvent that can be used is clearly dependent on the solubility of β-CD in the solvent system at the temperature used for the synthesis. Under the conditions used in the current study the minimum total volume was found to be 60 mL. Decreasing the solvent volume increases the amount of solute in the solution thereby producing more precipitate, resulting in a higher yield. Moreover, in the preparation of these complexes, as the total solvent volume is decreased keeping the amounts of thymol and β-CD the same, the concentrations of both thymol and β-CD will be subsequently

increased, noting that the concentration of free thymol cannot be increased as much as that of β-CD due to its very low aqueous solubility. For a 1:1 inclusion complex of specific association constant *K*, the concentration of complex is given by the following:

$$[\text{complex}] = K[\text{free guest}][\beta\text{-CD}] \qquad (3.5)$$

where [complex] is the concentration of the complex, [free guest] is the concentration of the free guest, and [β-CD] is the concentration of the free β-CD. Thus, it is expected that decreasing the total solvent volume, and thereby increasing the free guest and β-CD concentrations, will cause the dynamic equilibrium to be moved towards the direction of complexation resulting in a higher IE under these conditions.

3.4.3 Optimized Inclusion of Thymol, Carvacrol and Linalool

The optimum synthesis conditions for the inclusion of thymol in β-CD were applied to the inclusion of carvacrol and linalool in β-CD and a summary of the results is shown in Table 3.2. These parameters were calculated in accordance with the definitions given above in equations (3.1) to (3.3).

Table 3.2: Optimized yield and inclusion efficiency for β-CD complexes with uncomplexed AM agent and amount of AM agent adsorbed

AM Agent	Complex Yield/% (w/w)	Inclusion efficiency IE/% (w/w)	Overall IE/%	Amount of uncomplexed agent (UA)/ % (w/w)	Amount of adsorbed AM (AO)/ % (w/w)
Thymol	87 ± 3	99.8 ± 1.0	86.8	8.9 ± 1.8	0.27 ± 0.01
Carvacrol	84 ± 2	99.6 ± 0.9	83.7	8.4 ± 1.0	0.35 ± 0.09
Linalool	86 ± 2	99.3 ± 1.1	85.4	5.0 ± 0.3	0.30 ± 0.03

Similar to the case of thymol, both carvacrol and linalool were successfully complexed with β-CD at high yield using the optimized conditions with IE's close to 100% (w/w) suggesting this method is highly feasible for the inclusion of these agents. Guarda *et al.* (2011) reported the percentages of microencapsulated agents thymol and carvacrol in gum Arabic *via* oil-in-water emulsions to be 98% and 91% respectively, which are slightly lower than the efficiencies achieved in the present study.

Evaluation of the AM agents adsorbed onto the surface of the β-CD indicated that surface adsorptions of carvacrol and linalool were very small as was observed in the case of thymol. Analysis of the filtrates showed the quantities of carvacrol and linalool

remaining in solution after complexation were lower than that of thymol (see Table 3.2) which may be a result of the physical state of the agents with carvacrol and linalool being liquids at room temperature whereas thymol is a semi-crystalline solid. In the present study, the maximum theoretical IE for thymol was found to be 13.23 g of thymol per 100 g β-CD. The maximum IE that was experimentally achieved was 13.20 g per 100 g of β-CD and was achieved without the use of ethanol as a co-solvent. The high IE of thymol, and indeed the high IE's of carvacrol and linalool, obtained in the present study may be the result of two important factors. Firstly, the use of water only as the solvent produced both higher yields and IE's. Secondly, maintaining stirring of the mixture at 55°C for 4 h may have contributed to improved IE's of the agents.

The IE's obtained for each of these complexes is close to 100% (w/w) indicating that in all cases studied a 1:1 mole ratio of guest/wall exists. In the cases of the thymol/β-CD and linalool/β-CD complexes these results are confirmed by the respective x-ray crystallographic studies of Bethanis *et al.* (2013) who reported a 1:1 mole ratio for thymol/β-CD and Ceborska (2016) who reported a 2:2 molar ratio for the (-)-linalool/β-CD dimer. It is interesting to note, however, that the X-ray crystallographic study by Bethanis *et al.* (2013) suggests that a 1:2 carvacrol/β-CD ratio exists in the solid state where the complex was produced using a guest/wall molar ratio of 1.5:1. Under the synthesis conditions used in the current work, if a complex was formed having a 1:2 mole ratio of guest/wall then that would result in an observed IE of only 50% (w/w) so clearly a 1:2 complex was not obtained in the present study.

3.5 Summary

The natural AM agent thymol was successfully complexed with β-CD using the method of co-precipitation. The concentration of ethanol in the solution significantly influenced the complex formation with higher ethanol levels resulting in lower yields and lower IE's. The optimum conditions comprised a reaction temperature of 55°C, a stirring time of 4-5 h after the complex had formed, and a reduced total solvent volume. Using these optimized parameters, the average yields of the AM/β-CD complexes were 87, 84, and 86% (w/w) for thymol, carvacrol, and linalool respectively. For each complex, an IE that was close to 100% (w/w) was obtained suggesting that this optimized method is highly feasible for the complexation of these agents with β-CD for use in various applications.

Chapter 4 Thermal Analysis of β-CD Complexes

4.1 Overview

In the previous chapter, the optimization of β-CD complexes with thymol, carvacrol and linalool was presented. In this chapter, a novel technique for evaluating the stability of the formed complexes using thermogravimetric analysis with regard to the activation energies required to facilitate the release of the AM agents from the β-CD cavity is presented.

4.2 Introduction

Active food packaging materials containing additives such as AM and AO compounds are becoming increasingly popular and are widely viewed as an alternative to the use of many conventional food preservatives (Pezo *et al.*, 2006, Muriel-Galet *et al.*, 2012b, Makwana *et al.*, 2014, Sung *et al.*, 2014). Natural additives are generally preferred with EOs and EOEs among the more common, readily available substances that have been shown to impart effective AM activity (Rodríguez *et al.*, 2014). Basil, oregano and thyme are examples of culinary herbs that contain valuable EOEs including carvacrol, linalool, and thymol (Guarda *et al.*, 2011, Lopez *et al.*, 2012, Bethanis *et al.*, 2013). These EOEs in particular have been shown to be effective additives in packaging materials (Suppakul *et al.*, 2011a, Ramos *et al.*, 2012, Tawakkal *et al.*, 2016a).

The production of packaging films containing these additives generally requires some type of thermal extrusion which can result in unacceptable losses of the volatile EOEs (Kuorwel *et al.*, 2011b). One solution to this problem is the use of microencapsulation to facilitate the incorporation of the active AM compounds that would otherwise be depleted through thermal processing (Suppakul *et al.*, 2003, Makwana *et al.*, 2014). Bio-derived β-CDs are among the more common materials used for encapsulation with chemically modified derivatives such as hydroxypropyl-β-CDs becoming increasingly popular due to the improved water solubility promoted by the modification (Gould and Scott, 2005).

The thermal stability of encapsulated compounds used in packaging films is an important physicochemical property, particularly in the case of extruded films where high temperature processes are employed. Characterizing the thermal stability is therefore critical in the development of effective packaging materials and DSC and TG analysis are typical methods of thermal characterization (Hill *et al.*, 2013, Varganici *et al.*, 2015, Abarca *et al.*, 2016, Sun *et al.*, 2017). Encapsulated complexes will generally display mass losses in a TG curve over several distinct stages that are associated with processes such as the loss of water, loss of the volatile agent, and decomposition of the β-CD molecule (Abarca *et al.*, 2016). Typically, TG mass loss curves are reported to confirm the formation of complexes and to quantify the mass loss due to the separate processes (Giordano *et al.*, 2001, Zhu and Ping, 2014). However, there is considerably more information that can be extracted from TG curves than is usually reported including kinetic models and associated parameters (Reverte *et al.*, 2007). To this end, the current paper explores the kinetics and decomposition of three inclusion complexes containing EOEs that are proposed for use in AM packaging applications.

4.3 Materials and Methods

4.3.1 Materials

The following chemicals were purchased from Sigma-Aldrich Australia: β-CD (≥ 97% purity, 1.5% water content), carvacrol (98% purity), linalool (97% purity) and thymol (99.5% purity). Milli-Q water was used for the preparation of all complexes.

4.3.2 Complex Preparation

The inclusion complexes of β-CD with either carvacrol, linalool or thymol were prepared by the precipitation method. Briefly, β-CD (3.8352 g, 98.5% purity, 3.33 mmol) was dissolved in 60 mL of water and heated to 60°C on a hot plate. The solution of β-CD was then added to the carvacrol, linalool or thymol in a 1:1 mole ratio in each case. The complex was covered and stirred vigorously at 55°C for 4 h after which the heating was discontinued the complex was continuously stirred for a further 4-5 h until the temperature decreased to 26 ± 1°C. The complex was then cooled to 4°C and maintained at this temperature for 24 h. The precipitated complexes were recovered by vacuum filtration and dried in a vacuum desiccator for 1 h. The complexes were kept in the

desiccator for a further 12 h without vacuum to remove any residual water and until a constant mass was attained.

4.3.3 Thermogravimetric Analysis

Thermogravimetric analyses of the β-CD complexes were performed using a Mettler Toledo TGA/DSC1 STARe system TG analyser (Mettler Toledo, Melbourne, Australia). For all experiments, the samples were heated from 25 to 600°C at a heating rate of 10°C min^{-1} under a nitrogen atmosphere at a flow rate of 50 mL min^{-1}.

4.4 Results and Discussion

Figure 4.1 shows the thermograms of un-complexed (neat) β-CD and β-CD complexes with carvacrol (β-CD-Car), linalool (β-CD-Lin) and thymol (β-CD-Thy) where the mass has been normalized in each case for comparison. In the case of the neat β-CD there is an initial mass loss due to adsorbed water followed by a relatively flat response until the onset of decomposition that occurs at *ca*. 300°C (Abarca *et al.*, 2016). The onset of the major decomposition stage of the β-CD-Lin complex appears at a slightly lower temperature compared to the neat β-CD suggesting that the presence of the complexed linalool destabilizes the β-CD (Sbarcea *et al.*, 2016).

The effect of the guest molecules carvacrol, linalool and thymol on the temperature at which the onset of the major decomposition step occurs was investigated by determining the temperature corresponding to an arbitrarily-defined low degree of conversion, $\alpha = 0.1$, of each of the complexes and comparing these values with that obtained for neat β-CD. Figure 4.2 shows the typical plots from which these temperatures were determined. The onset temperatures determined from three separate measurements as such were found to be 306.9 ± 0.2, 308.9 ± 0.9 and 306.2 ± 0.2°C for the β-CD-Car, β-CD-Lin and β-CD-Thy complexes respectively. These are all significantly lower than the onset temperature for the neat β-CD which was found to be 317.7 ± 0.3°C from two separate runs. These results collectively suggest that the presence of the guest molecules has a destabilizing effect on the β-CD and thus confirms the observation made in the case of β-CD-Lin.

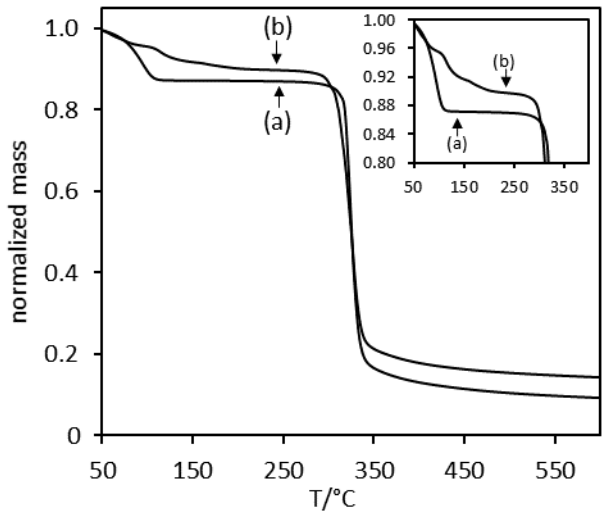


Figure 4.1: Mass-normalized thermograms of β-CD complexes (a) β-CD and (b) β-CD-Lin complex. Inset: Enlarged portion of the mass-normalized thermograms of: (a) β-CD and (b) β-CD-Lin complex in the region 25 to 310°C

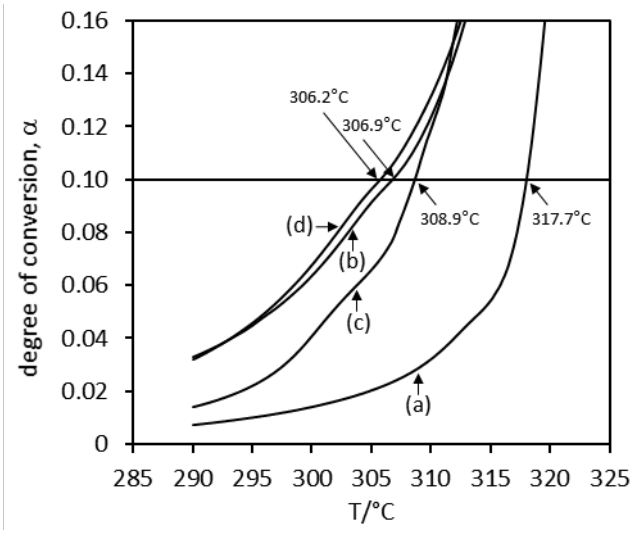


Figure 4.2: Degree of conversion, α, for β-CD complexes in the temperature range 290 to 320°C under for the thermal decomposition of: (a) β-CD, (b) β-CD-Car, (c) β-CD-Lin and (d) β-CD-Thy complexes

Close inspection and analysis of the thermograms shown in the inset of Figure 1 indicates that in the temperature range between *ca*. 25 to 300°C, the β-CD-Lin complex undergoes a mass loss of $\Delta m = 7.4 \pm 0.3$ % and that this appears to occur in various stages. A mass loss as such is not observed in the case of the neat β-CD suggesting that the mass

loss from the β-CD-Lin complex is primarily due to the loss of the linalool guest species. The staged loss of the guest molecule in the incipient stage of the decomposition further suggests the possibility of there being different sites either within or on the surface of the β-CD at which the linalool molecules may be located and where there may exist different chemical interactions between the wall and guest molecules (Poorghorban *et al.*, 2015).

The β-CD-Car and β-CD-Thy complexes were found to exhibit similar behaviour to the β-CD-Lin complex in that each of these showed a staged mass loss that occurred up to *ca*. 300°C with Δm = 7.2 ± 0.1 % and Δm = 7.5 ± 0.3 % being observed for the β-CD-Car and β-CD-Thy complexes respectively. Based on a 1:1 mole ratio of guest to wall, the theoretical percentage of carvacrol, linalool and thymol in the β-CD complexes on a mass basis is: 11.69%, 11.96% and 11.69% respectively. Whence, the observed mass losses in the early stages of the thermal decomposition of these β-CD complexes in each case accounts for a little more than half of the amount of guest species that is theoretically present and available. The remainder of the guest species is presumably lost at the major stage of decomposition of the β-CD itself and may explain why the β-CD is destabilized to some extent by the presence of these guest molecules, as observed in Figure 4.1.

The kinetic model and decomposition kinetics of β-CD and its complexes at temperatures above 300°C were analysed using two computer-based algorithms described and successfully applied elsewhere (Bigger *et al.*, 2015a, Bigger *et al.*, 2015b, Tawakkal *et al.*, 2016b). The first algorithm utilizes previously published characteristic parameters that define 15 kinetic models commonly used to analyse TG data (Dollimore *et al.*, 1992). The algorithm calculates values of the characteristic parameters for the kinetic run under consideration and quantitatively compares these with those defined by Dollimore *et al.* (1992) in order to determine a single parameter, ρ, ($0 \leq \rho \leq 1$) whose value is indicative of the goodness of fit of each of the kinetic models to the experimentally obtained TG profile. The second algorithm utilizes an iterative arithmetic method in order to extract the apparent activation energy, E_a, and the Arrhenius A-factor from the experimental TG data without having to make simplifying mathematical approximations and assumptions. In extracting these data, the algorithm iteratively solves the general kinetic equation that pertains to TG analysis:

$$g(\alpha) = (AE_a/R) \times p(x) \qquad (4.1)$$

where g(α) is a function that defines the kinetic model, α is the degree of conversion at time t in the process, A is the Arrhenius A-factor, E_a is the apparent activation energy for the process, and R is the ideal gas constant. The function p(x) represents the integral:

$$p(x) = \int_x^\infty [\exp(-x)/x^2]dx \qquad (4.2)$$

where $x = E_a/RT$ and T is the absolute temperature. Thus, if the first algorithm correctly identifies an appropriate kinetic model to fit the experimental data, a plot of g(α) *vs* p(x), which are two highly non-linear functions when considered separately, should be linear and pass through the origin.

Table 4.1 lists the values of the kinetic model fitting parameter, ρ, along with the apparent activation energies, E_a, that were derived from the analysis of the thermograms of the studied complexes at temperatures greater than *ca*. 300°C using the software algorithms (Bigger *et al.*, 2015a). The "R1", "R2", etc., designations indicate separate replicates that were performed on the samples in order to assess the reproducibility of the experimental data and the analysis of these. In each case the thermogram data in the domain $0.1 \leq \alpha \leq 0.8$ was analysed for consistency. It is believed that this range of data is sufficient to represent the bulk of the decomposition process whilst avoiding the large errors in numerical calculations that are invoked at very low and very high degrees of conversion. Values of the fitting parameter that indicate the best fitting kinetic models predicted by the software have been highlighted in Table 4.1 using bold font. These data consistently suggest that the decomposition kinetics are adequately fitted by the Avrami-Erofeev index 2 and index 3 models, although in some cases first and second-order kinetics appear to be reasonably good fits to the data as well. Nonetheless, in these latter cases the Avrami-Erofeev models also produce reasonable fits to the data. The values of the fitting parameter obtained for the neat β-CD samples, when compared to those corresponding values obtained across the range of the β-CD complexes under the Avrami-Erofeev models, remain consistently high. This suggests the decomposition kinetics for the neat β-CD and its complexes are similar and there is little evidence to suggest the need to consider there being a different kinetic model that applies in the case of the neat β-CD compared to that which applies in the cases of its complexes. This, in turn, suggests that the relatively small amount of the guest species that remains associated with the β-CD wall at *ca*. 300°C does not affect significantly the decomposition process.

Table 4.1: Values of the TG kinetic fitting parameters ρ, linear regression coefficient, R^2, and apparent activation energies, *Ea*, for the thermal decomposition of β-cyclodextrin and its complexes with carvacrol, linalool and thymol (degree of conversion range: $0.1 \leq \alpha \leq 0.8$)

Kinetic Model	β-CD R1	β-CD R2	β-CD-Car R1	β-CD-Car R2	β-CD-Car R3	β-CD-Lin R1	β-CD-Lin R2	β-CD-Lin R3	β-CD-Thy R1	β-CD-Thy R2	β-CD-Thy R3
Acceleratory											
Pn Power law	0.652	0.654	0.428	0.501	0.574	0.333	0.385	0.488	0.598	0.571	0.599
E1 Exponential law	0.548	0.547	0.532	0.533	0.536	0.527	0.531	0.532	0.538	0.536	0.539
Sigmoidal											
A2 Avrami-Erofeev	**0.894**	**0.902**	**0.893**	**0.922**	**0.907**	**0.910**	**0.979**	**0.989**	**0.916**	**0.913**	**0.895**
A3 Avrami-Erofeev	**0.897**	**0.905**	**0.893**	**0.918**	**0.903**	**0.942**	**0.974**	**0.988**	**0.912**	**0.909**	**0.891**
A4 Avrami-Erofeev	0.689	0.693	0.425	0.517	0.601	0.329	0.394	0.519	0.631	0.599	0.626
B1 Prout-Tompkins	0.736	0.742	0.523	0.608	0.671	0.459	0.466	0.557	0.698	0.671	0.687
Deceleratory											
Geometrical											
R2 Contracting area	0.723	0.730	0.793	0.789	0.741	0.906	0.918	0.875	0.745	0.750	0.722
R3 Contracting volume	0.772	0.780	0.857	0.850	0.794	0.933	0.954	0.944	0.798	0.803	0.772
Diffusion											
D1 One dimensional	0.669	0.674	0.740	0.731	0.686	0.838	0.844	0.800	0.688	0.693	0.669
D2 Two dimensional	0.639	0.651	0.774	0.768	0.721	0.881	0.891	0.847	0.724	0.729	0.702
D3 Three dimensional	0.539	0.554	0.758	0.750	0.676	0.865	0.879	0.847	0.661	0.687	0.630
D4 Ginstling-Brounshtein	0.583	0.596	0.690	0.696	0.661	0.781	0.795	0.770	0.666	0.668	0.645
Reaction Order											
F1 First order	0.796	0.804	0.880	0.876	0.818	0.963	0.987	0.977	0.823	0.828	0.795
F2 Second order	0.680	0.698	0.919	0.913	0.824	0.950	0.920	0.866	0.811	0.837	0.770
F3 Third order	0.377	0.374	0.633	0.565	0.534	0.617	0.517	0.485	0.490	0.529	0.512
R^2 for g(α) *vs* p(x) plot	0.9632	0.9545	0.9961	0.9946	0.9955	0.9840	0.9970	0.9939	0.9921	0.9912	0.9909
Apparent *Ea*/kJ mol^{-1}	156 ± 6			107 ± 7			96 ± 3			110 ± 3	

Of the two Avrami-Erofeev models identified in Table 4.1 as being appropriate fitting models for the data, the Avrami-Erofeev index 3 model (see eq. (4.3) gives slightly greater values of the fitting parameter across the range of samples tested and so that model was used to fit the experimental data.

$$g(\alpha) = [-\ln(1-\alpha)]^{1/3} \qquad (4.3)$$

The goodness of fit of the Avrami-Erofeev index 3 model can be verified by a plot of g(α) *vs* p(x) which is expected to be linear and pass through the origin as indicated above. Such a plot is shown in Figure 3 for the thermal decomposition of the β-CD-Lin complex and typifies the plots from which the linear regression coefficients, R^2, appearing in Table 4.1 were obtained.

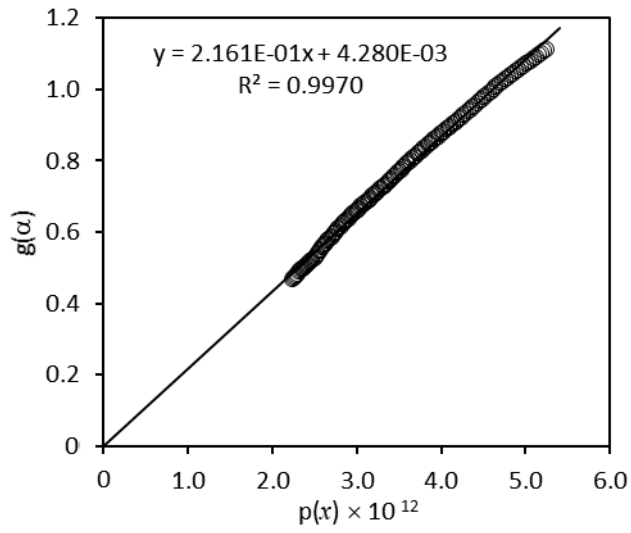


Figure 4.3: Plot of g(α) *vs* p(x) for β-CD-linalool thermal decomposition above *ca*. 300°C in the range 0.1 ≤ α ≤ 0.8

The values of the apparent activation energy, E_a, for the major decomposition process occurring above *ca*. 300°C (see Table 4.1) were derived by fitting the thermogram data in the range 0.1≤ $\alpha \leq$ 0.8 using eq. (4.3). The apparent E_a obtained for the neat β-CD (156 ± 6 kJ mol^{-1}) is significantly higher than each of the three E_a values obtained for the β-CD-Car, β-CD-Lin and β-CD-Thy complexes (107 ± 7, 96 ± 3 and 110 ± 3 kJ mol^{-1} respectively). This confirms the preliminary observation made in assessing the temperatures at which the onset of decomposition above *ca*. 300°C occurs (see Figure

4.2) in that the presence of the guest species destabilizes the β-CD. The mechanism of this destabilization is unclear at present, however, the formation of the inclusion complex would not be expected to result in chemical modification of the guest molecules (Braga *et al.*, 2003).

The mass losses that occur from these complexes at temperatures below 300°C was investigated in further detail in order to possibly elucidate the kinetic model and associated apparent activation energies associated with these. Figure 4 shows plots of the derivative of the mass with respect to temperature, d*m*/d*T*, as a function of temperature in the temperature range of 80 to 220°C.

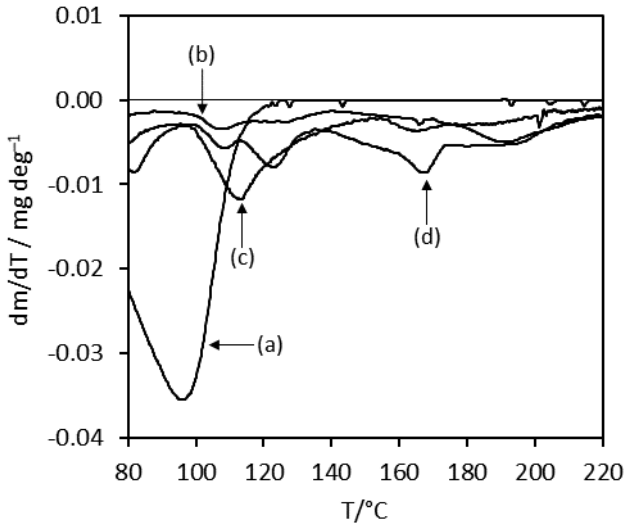


Figure 4.4: Plots of the derivative d*m*/d*T* as a function of temperature in the temperature range of 80 to 220°C for the thermal decomposition of: (a) β-CD, (b) β-CD-Car, (c) β-CD-Lin and (d) β-CD-Thy

The derivative plot for the neat β-CD reveals a broad peak centred at *ca*. 95°C which is presumably due to the adsorbed water within this compound (Abarca *et al.*, 2016). The derivative curve for each of the complexes at this particular temperature is at or near a minimum value in each case. This suggests that, in the case of the complexes, such water molecules have been displaced by the guest molecules and that any water molecules that may remain associated with the complex have been relegated to surface sites and are more readily lost at lower temperatures as observed in the thermograms. Thus, the mass losses from the complexes that are observed between *ca*. 95 and 300°C may be attributed solely to the loss of the guest species either from sites within the wall

or perhaps surface sites (Sun *et al.*, 2017). In the present study, this is unlikely to be the case since analysis of the complexes showed minimal adsorption on the surface (see Chapter 3).

The derivative plot for the β-CD-Car complex (Figure 4.4) shows numerous convoluted peaks below *ca*. 140°C but there exists a broad single peak from *ca*. 140 to 230°C that does not appear to be convoluted. In the case of the β-CD-Lin complex, there is a single peak in the range of *ca*. 95 to 150°C and possibly another broad but much less intense peak at higher temperatures up to *ca*. 220°C. The derivative plot for the β-CD-Thy complex, however, is much more complicated with a greater number of convoluted peaks than the other two complexes over the temperature range shown. Whence only the data associated with three separate runs of the β-CD-Car and β-CD-Lin complexes in the respective identified temperature ranges were selected to explore the kinetics of the mass losses occurring at these lower temperatures.

Figure 4.5 shows the mass loss during thermal decomposition and the corresponding derivative plot that was obtained from one of the β-CD-Lin samples which typifies the results obtained from the other two β-CD-Lin samples for temperatures below 300°C. The percentage mass loss for the β-CD-Lin over the temperature range shown in Figure 5 accounts for *ca*. 4.9% of the total mass of the sample. This, in turn, accounts for *ca*. 4.9% of the total of *ca*. 7.4% of the sample that is lost over the temperature range leading up to the major decomposition stage at *ca*. 300°C. The thermogravimetric data obtained over this temperature range were also analysed using the fitting protocol and software described above (Bigger *et al.*, 2015a) which resulted in a second-order kinetic model in accordance with eq. (4.4) being identified as the most appropriate fit for the data:

$$g(\alpha) = 1/(1-\alpha) \qquad (4.4)$$

The corresponding g(α) *vs* p(x) plot for the β-CD-Lin complex is shown in Figure 4.6 and its linearity confirms the data are fitted reasonably well by the second-order model. The apparent activation energy associated with this mass loss was found to be 38 ± 1 kJ mol^{-1}. This finding is consistent with the previous contention that these mass losses occurring at these lower temperatures may be associated with different types of binding interactions either within or on the surface of the wall molecules.

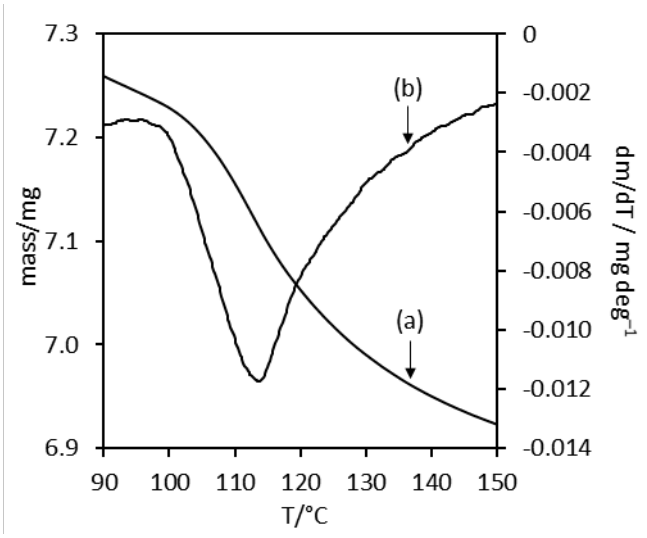


Figure 4.5: TG plots of the thermal decomposition of β-CD-Lin (a) mass and (b) the corresponding derivative d*m*/d*T* over the temperature range of 90 to 150°C

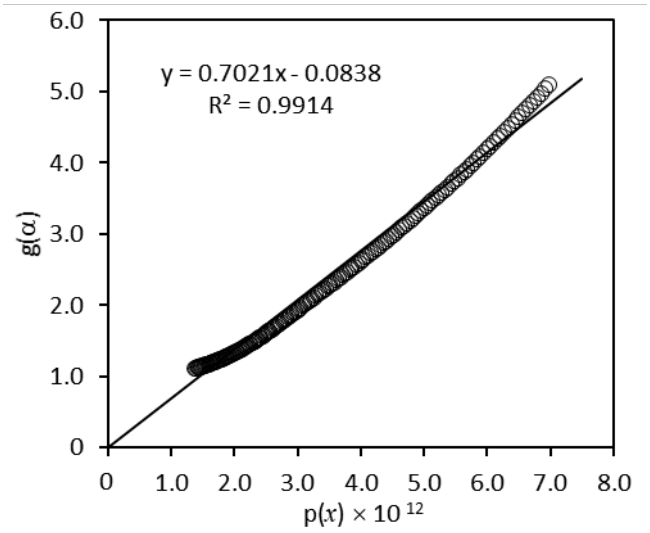


Figure 4.6: Plot of g(*α*) *vs* p*(x)* for the β-CD-Lin thermal decomposition in the range 0.1 ≤ α ≤ 0.8 above *ca*. 300°C.

The kinetics of the mass loss from the β-CD-Car (temperature range: *ca*. 138 to 231°C) as well as the β-CD-Lin (temperature range: *ca*. 92 to 152°C) complexes is summarized in Table 4.2. Each of the analyses were performed using data in the degree of conversion range $0.1 \leq \alpha \leq 0.8$ and accounts for 4.9 ± 0.1 % (β-CD-Car) and 4.9 ± 0.1 % (β-CD-Lin) of the total mass of the samples. The values of the fit parameter, ρ, that are

shown in bold are those associated with the best kinetic fits to the data as identified by the fitting algorithm. These values consistently suggest that a second-order kinetic model is appropriate to fit the data although in the case of the β-CD-Car complex a 3D-diffusion model or first-order model may also apply. Clearly, further studies aimed specifically at elucidating the most appropriate model need to be undertaken. However, for the purposes of comparing the samples in the current study, the second-order model, which provides an acceptably high value of the fitting parameter in each case, was selected as the model from which to derive the apparent activation energies for the mass losses from these complexes at the lower temperatures.

Table 4.2: Kinetic model fits and associated apparent E_a values for the low-temperature mass loss from β-cyclodextrin complexes with carvacrol and linalool during thermal decomposition (degree of conversion range: $0.1 \leq \alpha \leq 0.8$)

Kinetic Model	β-CD-Car R1	β-CD-Car R2	β-CD-Car R3	β-CD-Lin R1	β-CD-Lin R2	β-CD-Lin R3
Acceleratory						
Pn Power law	0	0	0	0.193	0.309	0.287
E1 Exponential law	0.516	0.516	0.519	0.528	0.531	0.529
Sigmoidal						
A2 Avrami-Erofeev	0.722	0.730	0.620	0.820	0.841	0.849
A3 Avrami-Erofeev	0.699	0.708	0.560	0.803	0.848	0.861
A4 Avrami-Erofeev	0	0	0	0.137	0.271	0.253
B1 Prout-Tompkins	0	0	0	0.277	0.382	0.377
Deceleratory						
Geometrical						
R2 Contracting area	0.757	0.766	0.685	0.838	0.801	0.842
R3 Contracting volume	0.819	0.822	0.757	0.848	0.835	0.862
Diffusion						
D1 One dimensional	0.531	0.542	0.479	0.762	0.758	0.792
D2 Two dimensional	0.891	0.891	0.802	0.821	0.785	0.823
D3 Three dimensional	0.938	0.938	0.830	0.810	0.754	0.793
D4 Ginstling-Brounshtein	0.909	0.909	0.813	0.729	0.683	0.717
Reaction Order						
F1 First order	0.960	0.959	0.844	0.868	0.854	0.884
F2 Second order	0.954	0.955	0.895	0.927	0.909	0.947
F3 Third order	0.742	0.740	0.812	0.731	0.643	0.717
R^2 for g(α) *vs* p(x) plot	0.8619	0.8635	0.8680	0.9839	0.9827	0.9905
Apparent *Ea*/kJ mol^{-1}		37 ± 1			69 ± 6	

The apparent activation energies for the mass losses from the β-CD-Car (37 ± 1 kJ mol^{-1}) and β-CD-Lin (69 ± 6 kJ mol^{-1}) occurring in the lower temperature ranges are significantly lower than the corresponding apparent activation energies for the major decomposition step for these complexes at *ca*. 300°C (i.e. 107 ± 7 and 96 ± 3 kJ mol^{-1}

respectively). This provides further evidence to suggest that the guest molecules in these complexes may be located at different sites that involve a different extent of chemical interaction between the guest and wall molecules.

4.5 Summary

The mass losses from the β-CD complexes under a linear temperature ramp below *ca*. 300°C do not account for the total mass of guest species present in the studied complexes. This may be attributed to the presence of different binding sites where some guest molecules are strongly bound to the wall and are only released along with the decomposition of the wall itself whereas other guest molecules are less strongly bound to the wall and are released at lower temperatures. The low-temperature release of guest species for the complexes was consistently found to follow second-order kinetics with apparent activation energies that were significantly lower than those obtained either for the major decomposition stage of the neat β-CD or the respective β-CD complexes. The presence of the guest molecules in the β-CD decreased the apparent activation energy observed for the major decomposition stage in each case. The major decomposition stage was consistently and adequately fitted by the Avrami-Erofeev index 3 kinetic model.

Close inspection of the derivative plots for the thermal decomposition of the β-CD complexes below *ca*. 300°C suggests the release of the guest species may occur in a number of separate stages. In some cases, these could not be fully resolved in the current work using the data analysis techniques available. Work is continuing in our laboratory to develop suitable techniques to de-convolute the kinetic profiles of these with a view to determining whether or not these stages follow similar kinetics and thereby extract the relevant kinetic parameters. The quantitative assessment of the mass losses occurring from these complexes under a temperature ramp has important implications in their application in polymer formulations for food-packaging materials. In particular, the current work has demonstrated that in each case only a little more than half of the available guest molecule is released from the complex at temperatures at which many commodity packaging polymers such as polyethylene are thermally processed commercially. This must be taken into account in the future use of these complexes in AM systems and ways found to increase the extent to which the active agent is released from the complex. The following chapters address this limitation.

Chapter 5 Release of AM Agents from β-CD Complexes

5.1 Overview

The previous chapter explored the kinetic release of the various AM agents from β-CD complexes under thermal conditions and this has clear implications for the retention of these complexes during thermal processing in the preparation of extruded films. Equally important is the release of the agents from the complexes in field applications in order to impart their activity against food spoilage and oxidation. This chapter investigates the kinetic release of the AM agents into food simulants from films with a comparison between freely added agents and those encapsulated in β-CD.

5.2 Introduction

Food packaging plays a crucial role in maintaining food quality, protecting food components, and extending the shelf-life of food products. The use of natural additives for preservation has extended into food packaging with the use of EOs and their extracts incorporated into packaging film becoming increasingly popular to impart AM activity (Rodríguez *et al.*, 2014). Over recent years, considerable studies have therefore focused on the incorporation of natural AM agents into packaging films to control or minimize food spoilage (Chalier *et al.*, 2007, Gutiérrez *et al.*, 2010, Becerril *et al.*, 2011, Kuorwel *et al.*, 2011a, Mascheroni *et al.*, 2011, Ramos *et al.*, 2012). However, the volatility of these agents can result in their rapid loss from the polymer matrix and many can be relatively unstable at the high temperatures required to process the polymer formulations (Han, 2005, Tawakkal *et al.*, 2016c). The concentration of the AM agent plays a critical role in determining its activity against targeted microorganisms so any losses during processing can be costly and reduce the efficacy of the film.

Reducing the thermal processing temperature during manufacturing of the film can potentially lead to higher retention of volatile AM agents. For example, the retention of linalool directly incorporated into LDPE film increased from 5% (w/w) to 34% (w/w) by decreasing the film processing temperature (Suppakul *et al.*, 2008, Suppakul *et al.*, 2011b, Suppakul *et al.*, 2011a). However, such a measure may reduce the film quality because most polymers require a high temperature to melt and adequately flow for

extrusion. Similarly, the work of Kuorwel *et al.* (2011a) who surface-coated AM agents onto a polymer substrate at low temperature also showed significant losses of the natural AM agents between 29% and 52% (w/w) from coated starch-based films. Although there have been considerable developments in AM packaging research over recent decades, there has been limited commercial success in this field mainly due to excessive AM losses during processing as well as possible food tainting effects. Nonetheless, incorporating natural AM agents by extrusion is considered to be the most effective industrial process for producing AM films (Del Nobile *et al.*, 2009).

In order to overcome these limitations, microencapsulation techniques have been suggested as a means to protect the loss of volatile compounds primarily during thermal processing (Madene *et al.*, 2006, Astray *et al.*, 2009, Eun-Ju *et al.*, 2010, Levi *et al.*, 2011). In addition, a considerable number of studies have investigated the benefit of different encapsulation techniques to attain a fast, slow and/or sustainable release rate of encapsulated agents for different applications (Wang *et al.*, 2009, Paramera *et al.*, 2011, Xiao *et al.*, 2011, de Oliveira *et al.*, 2014, Rassu *et al.*, 2014, Chen *et al.*, 2015b, da Rosa *et al.*, 2015, Tang *et al.*, 2015, Wang *et al.*, 2016, Zhao and Tang, 2016). In most of these studies, the focus has been on the benefits of using encapsulation techniques in various pharmaceutical, cosmetic, and food industry applications. However, little attention has been devoted to evaluating the release of encapsulated AM agents from packaging films for shelf-life extension of foods, either into food simulants or into real systems (Siro *et al.* (2006).

Given the growing trend to incorporate natural AM agents into food packaging materials and the limitations due to the inherent volatility of these agents, the aims of the current work were to: (i) develop LDPE food packaging films containing β-CD encapsulated AM agents thymol, carvacrol, and linalool; (ii) evaluate the stability of the encapsulated AM agents following film processing and storage; and (iii) investigate the release of the AM agents from the film into a fatty food simulant.

5.3 Materials and Methods

5.3.1 Materials

The films for the present study were prepared using LDPE (grade XJF143/1700, Qenos, Australia). Ethanol (absolute) was purchased from Merck Australia and the following chemicals were purchased from Sigma-Aldrich Australia: beta-cyclodextrin (β-CD, 98.5% purity, 1.5% (w/w) water content); thymol (99.5% purity); carvacrol (98% purity); and linalool (97% purity). Milli-Q water was used in all preparations.

5.3.2 Preparation of Inclusion Complex

The complexes were prepared according to the method reported in our previous study (Al-Nasiri *et al.*, 2018). Briefly, β-CD (3.8352 g, 98.5% purity, 3.33 mmol) was dissolved in 60 mL and the selected AM agent was added at a 1:1 mole ratio under vigorous stirring. The mixture was maintained under moderate stirring at 55°C for 4 h after which time the heat was removed and stirring continued until the temperature reached 26 ± 1°C. The β-CD/AM agent solution was then cooled to 4°C and maintained at this temperature for 24 h after which the precipitate was recovered by vacuum filtration and dried in a vacuum desiccator for 1 h. The vacuum was then removed and the complexes remained in the desiccator for a further 12 h to remove any residual water and until a constant mass was attained.

5.3.3 Incorporation of Free AM Agents in LDPE Films

A mass of 0.1 g of carvacrol, linalool or thymol was dissolved in 5 mL of 90% (v/v) ethanol/water and mixed well until the AM agent was completely dissolved. A mass of 9.9 g of powdered LDPE was added to the AM/ethanol mixture and the slurry was thoroughly mixed for a few minutes to ensure a homogeneous distribution of the AM agent on the polymeric particles. The slurry was left to dry in air for 210 min to evaporate the ethanol. A sample weighing *ca*. 3 g of the resultant solid mixture was placed between Mylar™ films that were positioned between a set of aluminium plates, ready for pressing. Film samples were created by pressing the solid mixture in a laboratory press (IDM Instruments Pty. Ltd., Australia, Model No. L0003), that was preheated to 120°C. The temperature of the upper and lower platens of the press was maintained at 120°C for 4 min under a pressure of 20 kPa. The plates were quench-cooled with water to 35°C, then

removed from the press and the film samples removed from between the aluminium plates. The films were peeled off gently from the Mylar™ after they had cooled completely. The films were wrapped with aluminium foil after pressing until they were analysed within 24 h. The thickness of the films was measured at five different locations using a hand-held digital micrometer (Schut IP54, The Netherlands) with a precision of 0.001 mm and the average thickness was calculated from these readings.

5.3.4 Incorporation of Inclusion Complex in LDPE Films

A dry mixture of 8.6 % of the β-CD/AM complex powder was dry mixed with LDPE powder for a few minutes using a spatula to obtain a homogenous distribution of the complex in the LDPE. A sample weighing *ca*. 3 g of the resultant solid mixture was heat pressed using the same conditions as described above.

5.3.5 Quantification of AM Agents in LDPE Films

Film samples were cut into *ca.* 1 × 1 cm and had a total mass of *ca*. 1 g each sample. The samples were extracted for 90 min under reflux using 50 mL of ethanol. An aliquot of the extract was directly analysed by GC. Gas chromatographic analyses were performed using a Varian Star 3400-CX gas chromatograph (GC) equipped with a fused silica capillary column DB-5 (30 m × 0.32 mm i.d., film thickness 0.25 μm, J & W Scientific, USA). The GC was operated using the following conditions: injection volume: 1.0 μL; initial column temperature: 80°C; heating rate: 5°C min^{-1}; up to 180°C, kept at this temperature for an additional 1 min; injector temperature, 250°C; split ratio 1:60; FID detector temperature: 300°C; and carrier gas: nitrogen. Quantification was performed using standard curves prepared using the different AM agents.

5.3.6 Retention of AM Agents in LDPE Films

The retention of the AM agents in the LDPE films was determined by quantifying the amounts of these agents remaining in the film following exposure in air at room temperature. The LDPE films incorporated directly with AM agents and films containing the complexes were prepared and the films were not wrapped with the aluminium foil after pressing. The films were left in the open atmosphere and random samples were taken and extracted as described above over period of 30 days. Samples were taken on day 6

and day 30 and the sample extracts were analysed using GC as described above (see section 2.5).

5.3.7 Migration of AM Agents from LDPE Films into Food Simulant

The release of AM agents was carried out using a total immersion migration test (Tawakkal *et al.*, 2016c). Samples of film weighing *ca.* 0.45–0.50 g were cut into *ca* 1.5 × 1.5 cm squares and were immersed into 50 mL of a food simulant solution comprised of 95% (v/v) ethanol in water. The vessel was tightly sealed and mildly agitated (100 rpm) in an incubator shaker (InnovaTM 4230, New Brunswick Scientific, USA) and the amount of AM agent released from the films was monitored by collecting a 5 mL sample of the simulant solution at different time intervals (i.e. 2, 4, 8, 24, 48 days) with fresh simulant solution added to the vessel in order to maintain a constant total volume. Aliquots of 1 μL were injected into the GC and the amount of AM agent released into the food simulants was quantified using the GC conditions given above. All measurements were performed in triplicate.

5.4 Results and Discussion

5.4.1 Retention of AM Agents in LDPE Films

Low-density polyethylene films containing free and encapsulated AM agents at 1% (w/w) were pressed at 120°C resulting in an average film thickness of *ca*. 0.2 mm. The films were extracted with ethanol by reflux extraction for 90 min and the average percentage of AM agents retained in each film after thermal processing, and after 6 and 30 days, is summarized in Table 5.1. The results show the highest to lowest retention is in the order of thymol > carvacrol > linalool with between 23% and 129% more AM agent retained when encapsulated in β-CD compared to the free addition of AM agent in the film. The retention of such agents is governed by the relative volatility and the affinity of the AM agent to the polymer backbone (Herath, 2010). Although thymol and carvacrol are isomers, the volatility of thymol is much lower than that of carvacrol and so a higher retention of thymol in the films is expected after processing (Guarda *et al.*, 2011). Other studies have reported retentions of 20-40% of thymol and carvacrol (Herath, 2010), 61-72% for linalool and thymol (Mistry, 2006), 5-66% for linalool and methyl chavicol (Suppakul *et al.*, 2006, Suppakul *et al.*, 2008), and 75% for carvacrol (Mascheroni *et al.*,

2011). In these cases, the use of ethylene vinyl acetate copolymer, changes in extrusion temperatures, and the use of a coating technique were reported to improve the retention.

Table 5.1 Quantification of free and encapsulated AM agents retained in LDPE films

AM Agent	Addition of AM Agent	Formulation mass/mg	Percentage retained		
			After processing	After 6 days	After 30 days
Thymol	Free	4.78 ± 0.20	79.1 ± 4.7	n.d.*	n.d.
	Encapsulated	5.24 ± 0.01	97.5 ± 1.6	94.4 ± 1.6	91.0 ± 2.4
Carvacrol	Free	5.17 ± 0.11	75.6 ± 0.6	n.d.	n.d.
	Encapsulated	5.24 ± 0.03	93.3 ± 1.9	83.3 ± 1.2	82.9 ± 2.2
Linalool	Free	5.62 ± 0.34	42.4 ± 1.3	n.d.	n.d.
	Encapsulated	5.29 ± 0.027	97.3 ± 0.9	84.1 ± 2.6	83.0 ± 0.8

*n.d. = not detected

In the present study, the increase in the retention of the AM agent in the film compared with other reported results might be due to the optimization of other conditions used during the production of the films and other method adaptations. For example, the temperature used to produce the LDPE/thymol and LDPE/carvacrol films in the laboratory hot press was 120°C which was the same as that used by Herath (2010). However, in the present study, a solution of 90% (v/v) ethanol in water was used to disperse the AM agents with an evaporation time of 3.5 h compared to a 70% (v/v) ethanol in water solution and a longer solvent evaporation time of 24-48 h (Herath, 2010).

The quantification of AM agents soon after processing is important in order to determine the efficiency of the encapsulation process and, as indicated in Table 5.1, the encapsulation technique using β-CD successfully improved the retention of the AM agents by providing sufficient protection during thermal processing of the films. Although the loss of thymol and carvacrol when added directly to the film was not as significant as the loss of linalool, encapsulation in β-CD significantly protected all of the AM agents during the heat pressing process. It is also important to evaluate the long-term stability of films during storage since many food packaging films may not be used immediately after they are extruded. This was achieved by exposing film samples where the AM agent was added directly and those where the AM agent had been encapsulated to the open

atmosphere at room temperature for up to 30 days. Following exposure, the samples were extracted and the levels of AM agent quantified.

The results presented in Table 5.1 show that for the films where the AM agent was added directly, the presence of AM agent was not detected after 6 days exposure. In contrast, for the films containing encapsulated AM agents, there were only small decreases in the concentrations of the AM agents up to 30 days. No significant losses were observed for any of the encapsulated AM agents between 6 and 30 days and the retention of encapsulated thymol was slightly higher than that of carvacrol and linalool. This may be due to the physical state of thymol which is a solid at room temperature whereas carvacrol and linalool are liquids at room temperature. The observed greater loss of AM agents between day 0 and day 6 is attributed to AM agent located closer to or on the surface of the film that is lost more quickly to the atmosphere upon exposure.

Compared to the free addition of linalool into the LDPE film, a relatively high retention was observed when linalool was encapsulated in β-CD before incorporation into the film. Moreover, the retention of linalool was similar to that of thymol and higher than that of carvacrol. Given that linalool has a higher vapour pressure than both thymol and carvacrol, a greater loss of linalool may have been anticipated. The high retention of encapsulated linalool may be due to factors such as a stronger binding between β-CD and linalool and a higher retention for linalool after film processing (see Table 5.2). However, the overall losses after film pressing to day 30 were *ca*. 7, 11, and 15% for thymol, carvacrol and linalool respectively which is in accordance with the previous retention order for the direct addition of the agents.

Table 5.2 Kinetic parameters of AM agents release from LDPE films

AM Agent	Addition of AM Agent	Percentage released after 30 days	First order rate constant, $k_1 \times 10^3$/h^{-1}	Initial release rate, $v_0 \times 10^3$/mg h^{-1}	Time to reach 99.9% release, $t_{99.9}$/h	Diffusion Coefficient, $D \times 10^{12}$/mg m^{-2} h^{-1}
Thymol	Free	94.0 ± 3.6	7.91	28.1	846	33.7
	Encapsulated	63.0 ± 0.1	2.29	7.4	2970	8.09
Carvacrol	Free	88.2 ± 1.8	8.98	31.0	752	34.6
	Encapsulated	65.8 ± 0.4	2.29	7.35	3000	7.41
Linalool	Free	99.6 ± 0.1	8.64	20.5	778	39.6
	Encapsulated	23.2 ± 0.1	2.21	2.6	3070	8.07

5.4.2 Migration of AM Agents from LDPE Films into Food Simulant

The migration of free and encapsulated AM agents from LDPE film into the fatty food simulant 95% (v/v) ethanol in water was investigated at a temperature of 4°C (Siro *et al.*, 2006, Ramos *et al.*, 2012, Tawakkal *et al.*, 2016c). This temperature was selected as it is a typical temperature for storage of refrigerated foods and this temperature was also used in the preparation of the complex (Al-Nasiri *et al.*, 2018).

Plots of the mass of AM agents (M_t) released from the film into the simulants from the AM films *vs* time (t) are shown in Figure 5.1. For the films containing the free AM agent (Figure 5.1(a)), the release of thymol and carvacrol showed similar release profiles with linalool reaching a lower asymptotic limit than either thymol or carvacrol due to lower retention of linalool in the produced films. A similar trend is observed for the release of the encapsulated agents (Figure 5.1(b)) but in this case, smaller amounts of each agent are released into the simulant over the same time period. Moreover, for the encapsulated AM Agents, no release was observed within the first 2 h which further suggests that the encapsulation process inhibits the release of the AM agents. The release of encapsulated thymol was first observed at 4 h whereas that of carvacrol and linalool was first measured at 8 h. The formation of the complexes between the AM agents and β-CD is influenced by the shape of the molecule where the hydrophobic groups of the AM agents bind with the hydrophobic cavity of β-CD (Hedges *et al.*, 1995). In addition, the hydroxyl group of the AM agents can project outside of the cavity where it can interact with hydroxyl groups on the outer surface resulting in strong binding between the AM agents and β-CD.

Migration experiments were continued for up to 30 days and the percentage of the AM agents released from the films is shown in Table 5.2. Based on these data and the data plotted in Figure 5.1, particularly in the cases of the more slowly releasing systems, it was evident that equilibrium release was not achieved. In order to estimate the mass of each agent released at equilibrium, the fit to a first-order kinetic model was used in accordance with:

$$\ln (1 - M_t/M_\infty) = -k_1 t \qquad (5.1)$$

where M_t is the amount of AM agent released from the film at time t, M_∞ is the amount of AM agent released from the film at equilibrium and k_1 is the first-order rate constant (Kuorwel *et al.*, 2013). A plot of ln $(1 - M_t/M_\infty)$ *vs* time should produce a straight line with a slope equal to $-k_1$. Values of M_∞ were optimized to obtain the best fit of the straight line with a maximum regression coefficient in each case. Figure 5.2 shows the plots of ln $(1 - M_t/M_\infty)$ *vs* time where the value of M_∞ has been optimized and in all cases, good linear fits are obtained. The initial rate of release, v_0, the product of $-k_1$ and M_∞ (Kuorwel *et al.*, 2013) was calculated from the regression data, and an estimate of the time to reach 99.9% release of the agents, $t_{99.9}$, was calculated from equation (5.1).

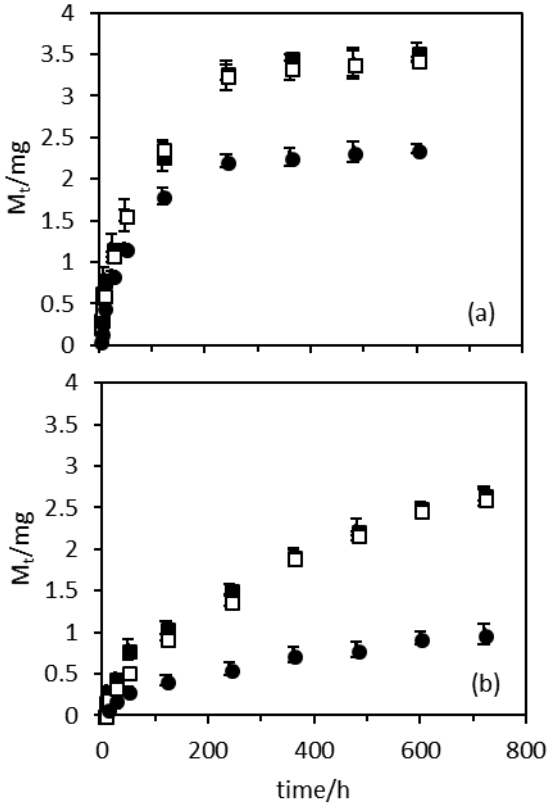


Figure 5.1: Plots of M_t *vs* time for release of AM agents thymol (■), carvacrol (□), and linalool (●) from LDPE where the AM agent is: (a) free and (b) encapsulated

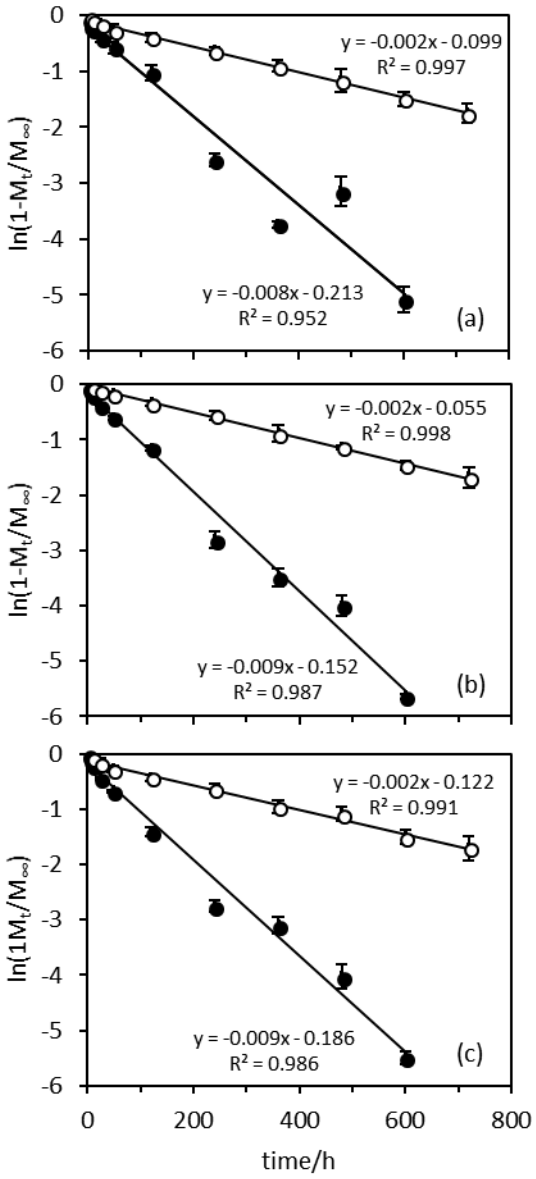


Figure 5.2: Plots of ln(1-M_t/M_∞) *vs* time for release of AM agents (a) thymol, (b) carvacrol, and (c) linalool where the AM agents are: (●) free and (○) encapsulated

Table 5.2 shows the values of k_1, v_0, $t_{99.9}$ for each system studied. In the case of the release of the free AM agents from the LDPE film, the values of k_1, v_0, $t_{99.9}$ are similar. For the encapsulated AM agents, the kinetic values are also similar but significantly different to those for the corresponding free AM agents. In particular, the $t_{99.9}$ values for the encapsulated AM agents are between 3.5 and 4 times greater than the values for the free agents. Herath (2010) found at 20°C, the release of free thymol and carvacrol into 95% (v/v) ethanol/water reached equilibrium in 60 and 40 min, respectively. Similarly,

Kuorwel *et al.* (2013) reported the release of carvacrol, linalool and thymol in isooctane at 15 and 35°C between 90 and 150 min with the release of carvacrol being consistently faster. A number of factors influence the migration process including migration temperatures, the chemistry of the AM agents, the type and properties of simulant (i.e. polarities, pH, viscosity), the polymer sorption to the simulant, and the number of voids and gaps in the polymer structure (Tovar *et al.*, 2005, Peltzer *et al.*, 2009, López De Dicastillo *et al.*, 2011, Aznar *et al.*, 2013, Echegoyen and Nerín, 2013, Carrizo *et al.*, 2014, Ramos *et al.*, 2014, Wrona *et al.*, 2017, Wu *et al.*, 2018). The slower release of free thymol is in agreement with study of Ramos *et al.* (2012) who reported a faster release of carvacrol from polypropylene films into 90% (v/v) ethanol at 40°C. However, the authors reported that the release of thymol was faster than carvacrol into distilled water and faster again into isooctane at 20°C (Ramos *et al.*, 2012).

Plots of M_t/M_∞ *vs* time are presented in Figure 5.3 with the solid lines representing the fit to Fick's second law of diffusion (Crank, 1979) in accordance with equation (5.2):

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2}\sum_{m=0}^{\infty}\frac{1}{(2m+1)^2}exp\left[\frac{-D(2m+1)^2\pi^2 t}{l^2}\right] \qquad (5.2)$$

where D is the diffusion coefficient, l is the film thickness, and t is time. The value of D was determined from an iterative evaluation of the fit of the release data to the theoretical curve such that the sum of the residuals was minimized. In all cases, the data are shown to adequately fit Fick's second law and the values of D are shown in Table 5.2. In the case of the free AM agents, the D values are similar for thymol and carvacrol with a significantly higher D value for linalool which is consistent with the first-order kinetic analysis. The D values are significantly lower for the encapsulated AM agents with the value for carvacrol slightly lower than that of thymol and linalool. The value of D is as important parameter to understand the release behaviour of active agents in order to maintain the activity of AM and/or AO agents from packaging films (Ramos *et al.*, 2012). It is evident from the results that encapsulation in β-CD significantly slows the release of the AM agents compared to those added directly to LDPE.

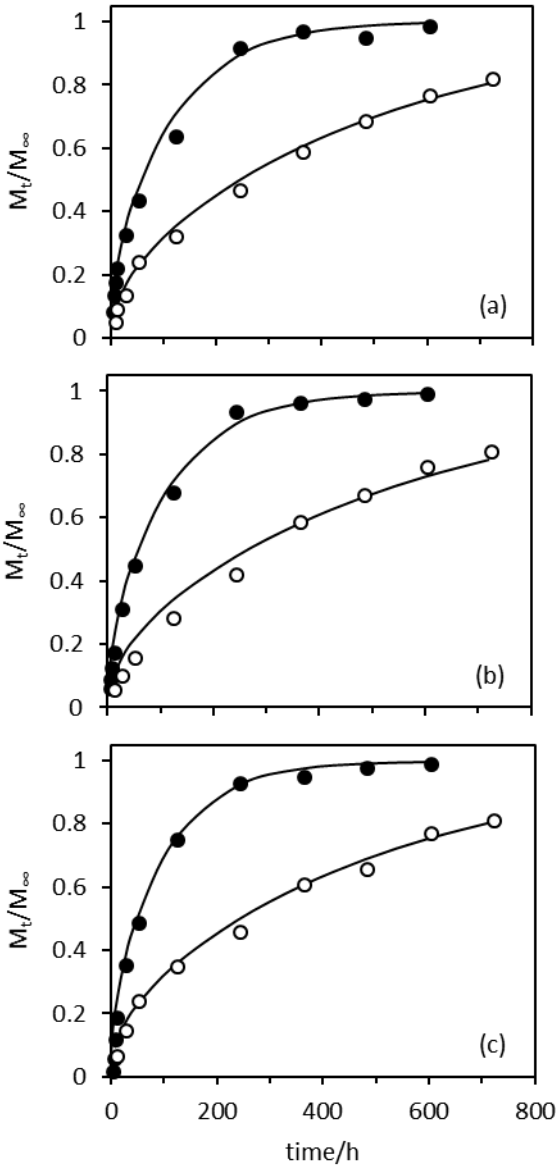


Figure 5.3: Plots of M_t/M_∞ *vs* time for release of AM agents (a) thymol, (b) carvacrol, and (c) linalool where the AM agents are (●) free and (○) encapsulated. Solid lines were obtained by fitting the data in accordance with Fick's second law

In a recent study, Kfoury *et al.* (2016) studied the structure of β-CD inclusion complexes with thymol and carvacrol to determine the exact positioning of the guest inside the β-CD cavity and utilized molecular modelling to rationalize the NMR results obtained in their study. The authors concluded the position of the hydroxyl group in carvacrol and thymol did not influence the stoichiometry of the inclusion complexes, however it affected the binding stability with the β-CD/carvacrol complex appearing to

be less stable than the thymol/β-CD complex. In the present study, the protrusion through the complex of the -OH group of an AM agent is presumed to increase its exposure to hydrogen-bonding interactions with polar food simulants such as ethanol. As a consequence, this renders the AM agent more readily extractable from the β-CD cavity. As shown previously (see Chapter 4), TG analyses of the complexes revelled a decreasing order of binding strength between β-CD and thymol followed by carvacrol and lastly linalool. This in agreement with the findings of Kfoury *et al.* (2016) and may be due to the positioning of the AM agent inside the cavity. Therefore, it might be expected that the migration rate will be higher for carvacrol than thymol and even higher for linalool. However, one must be cautious in comparing studies of migration of AM under the influence of a solvent system with studies of the release of AM agent from a complex during a thermal process such as that imposed during a non-isothermal TG experiment. Furthermore, in relation to migration studies AM solubility needs to be taken into account where thymol is slightly soluble in water whereas carvacrol and linalool are relatively insoluble although all are soluble in ethanol solutions (Kuorwel, 2011). The latter phenomena may be due to the higher affinity of thymol to the highly polar ethanol simulant solution thus resulting in its increased migration behaviour compared to the other AM agents (Ramos *et al.*, 2012). In addition, the complexation with β-CD is known to increase the solubility of the complexed guest molecule (Karathanos *et al.*, 2007, Hill *et al.*, 2013) and therefore all of the abovementioned factors can affect the release behaviour into food simulants. In any case, complexation of the AM agents in the present study effectively protects them from high temperature processing and facilitates the controlled release.

5.5 Summary

The encapsulation of AM agents in β-CD successfully protected the agents during thermal processing and improved their retention with regard to the long-term stability of the film formulation. The results show the highest to lowest retention is in the order of thymol > carvacrol > linalool with between 23% and 129% more AM agent retained when encapsulated in β-CD compared to the free addition of the same AM agent in the LDPE film. The migration of the AM agents into 95% (v/v) ethanol/water mixture indicated that the encapsulation process slowed the release rate. This slower release can potentially maximize the efficiency of the film by providing a more controlled or sustained release from the β-CD matrix.

Chapter 6 Influence of Glycerol on β-CD Complex Activity

6.1 Overview

In the preceding chapter, strategies are presented to assess whether LDPE films containing encapsulated AM agents could effectively release the AM agent from the β-CD matrix. Although the encapsulation process effectively slowed the AM agent release, it may be too slow to be effective in packaging applications. In this chapter, the potential for using additives such as glycerol to facilitate the release of AM agents from β-CD are explored with a focus on their activity against *E. coli*.

6.2 Introduction

Food products can be subjected to deterioration by microbial growth that can adversely affect their sensory qualities including their flavour, odour, colour, and texture (Singh and Singh, 2005). As a result, microbial contamination can significantly reduce the shelf-life of food products and the consumption of contaminated foods can impact on human health leading to serious food-borne diseases (Han, 2005). Over recent decades, there have been substantial advances in the field of active packaging designed to extend the shelf-life of food products and to maintain the integrity and the quality of the products. One of the key areas of active packaging utilizes the incorporation of natural AM additives, particularly those derived from EOs and their extracts (Ha *et al.*, 2001, Gutierrez *et al.*, 2008, Iturriaga *et al.*, 2012, Muriel-Galet *et al.*, 2012b). These developments further address consumer preferences for natural alternatives to conventional preservatives that would traditionally be added directly to food products.

Typically, natural AM agents derived from EOs are highly volatile and although this may be advantageous for some packaging systems, these agents can be rapidly lost at temperatures required to process packaging films when added directly into the polymer formulation (Han, 2005, Levi *et al.*, 2011, Chaliha *et al.*, 2013). This can add additional costs to film production with there being a need to add more AM agent to obtain an effective concentration of the additive in the final film. In addition, the incorporation of polar, low molecular weight molecules into non-polar polymer matrices can result in the rapid release of the additives from the matrix (Risch, 2000). These significant losses can

ultimately affect the resulting AM activity of the film given that the concentration of the AM additives, along with their release, play an essential role in determining their activity against targeted microorganisms (Hyldgaard *et al.*, 2014, Cerisuelo *et al.*, 2015).

Beta cyclodextrin (β-CD) is a cyclic oligosaccharide of high molar mass, 1134.98 g mol^{-1} that is comprised of seven glucose units (López-de-Dicastillo *et al.*, 2010). The structure of β-CD, described as 'empty capsules', provides effective protection for a wide range of organic molecules (Astray *et al.*, 2009). There have been an increasing number of studies focused on using β-CD to provide thermal stability to an extensive range of relatively unstable, volatile agents (Mourtzinos *et al.*, 2007, Mourtzinos *et al.*, 2008, Paramera *et al.*, 2011, Li *et al.*, 2015a). The hydroxyl functional groups of β-CD create a hydrophilic outer surface whereas the hydrogen and oxygen atoms of the glucose monomers provide a hydrophobic inner surface (Hedges *et al.*, 1995). These two features play a key role in the formation of β-CD complexes with other active agents where polar molecules react with the hydrophilic groups of β-CD to form hydrogen bonds and nonpolar organic molecules interact with its hydrophobic groups. The interaction between the β-CD molecule and active agents is governed by several factors such as van der Waal forces, hydrophobic interactions, and dipole-dipole interactions (Hedges *et al.*, 1995, Astray *et al.*, 2009). These interactions are strong enough to form a stable inclusion compound without influencing the release mechanism of the guest material so that the AM agent may become available for the desirable effect.

Several studies have reported the use and effectiveness of encapsulated agents against a wide spectrum of microbial pathogens and the controlled release of these agents in liquid media (Arana-Sánchez *et al.*, 2010, Hill *et al.*, 2013, Aytac *et al.*, 2014, Santos *et al.*, 2015). Examples of the use of encapsulated agents in films include the incorporation of β-CD/allyl isothiocyanate complexes into polylactic acid films (Wang *et al.*, 2017). At levels of 0.8% (w/w) of the natural EO component, the packaging film was found to be effective against *S. aureus, Salmonella, E. coli, B. subtilis and Penicillium, Aspergillus niger.* The assay for the AM activity experiment was carried out by a colony counting method where the overnight cultures were adjusted to a specific final optical density in liquid medium then serially diluted with the AM film placed onto an agar surface. Aloui and Khwaldia (2016) reviewed the various methods used to assess the activity of AM films and in most studies, the authors had noted that a typical colony

counting method (Aytac *et al.*, 2014) was used. In this method, known volumes taken from overnight cultures are placed into a liquid medium where the AM additives or film pieces are placed. After 24 h, a known volume of the medium is serially diluted and spread onto agar media for incubation and subsequent colony counting.

Given the limited literature on the use and effectiveness of β-CD/AM agent complexes in food packaging films, this study aimed at incorporating the AM agents thymol, carvacrol and linalool that were complexed with β-CD into LDPE films. In this chapter, the AM activity of the films *in vitro* as well as the effects of glycerol concentration on the release and AM activity of the packaging films was investigated.

6.3 Materials and Methods

6.3.1 Polymer and Chemicals

The films were prepared using LDPE grade XJF143/1700, obtained from Qenos, Australia. Ethanol (absolute) was purchased from Merck, Australia, and the following chemicals were purchased from Sigma-Aldrich, Australia: β-CD (98.5% purity and 1.5% (w/w) water content), thymol (99.5% purity), carvacrol (98% purity), linalool (97% purity), ethylene glycol, 1,2-dimethoxyethane, and polyethylene glycol 400. Milli-Q water was used in all preparations.

6.3.2 Media and Microorganism

Bacteriological agar (LP0011) and bacteriological peptone (LP0073) were purchased from Oxoid, UK. Lysogeny broth (LB, MA 755) was purchased from Amyl Media, Australia. The microorganism used was *Escherichia coli* (ATCC 25922) and was obtained from the culture collection of Food Science Australia, Werribee, Victoria, Australia.

6.3.3 Preparation of Inclusion Complex

The complexes were prepared in accordance with methods described in Chapter 3 (Al-Nasiri *et al.*, 2018) where β-CD was combined with the AM agents thymol, carvacrol, and linalool, using a 1:1 mole ratio. Briefly, the β-CD (3.8352 g, 3.33 mmol) was dissolved in 60 mL of water at 55°C then the AM agent was added and the reaction mixture maintained under stirring at 55°C for 4 h. The heating was then discontinued and

the reaction mixture was further stirred for 4-5 h and then cooled to 4°C where it remained for 24 h. The precipitated complexes were recovered by vacuum filtration and dried in a vacuum desiccator for 1 h. The vacuum was then removed and the complexes remained in the desiccator for a further 12 h.

6.3.4 Incorporation of Free AM Agents in LDPE Films

A mass of 0.1 g of AM agent was dissolved in 5 mL of 90% (v/v) ethanol/water and mixed until the agent was dissolved completely. Powdered LDPE (9.9 g) was added to the agent/ethanol solution and the slurry was thoroughly mixed for a few minutes to ensure a homogeneous mixing of the agent with the polymeric particles. The mixture was then left to dry in air for 210 min to evaporate the ethanol. A sample weighing *ca*. 3 g of the resultant solid mixture was placed between Mylar™ films that were positioned between a set of aluminium plates. Film samples were created by pressing the solid mixture in a laboratory press (IDM Instruments Pty. Ltd., Australia, Model No. L0003), that was preheated to 120°C. The upper and lower platens of the press were maintained at a temperature of 120°C for 4 min under a pressure of 20 kPa. The plates were quench-cooled with water to 35°C, then removed from the press and the film samples removed from between the aluminium plates. The films were peeled away gently from the Mylar™ after the film had cooled completely. The films were wrapped in aluminium foil after pressing and were analysed within 24 h. The thickness of the films was measured at 5 different positions using a hand-held digital micrometer (Schut IP54, The Netherlands) with a precision of 0.001 mm and the average thickness was calculated from the readings.

6.3.5 Incorporation of Inclusion Complex in LDPE Films

Films containing 1, 1.5, 2, 3 and 5% (w/w) of the AM agent present in the form of the β-CD complex were made by respectively producing films containing 8.6, 12.83, 17.11, 25.66, and 42.77 % (w/w) of β-CD complex. The procedure involved firstly weighing the appropriate amount of the β-CD complex and manually dry mixing this with the appropriate mass of LDPE powder for a few minutes, using a spatula to obtain a homogenous distribution of the complex in the LDPE. A sample weighing *ca*. 3 g of the resultant solid mixture was then pressed using the same optimized conditions that were used for producing films as described above.

6.3.6 AM Activity by Agar Plate Test Method

The film and AM agents were tested for their inhibition against the target microorganism *E. coli* (a Gram-negative bacterium) by using an agar disc diffusion method (Sung *et al.*, 2014). The test culture used in the microbiological assay was prepared from fresh cultures. A single colony of *E. coli* was incubated in LB media for 23 h after which 1 mL was taken and added to another 9 mL of LB and further incubated until a cell density of 10^6 CFU mL^{-1} was achieved. The time required to achieve a cell density of 10^6 CFU mL^{-1} was confirmed by the colony counting method (Feyzioglu and Tornuk, 2016). One mL of the culture was serially diluted into 9 mL of peptone 0.1% (w/v), then 100 μL of peptone water was placed onto the surface of an agar plate. The agar plates prepared as such were incubated for 24 h at 37°C and the colonies were then counted. Once the colony counts were confirmed, the prepared agar plates were surface seeded with 100 μL of the final test culture.

To evaluate the viability of each AM agent after its complete release from the β-CD matrix, a sample of each β-CD/AM complex was dissolved in water (*ca.* 86 mg mL^{-1}) and a 10 μL aliquot of the resulting solution was placed in the centre of an agar plate that was previously seeded with the test culture. The plates were incubated at 37°C for 24 h to assess the AM agent activity.

To evaluate the AM activity of the LDPE film formulations, samples of the films were cut into circles of 25 mm in diameter and aseptically placed on the agar plates which were then incubated for 24 h at 37°C. Evaluation of the inhibitory activity was carried out by measuring the inhibition zones with an average of 6 plates for the control (free agent) films and 16 plates for the β-CD/AM agent complex films. A Vernier calliper (Mitutoyo, Japan) was used to measure the zone of inhibition with an average of two measurements, taken 45° apart from each other, recorded for each test. Error bars and errors presented in the tables and text are based on the standard deviation from the mean.

6.3.7 Preparation and Testing of Films Containing Glycerol

Additional sets of films containing glycerol were prepared by adding different concentrations of glycerol into the film formulation (see Table 6.1). The films were produced as described above and the AM activity was also tested in accordance with the method presented above.

6.3.8 Release of Thymol from Films

Gas chromatographic analyses were performed using a Varian Star 3400-CX gas chromatograph (GC) equipped with a fused silica capillary column (DB-5, 30 m × 0.32 mm i.d., film thickness 0.25 µm, J & W Scientific, USA). The GC was operated using the following conditions: injection volume: 1.0 µL; initial column temperature: 80°C; heating rate: 5°C min^{-1}; up to 180°C, kept at this temperature for an additional 1 min; injector temperature, 250°C; split ratio 1:60; FID detector temperature: 300°C; and carrier gas: nitrogen.

6.4 Results and Discussion

6.4.1 Activity of Free and Encapsulated AM Agents *in vitro*

The films containing the AM agents either free or encapsulated at levels of 1% (w/w) showed no detectable inhibition against *E. coli (ATCC 25922)* in the agar disc diffusion test see (Table 6.1). At a concentration of 2% thymol and 3% (w/w) carvacrol in the films where the AM agents were free, a clear zone of inhibition around the films was observed suggesting this concentration was sufficient to inhibit the strain of *E. coli* used in this study. The appearance of the clear zone of inhibition is an indication that the AM additives were released from the film samples and diffused into the agar layer, preventing the development of microbial growth in the medium (Suppakul *et al.*, 2008). However, in the present study, films containing free linalool did not show any detectable inhibition against *E. coli*, even at concentrations in the film as high as 10% (w/w). Therefore, films containing free linalool prepared as such were not further investigated as potential AM packaging films. It is interesting to note that Suppakul *et al.* (2008) reported linalool and methyl chavicol at 1% (w/w) concentrations, with retained concentrations of 3.38 mg g^{-1} LDPE, showed inhibition against *E. coli* (FSA 1301) although in that study the strain of *E. coli* was different to that used in the present study.

Although the films with either free thymol or free carvacrol at 2% (w/w) concentration showed microbial inhibition, the films containing encapsulated thymol or carvacrol in the absence of glycerol failed to show inhibition even at concentrations as high as 5% (w/w) of these AM agents see (Table 6.1 and Figure 6.1). The addition of high concentrations of AM agents to polymeric materials can significantly affect the physical,

and optical properties of the ultimate packaging formulation as some of the AM agents may not adequately mix with packaging material matrix (Han, 2005). The optical properties in particular are important aspects in food packaging to allow the consumer to clearly see the contents of the package (Han, 2005). High concentrations of AM additives can result in a loss of transparency and a change in the color of the packaging material.

Table 6.1: Zone of inhibition for AM film formulations against *E. coli*

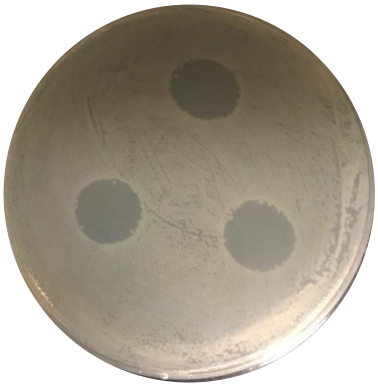
Mode of AM addition to LDPE Film	AM additive(s)	AM additive content /% (w/w)	Glycerol Content /% (w/w)	Zone of inhibition /mm
Free	Thymol	1	0	none
		2	0	30.82 ± 0.84
		2	2	27.70 ± 0.30
		3	0	34.50 ± 1.70
	Carvacrol	1	0	none
		3	0	33.42 ± 1.31
	Linalool	1	0	none
Encapsulated	Thymol	1	0.5	none
		2	0.5	none
		3	0.5	none
		1.5	2	<26[#]
		2	1	27.13 ± 0.19
		2	1.5	29.31 ± 0.48
		2	2	30.70 ± 0.72
		3	1	28.06 ± 0.25
		3	2	36.13 ± 0.68
		5	0	none
	Carvacrol	2	2	none
		3	1.5	26.44 ± 0.40
		3	2	29.61 ± 0.86
		5	0	None

Note: [#]zone of inhibition just visible around 25 mm diameter film.

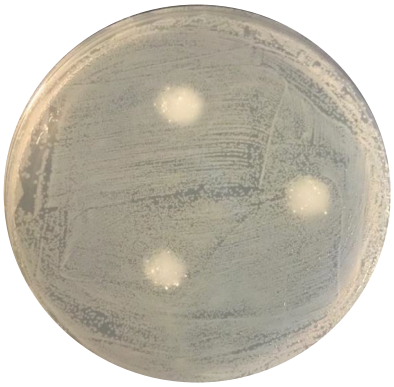
Further testing was performed to investigate possible reasons behind the low AM activity at such high concentrations of encapsulated agents. In particular, factors influencing the binding of the AM agent in the β-CD capsule was explored by microbial inhibition tests following dissolution of the β-CD and the subsequent complete release of the AM agent. As shown in Figure 6.1, inhibition against the targeted microorganism was observed for the complexes containing thymol and carvacrol with the exception of the complex containing linalool that did not show inhibition against the targeted

microorganism. The inhibition demonstrated by the released thymol and carvacrol suggests that the encapsulation process did not alter the physical or chemical properties of these AM agents.

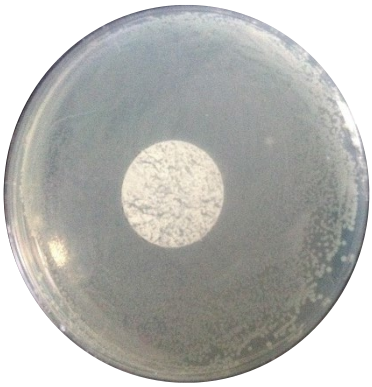
Dissolved β-CD complex with thymol



Dissolved β-CD complex with carvacrol

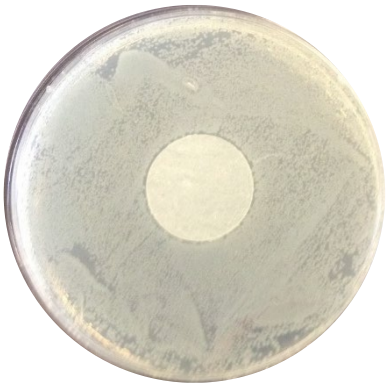


Dissolved β-CD complex with linalool

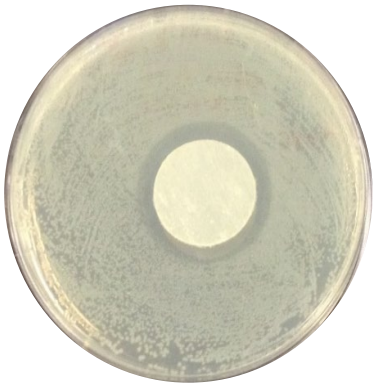


LDPE film with β-CD complex (5% (w/w) thymol)

LDPE film with β-CD complex (1% (w/w) thymol and 2% (w/w) glycerol)

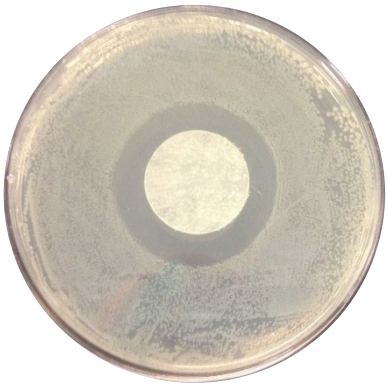


LDPE film with β-CD complex (1.5% (w/w) thymol and 2% (w/w) glycerol



LDPE film with β-CD complex (2% (w/w) thymol and 2% (w/w) glycerol)

LDPE film with β-CD complex (3% (w/w) thymol and 1% (w/w) glycerol)



LDPE film with β-CD complex (3% (w/w) thymol and 2% (w/w) glycerol)

Figure 6.1: *E. coli* growth plates with different treatments

In a similar study, Guarda *et al.* (2011) investigated the AM activity of thymol and carvacrol encapsulated in gum Arabic (GA) using the agar plate test. A bi-axially oriented polypropylene film was coated with the encapsulated agent emulsion, dried at room temperature and tested against a range of microorganisms. The encapsulated AM agents did not show inhibition at any of the concentrations that were tested (1%, 2%, 5% and 10%), however, inhibition was observed when both encapsulated thymol and carvacrol were mixed together at 10% each in the formulation. The authors suggested this is due to the relatively low release of the AM agents that resulted from their high attraction to the GA as this encapsulation matrix contains a fraction of glucuronic acid that develops negative charges around the particles in an oil emulsion (Guarda *et al.*, 2011). In addition, the release rate of the AM agent is decreased by arabinogalactan-protein fraction of the GA that has a high viscosity. Similar strong interactions between the AM agents and β-CD may be the cause of the lack of inhibition against *E. coli* observed in the present study whereby the AM agents are tightly bound in the β-CD capsule and unable to migrate from the structure.

6.4.2 Effects of Glycerol on the Release of Encapsulated AM Agents

The efficient migration of AM additives is important in order to protect foodstuffs against contamination (Peltzer *et al.*, 2009). The incorporation of other additives can potentially facilitate the release of some AM agents as long as they are compatible with the polymer matrix. For example, Weng and Hotchkiss (1993) reported that AM agents including sorbic acid, propionic acid and benzoic acid did not adequately incorporate into LDPE and did not diffuse in sufficient amounts to inhibit the growth of mould. By adding benzoic anhydride, better incorporation into the LDPE matrix was achieved as well as increased effectiveness of inhibiting mould growth. In the present study, as shown in Figure 6.2 and the associated kinetic parameters shown in Table 6.2, the addition of glycerol expedited the release of the AM agents from the β-CD cavity into 95% (v/v) ethanol food simulant. However, the release of free thymol in the presence of glycerol was impeded when compared to free thymol alone.

For films containing free thymol, the time to reach 99.9% release was 3.5 times less than that for films containing free thymol with glycerol (see Figure 6.2). In a similar study, Xiao *et al.* (2011) found that the release of thymol from zein microcapsules was influenced by the presence of surfactant (Tween 20) due to the reduced hydrophobic

interactions between thymol and zein. At high surfactant levels, the release rate of thymol was expected to be higher, yet the authors reported a converse observation. It was suggested that at high levels of Tween 20, thymol may be dissolved in the surfactant micelles that are much bigger than individual thymol molecules which can limit the diffusion through the capsule matrix resulting in a lower release of thymol. Nonetheless, Ramos et al. (2013) point out that the addition of plasticizers such as glycerol to polymer formulations can lead to a decrease in intermolecular forces along polymer chains thereby increasing chain flexibility and, presumably, facilitating the transport of small molecules such as thymol through the polymer matrix. In the present study, the former case is observed where the presence of glycerol inhibited the release of free from the LDPE matrix.

Table 6.2 Kinetic parameters of AM agent release from LDPE films

System	Percentage released after 30 days	First order rate constant, $k_1 \times 10^3$/h^{-1}	Initial release rate, $v_0 \times 10^3$/mg h^{-1}	Time to reach 99.9% release, $t_{99.9}$/h	Diffusion Coefficient, $D \times 10^{12}$/mg m^{-2} h^{-1}
Free thymol	94.0 ± 3.6	7.91	28.1	846	33.7
Free thymol with glycerol	92.9 ± 0.9	2.29	7.4	2970	8.09
β-CD/thymol with glycerol	99.9 ± 0.03	2.90	14.1	2290	15.4

In the case of films containing encapsulated thymol in the presence of glycerol at levels above 1% (w/w) inhibition of the target microorganism was observed (see Table 6.1) suggesting that the addition of glycerol to the formulation facilitates the release of the thymol from the complex. The release of encapsulated AM agents in the presence of glycerol in the formulation may be due to the high hydrophilicity and structure of the glycerol molecules which can, in turn, enhance the adsorption of water molecules by the β-CD capsule thereby decreasing the rigidity of the capsule (Cerqueira *et al.*, 2012). In addition, β-CD is also known to increase the water solubility of AM agents (Mourtzinos *et al.*, 2008) which may have contributed to the observed release of the AM agents from these formulations.

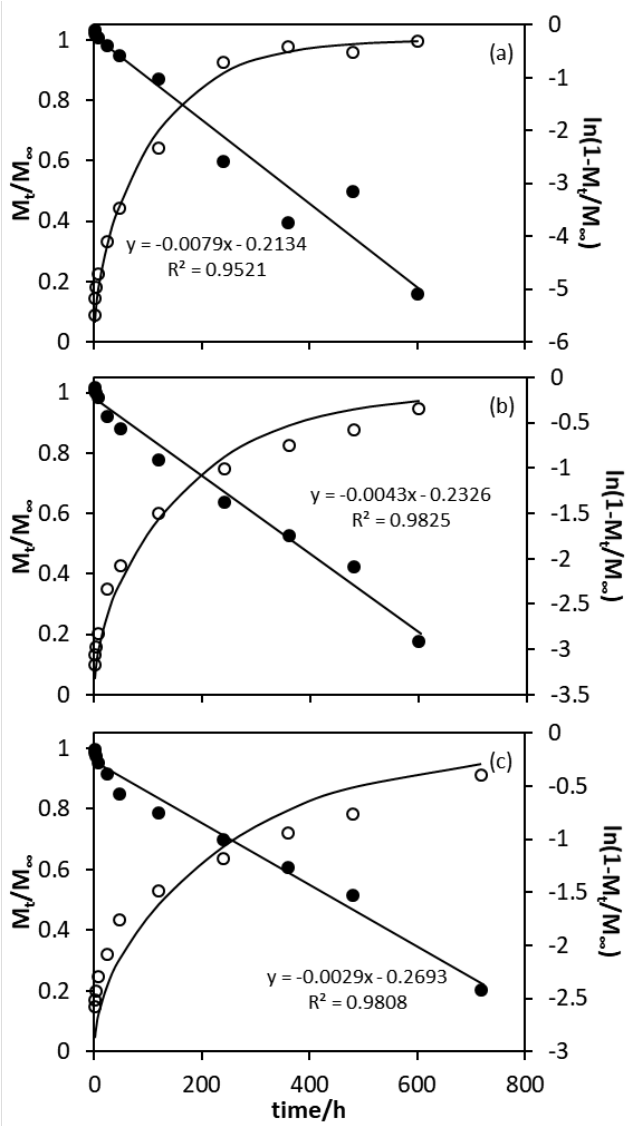


Figure 6.2: Plots of M_t/M_∞ and $\ln(1-M_t/M_\infty)$ *vs* time for release of AM agents (a) free thymol, (b) free thymol with glycerol and (c) β-CD/thymol with glycerol from LDPE films into 95% (v/v) ethanol at 4°C

At elevated temperatures such as those used during film processing, the addition of glycerol can result in some loss of the AM additives. The retention of encapsulated thymol after film processing was found to be 97.5 ± 1.6 % in the absence of glycerol in the formulation compared with a retention of 93.6 ± 1.1% in the presence of 2% (w/w) glycerol. The increased loss of AM agent during film processing in the presence of glycerol may be due to increases in the macromolecular chain mobility that glycerol imparts which facilitates the opening of the polymer matrix network and loss of AM additives (Kurek *et al.*, 2012, Koupantsis *et al.*, 2016).

6.4.3 The Effect of Glycerol on Free and Encapsulated AM Activity

The hydrophilic and hydrophobic groups of AM agents are the vital structural characteristics that determine the resulting AM activity (Davidson and Branen, 2005). The hydrophilic part enables the AM agent to be soluble in the water phase where the growth of microorganisms primarily occurs, and the hydrophobic properties enhance the lipophilicity that enable AM agents to react and act on the hydrophobic membrane of the microorganisms (Feyzioglu and Tornuk, 2016). Figure 6.1 shows that the addition of glycerol to the formulation facilitated the release of the AM agents from the film formulations containing encapsulated thymol and carvacrol in some cases resulting in a zone of inhibition.

In the case of films containing encapsulated thymol no zone of inhibition was noticed using a glycerol concentration of 0.5% (w/w), even when the AM agent concentration was increased to 3% (w/w). Increasing the glycerol concentration to 1% (w/w) and above resulted in the appearance of inhibition zones. In such cases, for a given thymol concentration of 2% (w/w) or 3% (w/w) the zone of inhibition increased with an increasing concentration of glycerol in the formulation. A similar effect was observed for formulations containing encapsulated carvacrol at a level of 3% (w/w). Increasing the concentration of glycerol may have resulted in changes in the polymer network creating mobile regions with greater interchain distances, thereby promoting water clustering and facilitating the AM agent release (Aloui *et al.*, 2011, Cerqueira *et al.*, 2012). Furthermore, the release of an AM agent from β-CD in the presence of glycerol, is also influenced by the bond between the AM agent and the β-CD. As a result, glycerol can replace the AM agent in the β-CD structure and facilitate its release from the film (Kurek *et al.*, 2012).

A comparison between control formulations containing 2% (w/w) free thymol alone and those control films containing 2% (w/w) free thymol in the presence of 2% (w/w) glycerol indicates that the addition of glycerol to the formulation decreases the zone of inhibition. This is in contrast to the greatly enhanced inhibition imparted by the addition of 2% (w/w) of glycerol to formulations containing 2% (w/w) thymol encapsulated in β-CD (see Table 6.1). This finding is consistent with the release results (see Figure 6.2) and may be due to interactions between glycerol and free thymol which decreases the activity of the thymol. These results are also in agreement with the work of Agustin and Padmawijaya (2017) who found that the addition of glycerol as a plasticizer

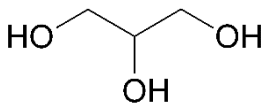
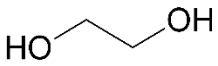
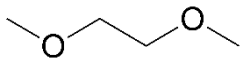
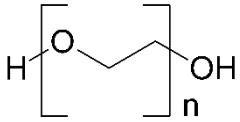
in chitosan decreased the AM activity of the chitosan film. The authors suggested that glycerol occupied the space between chitosan hydrogen bonds resulting in a weakened chitosan structure which consequently affected the AM activity of the chitosan.

The addition of glycerol in films containing β-CD provides more active sites in the β-CD by exposing its hydrophilic hydroxyl groups in which the water molecules could be adsorbed (Mali *et al.*, 2006). Although thymol and carvacrol are isomers, thymol showed consistently higher AM activity than carvacrol and this has also been reported previously by Liolios *et al.* (2009) and Ramos *et al.* (2012). This may be due to the higher solubility of thymol in the water-based agar compared to carvacrol as well as the faster diffusion of thymol.

6.4.4 Release of AM Agents in the Presence of Other Additives

In addition to glycerol, other additives were investigated as potential release facilitating compounds namely: ethylene glycol, 1,2-dimethoxyethane and poly(ethylene glycol) (PEG-400). Although 1,2-dimethoxyethane is not a food-grade additive, it was utilized to provide a better understanding of the reasons behind the effectiveness of glycerol addition in aiding the AM agent release from the β-CD in the film formulations. As shown in Table 6.3, film formulations containing the addition of the high molecular weight compound PEG-400 which contains only end-group hydroxyl moieties and 1,2-dimethoxyethane which does not contain hydroxyl groups, did not show any inhibition of microbial growth. In contrast, film formulations containing glycerol and ethylene glycol, which are both relatively low molecular weight compounds that contain hydroxyl functional groups, were found to be effective in facilitating the AM activity. It therefore appears that the effectiveness of glycerol is due to its physical size and chemical nature which can increase the molecular mobility of AM agents within the polymer matrix once they are released (Souza *et al.*, 2012). Furthermore, it is conceivable that glycerol is able to penetrate the β-CD *via* hydroxyl group interactions with the β-CD and thereby facilitate the displacement of the AM agent from the wall. The results in Table 6.3 are consistent with this proposed mechanism and with the notion that the low molecular weight additives will have greater mobility within the polymer matrix than the high molecular weight additives and as such are more efficient in facilitating the AM agent release from β-CD.

Table 6.3: The effect of additives on the thymol/β-CD AM activity

Compound	Structure	Molar mass/g mol^{-1}	Zone of inhibition/mm
Glycerol		92.09	36.13 ± 0.68
Ethylene glycol		62.07	31.56 ± 0.53
1,2-dimethoxyethane		90.12	none
Polyethylene glycol		380-420	none

6.5 Summary

Free thymol and carvacrol at 2% (w/w) showed inhibition against targeted bacterium whereas linalool did not show any detectable inhibition even at a concentration as high as 10% (w/w). Films containing encapsulated thymol or carvacrol did not show any detectable inhibition even at 5% (w/w) loading with respect to the AM agent. Glycerol was found to facilitate the release and subsequent AM activity of the produced films. The release rate and AM effectiveness of the encapsulated AM agents in LDPE films depended on the glycerol loading in the film formulation. The minimum concentration of glycerol required to produce AM activity in films containing the encapsulated AM agents was found to be 1% (w/w). Increasing the concentration of glycerol from 1 to 2% (w/w) increased the zone inhibition by 28.8% which supports the notion that glycerol facilitates AM release from the β-CD complexes by exposing hydrophilic hydroxyl groups in the complex enabling water molecules to be adsorbed. The control films containing the free AM agent in the presence of glycerol showed a lower extent of AM release than films containing the AM agent in the form of the β-CD complex suggesting that glycerol interacts directly with the free AM agent in the film. In addition, β-CD increases the solubility of thymol and therefore increased its release rate and AM activity. The results suggest glycerol can be used to facilitate AM agent release from β-CD complex, control its rate of release and therefore its activity.

Chapter 7 Activity of β-CD Complexes on Real Foods

7.1 Overview

In this chapter, the previously optimized formulations of thymol/β-CD in LDPE films were tested on real food products, namely minced beef and chicken fillet, that are susceptible to microbial spoilage. Since the meat products are also high in fat content, particularly minced beef, the potential for the system to impart AO activity is also investigated *in vitro* and *in vivo*.

7.2 Introduction

Monitoring microbial and non-microbial degradation in meat and poultry products is vitally important to meet increasing global demands for fresh protein-based foods. Meat and poultry products typically contain high levels of polyunsaturated fatty acids which can undergo oxidation under ambient conditions (Botsoglou *et al.*, 1994, Pignoli *et al.*, 2009). Although the presence of oxygen contributes to maintaining the red colour of meat (López-De-Dicastillo *et al.*, 2012), browning as a result of oxidation can result over time and this will generally be rejected by consumers. In addition to colour changes, oxidative processes can result in other sensorial changes to aroma and off-flavours (Nerín *et al.*, 2006, Guyon *et al.*, 2016). In the case of minced meats, the increased surface area can result in increased oxygen exposure and therefore accelerate oxidation (Tang *et al.*, 2001, Bao *et al.*, 2016). Minced meats are also susceptible to microbial spoilage due to the higher levels of moisture and nutrients (Andrés *et al.*, 2017) as well as the increased handling to process fresh cuts into minced products. It is therefore not surprising that minced meats are more susceptible to colour deterioration and oxidation than their uncut counterparts (Gomez *et al.*, 2016).

Active packaging technologies such as modified atmosphere packaging with high levels of oxygen are commonly used to preserve the colouring of red meat. However, high oxygen concentrations can initiate the oxidation of lipids and proteins and in the case of the latter, result in the formation of protein radicals which may lead to carbonylation of amino acid side chains, loss of free thiol groups and the formation of protein cross-links (Bao and Ertbjerg, 2015, Bao *et al.*, 2016). In the case of lipid oxidation, secondary

oxidation products can include aldehydes, ketones, alcohols, acids, and hydrocarbons which can adversely affect the organoleptic and nutritional properties of the meat products (Guyon *et al.*, 2016, Jung *et al.*, 2016).

There are several methods used to measure the level of lipid oxidation in meat and other food products. In one common method, malondialdehyde (MDA), which is a by-product of lipid oxidation, is reacted with thiobarbituric acid reactive substances (TBARS) under acidic conditions forming a pink/red colour and the concentration of MDA is measured spectrophotometrically at 532–535 nm (Jung *et al.*, 2016). Known as the TBARS method, this testing has been widely utilized for measuring the oxidation of various meat products and in one example, the TBARS value of minced beef stored at 3°C and in 0% oxygen increased from 0.03 to 0.34 mg MDA kg^{-1} over 6 days of storage (Bao *et al.*, 2016). The same study showed that lipid oxidation increased with increasing oxygen levels in modified atmospheres with the TBARS value increasing from 0.17 to 1.24 mg MDA kg^{-1} after 6 days of storage in the presence of 80% oxygen.

Synthetic chemical AOs such as butylated hydroxytoluene (BHT), butylated hydroxyanisole, propyl gallate, tertiary butylhydroquinone, trihydroxy-butyrophenone, nordihydroguaiaretic acid and ethoxyquin are commonly used to control oxidation processes (Nerín *et al.*, 2008) in meat products (Tang *et al.*, 2001, El Abed *et al.*, 2014). Nitrite is another common meat additive used for multiple purposes including controlling microbial growth, imparting a notable reddish–pink colour, and controlling lipid oxidation (Jung *et al.*, 2016, Ko *et al.*, 2017). However, the aforementioned and other synthetic chemical additives are perceived negatively by consumers due to reports of carcinogenic side-effects. For example, residual nitrite in meat products may react with certain amines in food to produce *N*-nitroso compounds, such as nitrosamines, which are known carcinogens (Young Mi *et al.*, 2017).

Natural AO and AM agents are increasingly popular with consumers and researchers with those derived from EOs particularly attractive (Nerín, 2012). For example, many research studies have studied thymol, a herbal extract, for its beneficial AM and AO properties for a range of different applications (Liolios *et al.*, 2009, Guarda *et al.*, 2011, Wattanasatcha *et al.*, 2012, Wu *et al.*, 2012, Li *et al.*, 2013, Zhang *et al.*, 2014b, Chen *et al.*, 2015b, da Rosa *et al.*, 2015). The limitations of using natural volatile

AM agents include their excessive loss during processing of food packaging, high evaporation rate and possible food tainting effects (Bhandari *et al.*, 2001, Sung *et al.*, 2014, Olmedo *et al.*, 2015, Fang *et al.*, 2017, Soroush and Hossein, 2017). The encapsulation of such natural agents for incorporation in food packaging films is an effective technology that can both protect volatile substances from processing losses and control the release to impart AM and AO activity (Al-Nasiri *et al.*, 2018).

Currently, there are few reports on the use of inclusion complexes in real food applications. Therefore, the objectives of this study were to evaluate the effectiveness of thymol encapsulated in β-CD in LDPE films to impart AM and AO activities on minced beef and chicken breast fillets.

7.3 Materials and Methods

7.3.1 Chemicals and Bacteria

The following chemicals were purchased from Sigma-Aldrich (Australia): beta-cyclodextrin (β-CD, ≥ 97% purity, 1.5% water content), thymol (99.5% purity), butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA), malondialdehyde precursor 1,1,3,3-tetraethoxypropane (TEP), 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH), and ethanol. Trichloroacetic acid (TCA) was purchased from Merck (Australia) and Milli-Q water was used in all preparations. Specific *E. coli* and *E. coli*/coliform 3M Petrifilms™ were purchased from Thermofisher Australia and all other media and microorganisms used were the same as those described in Section 6.3.2.

7.3.2 Preparation of Complex

The thymol/β-CD complex was prepared according to the method described previously in Chapter 3 .

7.3.3 Incorporation of Inclusion Complex in LDPE Films

The thymol/β-CD inclusion complex was incorporated into LDPE films according to the method described previously in Chapter 5. Film formulations containing 1% and 3% (w/w) levels of thymol with respect to LDPE were prepared both with and without glycerol.

7.3.4 Antioxidant Activity of Film using DPPH Assay

The AO activity of the LDPE film samples was assessed using the DPPH method where the film samples were immersed directly in the ethanolic DPPH solution. The solution was prepared by dissolving *ca*. 10 μg g^{-1} of DPPH in ethanol which was further diluted to obtain an initial absorbance of *ca*. 0.9. Sample strips of film were immersed into 10 mL of the DPPH solution and were stored in darkness prior to measuring their absorbance at 515 nm using an Aqualog® benchtop spectrofluorometer (Horiba Instruments Inc., California, USA). The absorbance was measured at 2 h, 4 h and 24 h after immersion with the sample aliquots returned to the test tubes following the absorbance measurement. The AO activity of the samples was determined as the percentage of inhibition of DPPH, AO%, calculated using $AO\% = [(A_O - A_S)/A_O] \times 100$ where A_O is the absorbance of the blank and A_S is the absorbance of the sample. All samples were tested in triplicate and the results expressed as the mean ± standard deviation of the measurement.

7.3.5 Antioxidant Testing on Minced Beef using TBARS Assay

The AO analysis protocol used was described previously (Botsoglou *et al.*, 1994) with minor changes to the filtration process. Briefly, minced beef samples of *ca*. 5 g were wrapped tightly with the prepared LDPE films containing the thymol/β-CD complex and were then placed in polyethylene bags and heat sealed. The packaged meat samples were stored at 4 ± 1°C for up 12 days with samples removed every 3 days for analysis. Samples weighing 2.5 g were transferred into a 50 mL centrifuge tube to which 8 mL of 5% (w/v) aqueous TCA and 5 mL of 0.8% (w/v) BHT in hexane was added. The samples were processed using an Ultra-Turrax® homogenizer for 30 s at high speed prior to centrifugation for 3 min at 3000 rpm. The top (hexane) layer was discarded and the aqueous bottom layer was filtered under vacuum prior to making up the volume to 10 mL with 5% (w/v) TCA. The extract was then filtered using a Phenex RC 0.45 μL syringe filter to remove any residues, coloured species and/or particles that could otherwise interfere with the absorbance measurement and a clear solution was obtained. A 2.5 mL aliquot was pipetted into a screw-capped tube to which 1.5 mL of 0.8% (w/v) aqueous TBA was added. The tubes then were incubated for 30 min in a water bath at 70°C and cooled under running water. A blank sample was prepared using equal amounts of TCA and TBA which was also incubated in a water bath at 70°C and mixed until all the solution

was clear. The absorbance of the samples was measured at 532 nm using a Shimadzu UV spectrophotometer against the prepared blank.

Standards of different MDA concentrations were prepared by transferring 1 mL of the standard solutions into screw-capped tubes which were diluted with 1.5 mL of TCA and 1.5 mL of TBA. The tubes were then incubated at 70°C in a water bath for 30 min prior to cooling and measuring the absorbance at 532 nm as described previously. The calibration curves were obtained by plotting the absorbance *vs* concentration in ng mL^{-1} with the concentration of MDA in the sample extracts calculated from the calibration curve. The concentration of MDA was determined in samples using the formula: MDA = $V_R C_{MDA} D_E / W_S$ mg kg^{-1} where, V_R is the total volume of the reaction sample (i.e. 4 mL), C_{MDA} is the MDA concentration in the sample extract obtained from the calibration curve (converted to mg), D_E is the dilution factor of the extract in the original sample, and W_S is the mass of the meat sample (kg).

7.3.6 Antimicrobial Activity of Film on Meat Products

The methods of inoculation, packaging and analysis were in accordance with those described by Sung *et al.* (2014) with some modifications. Minced beef slaughtered according to Halal requirements was purchased from a local supermarket and samples weighing *ca.* 5 g were exposed to UV light for 30 min to sterilize the surface. For the inoculum, *E. coli* was suspended and activated twice in nutrient broth at 37°C for 24 h to obtain fresh bacterial cultures with a concentration of 10^6 CFU mL^{-1} (Feyzioglu and Tornuk, 2016). The sterilized meat samples were then inoculated with 0.1 mL of the freshly grown *E. coli* solutions that were spread evenly over the surface to obtain a concentration of around 10^5 CFU g^{-1}. The maximum bacterial level in meat that is considered to be acceptable for meat and meat products is between 6 (Khan *et al.*, 2016) and 7 $\log_{10}$ CFU g^{-1} (Andrés *et al.*, 2017).

The samples were then placed inside a biosafety cabinet for 30 min to facilitate bacterial attachment before being wrapped tightly in the prepared thymol/β-CD LDPE films, packaged into sterile polyethylene bags and sealed using a plastic bag sealer device. Control minced beef samples were packaged directly into sterile bags prior to heat sealing with all samples then stored immediately at 4 ± 1°C for 12 days. Duplicate samples were prepared for analysis. Bacterial counts were enumerated immediately after inoculation

and periodically after 4, 8 and 12 days of inoculation by using the serial dilution method. On the sampling days, two packages from each formulation were aseptically opened, 50 mL of 0.1% (w/v) saline peptone water was added, and the mixture was homogenized using a laboratory paddle blender masticator for 3 min. Serial dilutions were performed by withdrawing 1 mL of sample into 9 mL of the peptone water to make up 10 mL. Following this, 1 mL of each homogenate dilution was placed on 3M Petrifilm™ count plates and incubated for 24 h at 37ºC prior to enumeration. The final bacterial cell density was expressed in units of $\log_{10}$ CFU g^{-1}.

Raw chicken breast fillets were purchased from the same butcher and in this case, the skin was removed, and the chicken cut into cubes of *ca*. 7-9 g. The same procedures were followed as described above for the minced meat.

7.4 Results and Discussion

7.4.1 Antioxidant Activity of Films by DPPH Inhibition

The AO activity of encapsulated thymol released from LDPE films into ethanol was evaluated using the DPPH radical assay. As shown in Table 7.1, the highest inhibition (70.3 ± 3.3) was observed after 24 h for the film containing thymol/β-CD with 2% (w/w) glycerol. Ramos *et al.* (2014) reported the AO activity of 8% (w/w) thymol from polypropylene film released into isooctane resulted in 42.2% DPPH inhibition. The high AO activity in this study using encapsulated thymol at a lower concentration may be due to the complexation process providing both stabilization and increasing solubility into the DPPH/ethanol solvent (Celebioglu *et al.*, 2018).

The control films containing just β-CD or β-CD with 2% (w/w) glycerol also showed some DPPH inhibition. This result contrasts with the work of Santos *et al.* (2015) who reported that free β-CD did not show AO activity when compared to β-CD/carvacrol inclusion complexes using the Trolox equivalent AO capacity method. The difference in results may be due to the different methods that have been used. Nonetheless, in the case of the DPPH assay, Aree and Jongrungruangchok (2018) observed some inhibition with free β-CD and suggested that it probably resulted from the partial encapsulation of DPPH in the β-CD cavity. Moreover, cyclodextrins are reported to act as secondary AOs, preventing food browning and improving the AO capacity of foods (Aree and

Jongrungruangchok, 2018) which supports the present findings of AO activity for β-CD alone and/or with the addition of glycerol. Films containing encapsulated thymol with and without the addition of glycerol both showed AO activity, with the presence of glycerol in the film resulting in between 5 and 25% greater inhibition compared to films without glycerol. The same trend was observed for the films containing free β-CD with and without glycerol although the extent of the inhibition was considerably lower in comparison with the systems containing thymol.

Table 7.1: DPPH AO activities of thymol/β-CD/LDPE films for thymol released into ethanol up to 24 h

	Percentage DPPH inhibition		
	2 h	**4 h**	**24 h**
β-CD	4.20 ± 0.50	5.40 ± 0.10	8.0 ± 0.40
β-CD/glycerol(2%)[#]	7.30 ± 1.52	7.90 ± 1.60	10.10 ± 1.30
β-CD/thymol(3%)	19.90 ± 0.80	35.23 ± 2.30	66.90 ± 1.44
β-CD/thymol(3%)/glycerol(2%)	25.04 ± 1.70	41.70 ± 0.94	70.30 ± 3.30

[#]percentages shown are (w/w)

Factors such as concentration, film thickness (Bentayeb *et al.*, 2009), temperature, pH and light can all influence the AO activity of a given system (Ramos *et al.*, 2014). The choice of DPPH solvent for the analysis is also very important since β-CD is readily soluble in absolute ethanol, which was used in the present study. The use of ethanol facilitates the release of thymol from the β-CD cavity in the liquid environment and this enables a faster reduction of DPPH which is enhanced when glycerol is present in the film formation. Films containing 1% (w/w) encapsulated thymol that were stored at room temperature for 20 months also showed about 22% reduction in the DPPH assay which confirms that encapsulation can maintain some AO activity.

7.4.2 Antioxidant Activity by TBARS Assay on Minced Beef

The oxidation of lipids and proteins can result in the development of off-flavours, and increase the toughness of meat products (Bao and Ertbjerg, 2015, Bao *et al.*, 2016). Minimizing or delaying oxidation can therefore be highly beneficial to maintaining the quality of fresh meats. The ability of the thymol/β-CD/LDPE film containing 1 and 3% (w/w) thymol (relative to LDPE) with 2% glycerol to influence the oxidation of minced beef lipids was assessed using the TBARS assay and the results over 12 days of storage

at 4°C are shown in Table 7.2. The MDA level was initially 0.34 ± 0.08 mg MDA kg^{-1} for the fresh minced beef sample. During the storage period, the TBARS values increased in the samples packaged in the control films including those films containing β-CD alone and β-CD/glycerol. In contrast, the TBARS values were much lower for all samples packaged in films containing encapsulated thymol with the lowest value obtained for the minced meat packaged with the complex containing 3% (w/w) thymol with glycerol. A reduction of MDA in minced sheep meat stored at 4°C was recently reported by Ahmad Mir *et al.* (2017) where the TBARS values were 0.87, 0.77, 0.83 and 0.85 mg MDA kg^{-1} for the control, cumin, clove and cardamom respectively. Clearly, the effectiveness of EOs and their extracts depends on the type of oil that is used (Pezo *et al.*, 2008). It also depends on the levels of phenolic compounds (Seydim and Sarikus, 2006, Bentayeb *et al.*, 2009, Colon and Nerin, 2012, Akrami *et al.*, 2015, Moudache *et al.*, 2016).

Table 7.2: TBARS values of raw minced beef packaged in LDPE films containing thymol/β-CD/glycerol and refrigerated for up to 12 days

	TBARS value/mg MDA kg^{-1}		
	Day 4	**Day 8**	**Day 12**
Control film	0.71 ± 0.02	0.83 ± 0.04	0.89 ± 0.13
β-CD	0.59 ± 0.14	0.80 ± 0.02	0.88 ± 0.16
β-CD/glycerol(2%)[#]	0.67 ± 0.15	0.80 ± 0.10	0.86 ± 0.05
β-CD/thymol(3%)	0.18 ± 0.05	0.20 ± 0.02	0.30 ± 0.01
β-CD/thymol(1%)/glycerol(2%)	0.31 ± 0.03	0.32 ± 0.03	0.32 ± 0.03
β-CD/thymol(3%)/glycerol(2%)	0.28 ± 0.01	0.19 ± 0.01	0.22 ± 0.01

[#]percentages shown are (w/w)

After four days, the film containing 3% (w/w) encapsulated thymol without glycerol produced higher AO activity than the film containing the same level of thymol but containing glycerol which suggests that either glycerol inhibited the thymol AM release in the early stages or some other factors influenced the release. However, at day 12, the results further suggest that the presence of glycerol resulted in a significantly lower TBARS value compared with the same level of encapsulated thymol without glycerol. Whence it appears that the retention of thymol in the system by glycerol may be beneficial in the longer-term effectiveness of these formulations. This result also supports the notion that glycerol facilitates the longer-term release of thymol from the β-CD complex as observed in Chapter 6. Furthermore, the interactions existing among LDPE, thymol, β-

CD, glycerol, water molecules, etc., in the current system are complex and thus the nature of the system itself needs to be considered. Moisture content, for example, is crucial since the exposure of the complex to water molecules weakens the β-CD/AM agent interactions and facilitates the release of the encapsulated agent. However, it was suggested that at a_w values less than 0.6 glycerol could serve as a reasonably good protective layer against moisture sorption for food products (Cheng *et al.*, 2006). It seems at low water levels during the first few days glycerol competes with water for active sites on the β-CD, resulting in a low extent of water sorption on the surface of the β-CD. Hence, in the first few days less thymol is released from the film containing the complex in the presence of glycerol thereby imparting a lower AO activity compared with the film formulation containing no glycerol. Since water loss is one of the main deleterious factors that can affect food quality (Ayala-Zavala *et al.*, 2008), it is likely that the minced beef samples released moisture during the 12 day storage period. Consequently, the glycerol- β-CD interactions may be diminished at high moisture content allowing the matrix to sorb more water and release thymol resulting in higher AO activity. In general, a TBARS value of 0.5 mg MDA kg^{-1} indicates some oxidation (Reitznerova *et al.*, 2017) whereas values above 1 mg/kg (Moon *et al.*, 2016) and 2 mg MDA kg^{-1} (Gomez *et al.*, 2016) are indicative of unacceptable oxidation and the formation of off-odours in meat. The TBARS values in this study were below 0.5 mg MDA kg^{-1} for meat packaged in LDPE films containing the β-CD/thymol complex at the end of the analysis period.

In the case of films containing β-CD alone, the films showed a 5.6% and 16.9% reduction in the TBARS value in the presence and absence of glycerol respectively compared to the control film. In these cases, the complex may have absorbed some of the moisture content released from the minced beef in the first four days thereby reducing the oxidation of the meat. In the presence of glycerol, interactions between glycerol and β-CD may have reduced the available active -OH groups on the β-CD for water adsorption (Cheng *et al.*, 2006). In this case more water is available resulting in changes to the meat quality that, in turn, lead to slightly higher extent of oxidation. At the end of the experiment, both β-CD films produced similar TBARS values to the control film which supports the contention that in the early stages, environmental factors may have contributed to the lipid oxidation.

7.4.3 Comments on the TBARS Assay

Over recent years, there has been some conjecture on the use of the TBARS assay to measure lipid oxidation with large variations in TBARS values having been reported. In one example, the TBARS value of freshly ground beef with a control LDPE film reached more than 20 mg kg^{-1} in less than 20 days storage at 3°C, whereas the same beef packaged with film containing 1% (w/w) grapefruit seed extract reached a value of 10 mg MDA kg^{-1} within 15 days (Ha *et al.*, 2001). Nishad *et al.* (2018) reported an initial TBARS value of 0.22 mg kg^{-1}in minced goat meat which increased to 1.64 mg kg^{-1} at the end of the test storage period in the presence of nutmeg and citrus peel extracts. Other studies have reported minimal differences in meat lipid oxidation measured with the TBARS assay. For example, Oussalah *et al.* (2004) reported the incorporation of different EOs at 1% (w/v) in milk protein-based edible films applied to beef muscle slices stored at 4°C did not protect the meat samples against lipid oxidation. The authors concluded that lipid oxidation in beef muscle increased significantly with pimento and oregano/pimento EOs which is inconsistent with other research studies. Coskun *et al.* (2014), reported TBARS values of oregano or thyme EOs incorporated into soy-based edible films applied on ground beef patties at 4°C for 12 days reached 1.6, 1.4 and 1.3 mg MDA kg^{-1} at the end of storage at day 12 for control, oregano, and thyme films respectively. The authors concluded that the incorporation of EOs initially slowed lipid oxidation, however, the results were statistically insignificant when compared to the control films after day 3 of refrigerated storage. Differences in TBARS values between studies can be a result of factors such as animal species, breed, health, muscle type, diet (fat, food additives, etc.), post-slaughter processes (Guyon *et al.*, 2016), and the nature of processing (i.e. mincing, cooking etc.) (Tang *et al.*, 2001, Papastergiadis *et al.*, 2012). The reactivity of TBA with various carbonyl compounds in oxidized food in some cases can also result in an overestimation of MDA content and the subsequent TBARS value (Jung *et al.*, 2016).

In the present study, it was observed *via* visual inspection that there were changes in the colour of the minced beef between samples packaged in the LDPE/thymol/β-CD/glycerol and those packed in the control LDPE/β-CD film without thymol. The sample packaged in the presence of thymol maintained the red colour whereas those packaged without thymol had lost the red colouration and had slightly browned (data not

shown). However, the initial measurement of the MDA content suggested a high level of oxidation for the samples that had maintained their red colouration and did not appear visually to be oxidized or browned. This may suggest a problem with the quantification of MDA in meat products using the TBARS assay as a result of the formation of a yellow or orange chromogen from the reaction of TBA with various ingredients in meat products (Jung *et al.*, 2016). This may produce misleading results as these chromogens are not spectrally distinct from MDA at 532 nm (Jung *et al.*, 2016). Although filtration of the sample extract is recommended, some particles may still pass through the filter causing high turbidity which can potentially interfere with the results. Vera *et al.* (2016), reported that the presence of nanoparticles can result in spectral interferences using the DPPH method. In order to overcome these drawbacks, ultrafiltration and/or dialysis to remove the nanoparticles from the solution are required.

Some studies have suggested the use of Raman spectroscopy (Moudache *et al.*, 2017), reverse-phase high-performance liquid chromatography (HPLC) (Reitznerova *et al.*, 2017), HPLC coupled with fluorescence detection (Papastergiadis *et al.*, 2012), and HPLC coupled with UV/VIS detection systems (Jung *et al.*, 2016) to detect the MDA content directly in meat products without interference from other ingredients. Reitznerova *et al.* (2017) found that MDA concentrations measured by the TBARS spectrophotometric method were found to be overestimated by more than 25% in raw meat in contrast with reverse-phase HPLC measurements. In the present study, the potential for interference was overcome by passing the sample extract through a 0.45 µL syringe filter after the vacuum filtration step and a clear filtrate was obtained prior to the reaction with TBA. Before the syringe filtration process was applied, the absorbance of the sample extracts was 40-60% higher which suggests that interfering compounds were removed by the second filtration.

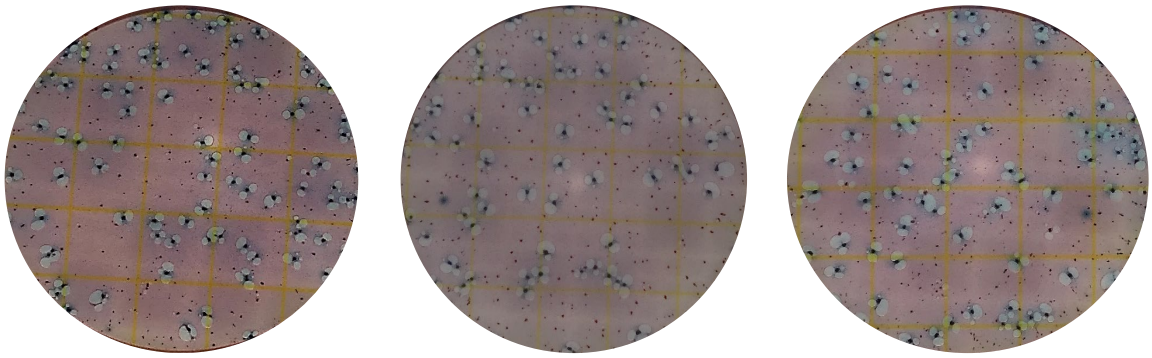
7.4.4 Antimicrobial Activity on Minced Beef

Levels of *E. coli* on inoculated minced beef were initially 4.90 ± 0.02 $\log_{10}$ CFU g^{-1} and as shown in Table 7.3, *E. coli* growth only increased slightly after 4 days then decreased to levels not significantly different from the initial level of inoculation for all samples. Examples of the growth plates are shown in Figure 7.1 and Figure 7.2 for minced beef packaged with the control film and the encapsulated thymol film respectively. In each case, *E. coli* is detected as blue colonies and all samples showed a similar level of

growth. One possible reason for the lack of *E. coli* growth on the minced beef compared to the day 1 level is related to the optimal growth temperature which is in the range of 35-40°C and the inherent survivability of this species under refrigeration (3-7°C) for up to 5 weeks of storage (Sung et al., 2014). Huang (2010) conducted a kinetic study to investigate the growth of *E. coli* O157:H7 in mechanically tenderized beef meats that stored at different temperatures and reported no growth at 5°C and only minimal growth at 10°C. This is also in agreement with Vold *et al.* (2000) who found that ground beef stored at 8°C showed no growth of *E. coli* O157:H7 when samples were stored anaerobically although samples stored aerobically increased in concentration from 10^3 to 10^4 CFU g^{-1} during the 7th day of storage. Silva *et al.* (2018a) studied a chicken breast packaging system using absorbent pads containing pinosylvin inclusion complexes and reported that the microbial growth of *Campylobacter jejuni* ATCC 33560, *Campylobacter coli* ATCC 33559, and *Campylobacter jejuni* 930/12 remained relatively unchanged up to the third day of refrigerated storage at 4°C. A slight increase after 5 days was then followed by a substantial increase after 7 days of storage. This is in contrast with other studies that have reported an increase in *E. coli* with longer storage times including Khan *et al.* (2016) who showed that *E. coli* levels in meat samples increased from 3.0 to 5.1 $\log_{10}$ CFU g^{-1} when vacuum packaged and stored at 4°C for 35 days. In another example, Nadarajah *et al.* (2005) showed that the total *E. coli* O157:H7 count on ground beef increased from 3.6 to 7.3 $\log_{10}$ CFU g^{-1} after 21 days at 4°C.

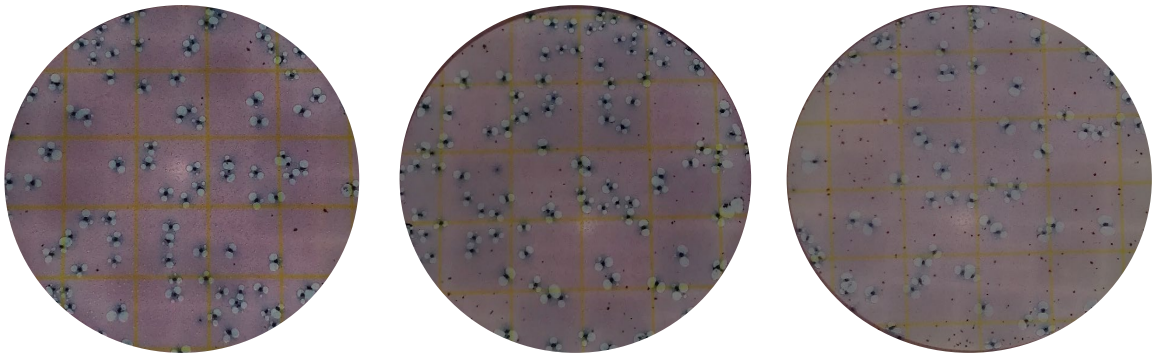
Table 7.3: Levels of *E. coli* on inoculated minced beef stored for 12 days under refrigeration (4 ± 1°C)

	Levels of *E. coli* on minced beef/$\log_{10}$ CFU g^{-1}		
	Day 4	**Day 8**	**Day 12**
Control film	4.93 ± 0.05	4.82 ± 0.01	4.87 ± 0.01
β-CD/glycerol	4.90 ± 0.01	4.85 ± 0.04	4.82 ± 0.06
β-CD/thymol/glycerol	4.98 ± 0.01	4.84 ± 0.08	4.82 ± 0.01



Day 4 **Day 8** **Day 12**

Figure 7.1: Growth of *E. coli* on meat samples packaged with LDPE control film with β-CD and glycerol



Day 4 **Day 8** **Day 12**

Figure 7.2: Growth of *E. coli* on meat samples packaged with LDPE/ thymol/β-CD/glycerol films

Ayala-Zavala *et al.* (2008) hypothesized that the water availability and the interaction with β-CD results in displacement of the AM agents resulting in its release from the cavity. However, previous analysis of the encapsulated AM agents thymol and carvacrol incorporated into LDPE films *in vitro* did not result in the inhibition of the growth of *E. coli,* even at levels as high as 5% (w/w) of the AM agents (see Chapter 6). In these cases, the strong interaction between β-CD and the AM agents, the low water availability in the Petri dish environment, and high growth rate of the bacteria at 37°C rendered the films inactive. Although the addition of glycerol facilitated the release of the AM agents and consequently showed inhibition at a level of 2-3% (w/w) encapsulated

thymol, *in vitro* in the agar disc test (see Chapter 6), the same film formulation was ineffective on the same bacterium on minced beef as shown in Table 7.3. Nonetheless, the same film formulation imparted a significant AO activity with levels of thymol as low as 1% (w/w) (see Table 7.2). Clearly, in this case the concentration of the active agent required to achieve AM effectiveness (Goñi *et al.*, 2009, Otero *et al.*, 2014) appears to be significantly higher than the concentration required to achieve AO effectiveness.

Gram-negative bacteria such as *E. coli* are generally found to be less sensitive to spice and herb extracts than Gram-positive bacteria (López *et al.*, 2005, Kanatt *et al.*, 2008, Radha krishnan *et al.*, 2014, Sung *et al.*, 2014, Moradi *et al.*, 2016). Many studies have reported higher concentrations of naturally-occurring AM compounds are required to work effectively against Gram-negative species *in vitro* or *in vivo*. For example, Sung *et al.* (2014) incorporated 8% (w/w) *Allium sativum* EO into LDPE/ethylene-vinylacetate and reported a reduction of about 1.98 $\log_{10}$ CFU g^{-1} for *L. monocytogenes* inoculated on cooked beef stored at 4°C. However, for the samples inoculated with *E. coli,* the difference in counts between the control film and AM films was low at ≤0.2 $\log_{10}$ CFU g^{-1} using the same film formulation. Moradi *et al.* (2016) studied the activity of cast corn zein films containing 1% (w/w) monolaurin and 3% (w/w) *Zataria multiflora* Boiss EO on minced beef inoculated with *L. monocytogenes* and *E. coli.* The films were found to be more effective against *L. monocytogenes* than *E. coli*, but only within the first 3 days of storage. In contrast, Muriel-Galet *et al.* (2012a) reported that EVOH/PP films containing 5 and 10% (w/w) of oregano EO or citral were found to favour the inhibition of Gram-negative bacteria.

It was generally found samples that had lower levels of coliforms had higher levels of *E. coli* and vice versa. Moreover, the growth of coliforms resulted in some difficulty in counting *E. coli*. Increasing the temperature to 10°C to speed up the growth of *E. coli* was also investigated, however, the coliform numbers also increased impacting the growth of *E. coli*. This has been observed previously by (Vold *et al.*, 2000) who found the presence of a large number of background bacteria in ground meat at 12°C inhibited the growth of *E. coli* O157:H7 both aerobically and anaerobically. In the present study, coliforms were detected on the minced beef samples from 8 days onwards with levels of 3.0 and 2.8 $\log_{10}$ CFU g^{-1} found on the control sample and samples packed with the 3% (w/w) encapsulated thymol and 2% (w/w) glycerol film respectively. In general, the

encapsulated thymol film at 3% (w/w) with glycerol ~~effectively~~ reduced the growth of coliforms on minced beef by 0.2 and 0.3 $\log_{10}$ CFU g^{-1} on days 4 and 8 respectively compared to the control samples. Since *E. coli* is a type of coliform, the presence of coliforms, in general, can indicate contamination of meat products.

7.4.5 Antimicrobial Activity of Films on Chicken Breast Fillets

Levels of *E. coli* on inoculated chicken fillet samples were initially 4.80 ± 0.02 $\log_{10}$ CFU g^{-1} and, as shown in Table 7.4, there was a reduction in *E. coli* levels in both the control and encapsulated film samples within the first 4 days of analysis. This is in agreement with Lourenço *et al.* (2013) who reported that microbial counts on chicken breast fillets inoculated with 4.2 $\log_{10}$ CFU g^{-1} of *E. coli* (ATCC 25922) and 4.9 $\log_{10}$ CFU g^{-1} *S. aureus* (ATCC 9801) that were stored at 4°C for two days reduced to 3.9 and 4.2 $\log_{10}$ CFU g^{-1} respectively. Higueras *et al.* (2014) reported that carvacrol incorporated into chitosan/hydroxypropyl-β-CD films that were obtained by casting inhibited lactic acid bacteria and yeast/fungi growth in packaged chicken breast fillets. However, the authors also found that the carvacrol resulted in an unacceptable sensory perception of the chicken fillets due to a rapid release of the AM agent from the film. In the present study, the low AM activity of encapsulated thymol in minced beef (see Section 7.4.4) is in contrast to its activity on chicken fillets and this may be due to the higher fat content in the beef resulting in reduced thymol activity in comparison to the leaner chicken breast fillet (Burt, 2004, Gutierrez *et al.*, 2008).

Table 7.4: Levels of *E. coli* on inoculated chicken fillets stored for 12 days under refrigeration (4 ± 1°C)

	Levels of *E. coli* on chicken fillet ($\log_{10}$ CFU g^{-1})		
	Day 4	Day 8	Day 12
Control film	3.94 ± 0.02	3.76 ± 0.06	3.22 ± 0.26
β-CD/thymol/glycerol	3.85 ± 0.05	3.01 ± 0.31	2.55 ± 0.26

7.4.6 Effect of Total Coliforms on *E. Coli* Growth

In addition to measuring the direct levels of *E. coli*, 3M Petrifilms™ were used to distinguish the presence of *E. coli* from other coliforms. In the case of the chicken fillet samples, no coliforms were detected on the samples up to 8 days. At 12 days, however, coliforms were detected on samples packaged in the control and encapsulated thymol

films at levels of 3.0 and 2.8 $\log_{10}$ CFU g^{-1} respectively. Examples of the plates showing coliform growth on the control mince beef samples are shown in Figure 7.3 where the coliforms are shown as red/blue colonies. Coliforms were detected from day 8 onwards on all minced beef samples, however, the film containing encapsulated thymol reduced the coliforms by 0.18 $\log_{10}$ CFU g^{-1}.

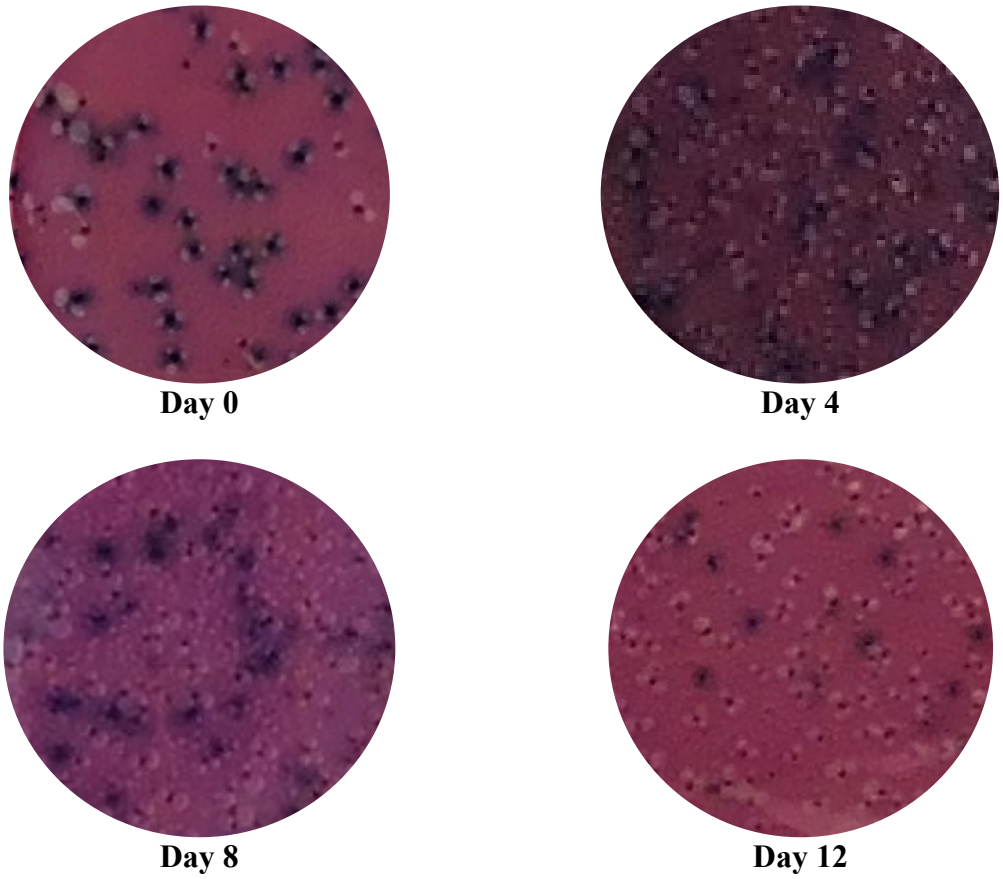


Figure 7.3: Growth of coliforms on control minced beef

Both the AM and AO activities are concentration-dependent. However, the concentration of a given agent required to impart AO activity is much lower than that required for it to impart AM activity. For example, Tawakkal *et al.* (2017) found composite films comprised of poly(lactic acid) (PLA), kenaf fibers and thymol reduced the population of *E. coli* on inoculated chicken slice samples when placed in direct contact with the film when the concentration of thymol was high as 30% (w/w). In comparison, Celebioglu *et al.* (2018) using the DPPH assay found that 4 mg mL^{-1} thymol complexed

with the modified cyclodextrins hydroxypropyl-β-CD, hydroxypropyl-γ-CD and methyl-β-CD quenched free radicals to 95, 93 and 95% respectively. Furthermore, it was found complexed thymol at levels of 0.773, 0.834, and 0.476 mg mL^{-1} in the respective complexes were enough to inhibit 50% of free radical formation.

7.5 Summary

Encapsulated thymol films containing glycerol displayed slightly higher AO activity than films without glycerol measured using the DPPH assay at 24 h which confirms the ability of glycerol to facilitate the release of thymol in pure ethanol. In accordance with these findings, LDPE films containing encapsulated thymol were effective in minimizing lipid oxidation in minced beef samples stored for up to 12 days under refrigeration as shown by the TBARS assay. The same film formulation containing encapsulated thymol did not show inhibition against *E. coli* on minced beef, however, a 0.7 $\log_{10}$ CFU g^{-1} reduction in growth over 12 days at 4°C against *E. coli* on chicken breast fillets was observed. The presence of coliforms and the incubation temperature were found to influence the growth of *E. coli*, particularly in the case of minced beef. The results suggest that LDPE/thymol/β-CD films have the potential for use in the packaging of meat products with the ability to impart both AM and AO activity.

Chapter 8 Conclusions and Recommendations

8.1 Overall Conclusions

In this study, three natural AM agents: thymol, carvacrol and linalool were successfully complexed with β-CD using a co-precipitation method. An optimization procedure was developed to obtain high yields and IEs and the thermal stability was studied to assess any potential losses that might be incurred at high temperatures. The optimized complexes were then incorporated into LDPE film, with and without additives, and the films were tested in vitro and in vivo for their AM and AO efficacy.

It was found that the use of ethanol as a co-solvent significantly influenced the complex formation of thymol/β-CD whereby the use of higher ethanol volumes resulted in lower yields and IE's. Heating the reaction mixture at 55°C for 4 h, a stirring time of 4-5 h after the complex had formed, and a reduced total solvent volume without the use of the co-solvent resulted in average yields of 87, 84, and 86% (w/w) for thymol, carvacrol, and linalool respectively with an IE that was close to 100% (w/w). This suggested that the optimised method is highly feasible for the complexation of these agents with β-CD for use in various future applications.

Thermal analysis showed that the mass losses from the β-CD complexes under a linear temperature ramp below *ca*. 300°C do not account for the total mass of guest species present in the studied complexes. This may be attributed to the presence of different binding sites where some guest molecules are strongly bound to the wall and are only released along with the decomposition of the wall itself whereas other guest molecules are less strongly bound to the wall and are released at lower temperatures. For each of the AM agents studied, the major decomposition stage was consistently and adequately fitted by the Avrami-Erofeev index 3 kinetic model. This work demonstrated that in each case, only a little more than half of the available guest molecule is released from the complex at temperatures at which many commodity packaging polymers such as polyethylene are thermally processed commercially.

Incorporation of the encapsulated AM agents into LDPE film resulted in high retention of the agents during thermal processing using a laboratory press. High retention

of the AM agents in the film was also maintained during long-term storage at room temperature. The results showed that the highest level of retention was obtained for thymol followed by carvacrol and then linalool with between 23 and 129% more AM agent retained when encapsulated in β-CD compared to the free addition of AM agent in the LDPE film. The encapsulation process also slowed the release into a 95% (v/v) ethanol/water food simulant. This slower release can potentially maximize the AM efficiency of the film by providing a more controlled or sustained release and therefore maintaining the AM agent's availability.

In vitro investigations of the AM agents incorporated into LDPE films showed that 2% (w/w) free thymol and 3% (w/w) free carvacrol inhibited the growth of *E. coli* whereas linalool did not show any detectable inhibition, even at a concentration as high as 10% (w/w). In contrast, thymol and carvacrol encapsulated in β-CD and incorporated into LDPE film did not show any detectable inhibition even at 5% (w/w) of the agent with respect to the film. Glycerol was then added to the film formulation in order to facilitate the release of the encapsulated AM agents from the film and the subsequent release improved with increasing glycerol concentration. The minimum concentration of glycerol required to produce AM activity in films containing the encapsulated AM agents was found to be 1% (w/w) and increasing the concentration of glycerol from 1 to 2% (w/w) increased the zone of inhibition against *E. coli* by 28.8%. Using glycerol in films containing free thymol showed a lower extent of AM release than films containing complexed thymol suggesting that the glycerol interacts directly with the free AM agent in the film inhibiting its release and activity. Conversely, β-CD increases the solubility of the AM agent in 95% (v/v) ethanol in water food simulant, and therefore increases its release and resulting AM activity which is further enhanced in the presence of glycerol.

In vivo investigations of films containing β-CD complexes with 3% (w/w) thymol relative to the film did not show inhibition against *E. coli* on minced beef. However, the same film formulation worked effectively against *E. coli* inoculated on chicken breast fillets resulting in a 0.7 $\log_{10}$ CFU reduction in growth over 12 days at 4°C. It was found that in the case of the minced beef, factors such as temperature and the presence of coliforms affected the growth of *E. coli*. The AO activity measured using the DPPH assay of the different LDPE films and the TBARS assay of packaged minced meat showed that the films containing encapsulated thymol were effective in minimizing lipid oxidation

over the storage period. The results suggest that LDPE/thymol/β-CD films with high loading efficiency, longer storage stability and sustained release behaviour can be used for food packaging applications where AM and AO activity are required.

8.2 Recommendations for Future work

Encapsulation of active substances in β-CD offers many advantages for the development of AO and AM food packaging films. The optimization of inclusion complexes is particularly important to ensure the subsequent controlled release of volatile agents from the film is suitable for their application in food preservation. It is also vitally important to test the efficacy of active packaging materials on real foods where complex systems can behave differently compared to simpler *in vitro* tests (Rodríguez *et al.*, 2007). Moreover, lower concentrations of volatile agents can be added to films without the considerable losses that are generally incurred when they are added directly without encapsulation. These factors are all important considerations when developing new packaging films which should be both effective and economically viable.

This work evaluated the production of films on a small scale using a laboratory press. Pilot or full-scale film extrusion could be investigated in future studies in order to evaluate the influence of the β-CD complex on the physicomechanical properties of the film. This would also require larger quantities of the complex to be produced and would enable further evaluation of the losses of the AM agents during processing.

It is common for AM agents to be studied singularly with fewer studies evaluating combinations of one or more agents. Moreover, it is typical that only one aspect of the additive is studied such as its AM activity. The later results of the present study showed that thymol imparts both AM and AO activity but to varying degrees. Future work could fully evaluate the potential for combined AM and AO activity for common EOEs encapsulated in β-CD and incorporated into packaging films. Combinations of two or more encapsulated agents could also be studied to expand the efficacy of AM films based on the target spoilage microorganisms for selected food products.

The literature review identified a wide range of encapsulation wall materials, of which β-CD is a common choice for many applications. Future work could investigate other wall materials in terms of the stability and release of the encapsulated agent, the

compatibility with different film matrices, as well as a very broad range of AM and AO agents using the methods developed in this work. Such investigations may also include the use of encapsulation to protect non-volatile, thermally-sensitive agents as well as the encapsulation of other volatile agents to prevent losses.

In this study, LDPE was used as the film matrix but there are a wide range of other polymers that serve as active packaging materials. It is becoming increasingly important to evaluate biopolymers for their potential as packaging films including PLA, polysaccharides (i.e. chitosan, carrageenan, alginates), PHAs, starch films, and various composites. These could be further investigated in terms of their ability to carry encapsulated AM/AO agents to develop biodegradable, sustainable active packaging films.

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APPENDIX A

Journal publication attachment

Ghofran Al-Nasiri, Marlene J. Cran, Andrew J. Smallridge & Stephen W. Bigger (2018): Optimisation of β-cyclodextrin inclusion complexes with natural antimicrobial agents: thymol, carvacrol and linalool, ***Journal of Microencapsulation*** 35(1):26-35.

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