Development and Evaluation of Low Density Polyethylene-Based Antimicrobial Food Packaging Films Containing Natural Agents

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Declaration

I, LiyanaArachchige Sanjeewani Rupika Herath, declare that the PhD thesis entitled "Development and Evaluation of Low Density Polyethylene-Based Antimicrobial Food Packaging Films Containing Natural Agents" is no more than 100,000 words in length exclusive of tables, figures, appendices, 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.

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Abstract

Antimicrobial (AM) films containing naturally-derived AM agents, thymol or carvacrol, were developed. These agents were incorporated into a low-density polyethylene (LDPE) substrate using the techniques of both compression moulding and extrusion film blowing. Different film formulations containing additive polymers, ethylene vinyl acetate (EVA) and polyethylene glycol (PEG), were investigated for their potential to enhance the retention and to control the release of the AM agents from the films. The physical and mechanical properties of the films were evaluated in order to assess the effect that the addition of AM agent has on the ultimate properties of the film. The extruded films were also studied for AM agent release and retention during storage. Films were tested for AM activity *in-vitro* enabling the optimum concentrations for AM activity to be obtained and the effect of AM films on the bacterial inhibition in liquid media to be successfully modelled. The effects of combined AM systems in LDPE/EVA-based films were also studied. Having identified the AM activity in laboratory media, the films were then used to package Cheddar cheese in order to assess the usefulness of AM films in the enhancement of food preservation. The AM films had a positive effect on the microbial and physio-chemical attributes of Cheddar cheese under actual storage conditions. The addition of higher concentrations of AM agents clearly imparted a noticeable odour to Cheddar cheese during storage. The shelf life extension of Cheddar cheese by AM films was affected by the type and concentration of the AM agent.

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List of Abbreviations and Nomenclature

ACM	Australian Collection of Microorganisms
ADI	Acceptable daily intake
AM	Antimicrobial
ANOVA	Analysis of variance
AP	Active Packaging
ASTM	American Society for Testing and Materials
CDs	Cyclodextrins
CFU	Colony forming units
COE	Council of Europe
CRD	Completely randomised design
DOT	Detection odour threshold
DSC	Differential scanning calorimetry
EC/C	*E. coli*/coliform
EO	Essential oil
EU	European Union
EVA	Ethylene vinyl acetate
EVOH	Ethylene vinyl alcohol
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FEMA	Flavor and Extract Manufacturers Association
FIC	Fractional inhibitory concentration
FID	Flame ionization detector
FT-IR	Fourier Transform Infrared (spectroscopy)
GC	Gas Chromatography
GLM	General linear model
GRAS	Generally Recognized as Safe
HDPE	High-density polyethylene
HLB	Hydrophilic–lipophilic balance
JECFA	Joint Expert Committee on Food Additives
LB	Lactic acid bacteria
LDPE	Low-density polyethylene
LLDPE	Linear low density polyethylene
MAB	Mesophillic aerobic bacteria
MAP	Modified atmosphere packaging
MBC	Minimum bactericidal concentration
MD	Machine direction (film)
MEC	Maximum effective concentration
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
NSLAB	Non-starter lactic acid bacteria
OEO	Essential oil of oregano
PE	polyethylene
PEG	Polyethylene glycol

PET	Polyethylene-terephthalate
PP	Polypropylene
PS	Polystyrene
PT	Probable threshold
PVC	Polyvinyl chloride
PVDC	Polyvinylidene chloride
RMSE	Root mean square error
TAB	Total aerobic bacteria
TD	Transverse direction (film)
TEO	Essential oil of thyme
UNSW	University of New South Wales
VP	Vacuum packaging
WHO	World Health Organization
YM	Yeast and moulds

a_w	Water activity
b	Short-term/long-term boundary
D	Diffusion coefficient
E_a	Activation energy of diffusion
ΔH	Enthalpy of melting
k_1	Diffusion rate constant
k_2	Kinetic rate constant
l	Film thickness
LD_{50}	Dose lethal to 50% of animals in mg/kg of body weight
m_t	Amount of AM agent released from the film
m_∞	Equilibrium amount of AM agent released from the film
$P_{o/w}$	Partition coefficient in octanol/water
R	Ideal gas constant
R^2	Correlation coefficient
T	Temperature
t	Time
T_m	Crystalline melting temperature
v_0	Initial rate of release of the AM agent

Chapter 1. Introduction

In this chapter a brief review of the developments in food packaging and new food packaging technologies are presented with a special emphasis on antimicrobial (AM) packaging. The concept of AM packaging is explored and current AM packaging systems and the future development requirements are also discussed.

1.1 Background

1.1.1 Role of Packaging for Foods

Packaging has become an essential element in today's society and at present, approximately fifty percent of the packaging market is for food. Forecasts suggest that the sector will continue to grow in size and importance (Kerry and Butler 2008; Robertson 2006). In particular, food packaging has experienced an extraordinary expansion, because most commercialized foodstuffs, including fresh fruits and vegetables, are being marketed already packaged (López-Rubio *et al.* 2008).

In the past, food packaging was limited to simple methods such as wrapping items in newspaper, and storing items in preserving jars and tin boxes. Advances in the preservation and storage of food have led to significant changes in packaging and although these changes have evolved in various forms, every package must still meet some basic functions. Food packaging has to protect food from environmental conditions, make distribution easier, and provide information and convenience to the consumer (Ahvenainen 2003; Bureau and Multon 1996; Han 2005b; Robertson 2006). Beyond these basic functions, food packaging also has to fulfil many demands and requirements such as sales promotion, to be of reasonable cost, technical feasibility, compatibility with the contents and must address effectively and economically environmental issues (Ahvenainen 2003).

1.1.2 New Food Packaging Technologies

Food packaging is quite different from the packaging of durable products due to the perishable and heterogeneous nature of foods. In addition, safety aspects need to be taken into account and most foods have a relatively short shelf life (Han 2000; López-Rubio *et al.* 2008). During the last few decades, the greatest changes ever witnessed have occurred in mankind's eating or consumption habits and in the production and distribution of food. Consumer trends for better quality, fresh and convenient food products have intensified (Bureau and Multon 1996; López-Rubio *et al.* 2008). Traditional systems are reaching their limits with regard to further extending the shelf life of packaged food. Therefore much innovation can be witnessed and a variety of new packaging technologies such as active, intelligent or smart packaging have been developed in response to this change (Ahvenainen 2003; Appendini and Hotchkiss 2002; Han 2005b; Kruijf *et al.* 2002; López-Rubio *et al.* 2004; Sonneveld 2000; Vermeiren *et al.* 1999).

1.1.3 Active Food Packaging

Active packaging (AP) is an innovative food packaging concept that has been introduced and developed as a series of responses to the continuous changes in consumer demands and market trends (Miltz *et al.* 1995; Rooney 1995b; Vermeiren *et al.* 1999). Active packaging performs some additional desired functions in comparison with traditional passive packaging, that provides primarily barrier and protective functions (Ahvenainen 2003; Appendini and Hotchkiss 2002; Kerry and Butler 2008; Quintavalla and Vicini 2002; Robertson 2006; Vermeiren *et al.* 1999). The AP systems change the condition of the packaged food to extend the shelf life or to improve safety or sensory properties while maintaining the quality (Ahvenainen 2003; Brody 2008; Kruijf *et al.* 2002).

Active packaging interacts with the internal gaseous environment and/or directly with the product, to produce a beneficial outcome. The internal atmosphere may be controlled by substances that absorb (scavenge) or release (emit) gases or vapours.

Some examples of AP systems include oxygen scavengers, moisture scavengers, carbon dioxide scavengers or emitters, humidity absorbers or controllers, ethylene scavengers, aroma emitters or absorbers and AM systems (Brody 2008; Han 2005b; López-Rubio *et al.* 2004). These concepts and techniques have been thoroughly reviewed in the scientific literature with an attempt to identify the role of AP in food preservation (Ahvenainen 2003; Brody 2008; Han 2005b; Kerry and Butler 2008; Kruijf *et al.* 2002; López-Rubio *et al.* 2004; Miltz *et al.* 1995; Ozdemir and Floros 2004; Rooney 1995b; Suppakul *et al.* 2003; Vermeiren *et al.* 1999).

1.2 AM Packaging

1.2.1 Food Safety and AM Packaging

Deterioration of food products is caused both biologically and chemically. Biological deterioration of foods is primarily due to the growth of microorganisms spoiling the food or making it unsafe for consumption (Quintavalla and Vicini 2002). Microbial growth can significantly lower the safety of foods and damage the food quality. Microbial spoilage due to bacteria, yeasts and moulds results in degraded quality, shortened shelf life and changes in microflora that could induce pathogenic problems. Contamination of food products by pathogenic microorganisms or microbial-toxins can cause major problems of food safety and serious food-borne illnesses (Han 2005a).

Packaging, when regarded as a food preservation technology, can retard food product deterioration and extend the shelf life (López-Rubio *et al.* 2004). For minimally processed or fresh food systems, packaging is one of the major, and only available, protection barrier whereas in the case of processed foods packaging provides further preservation (Tewari 2002). Despite the methods available for food protection, microbial growth on foods remains a major cause of spoilage and contamination (Han 2005a). Globally, the international food trade is disrupted by the increasing burden of foodborne illnesses and new and emerging foodborne hazards (FAO/WHO 2008). Recent trends in global food production, processing, distribution and preparation have increased the risk of foods being contaminated with pathogens (Theron and Lues 2007).

This has created an increasing demand for innovative ways to inhibit microbial growth in foods to ensure a safer global food supply while maintaining quality and freshness (Appendini and Hotchkiss 2002).

Antimicrobial packaging was initiated as a new and a promising applications of AP (Floros *et al.* 1997) to reduce, inhibit or retard microbial growth on food products (Appendini and Hotchkiss 2002; Kim *et al.* 2008). It performs an extra AM function to provide an increased margin of safety and quality while satisfying the conventional protection function of foods (Appendini and Hotchkiss 2002; Han 2003). Antimicrobial packaging also prolongs the shelf life of the packed food, usually by extending the lag phase and reducing the growth rate of microorganisms (Han 2000) and could therefore also play a role in food security assurance (Han 2005a). The AM package acts on the surface of foods, where a great portion of spoilage and contamination occurs (Appendini and Hotchkiss 2002; Vermeiren *et al.* 2002; Vermeiren *et al.* 1999). It can also act on microorganisms that may be present in or on the packaging material itself (Appendini and Hotchkiss 2002).

Foods such as meat, fish, poultry, bakery products and dairy products that are prone to microbial spoiling on the surface as well as fruits and vegetables have been the focus for AM packaging systems (Day 2008; Labuza and Breene 1989). In recent years there has been a great interest in AM food packaging due to increased foodborne microbial outbreaks triggered in minimally processed fresh and refrigerated products (Devlieghere *et al.* 2004). Traditional strategies for obtaining AM activity such as direct incorporation in initial food formulations (Skandamis and Nychas 2000; Valero and Giner 2006), washing (Bagamboula *et al.* 2004; Molinos *et al.* 2008), and dipping or spraying (Mahmoud *et al.* 2004; Roller and Seedhar 2002) are not always effective. The protective ability of the AM agent can cease if neutralized in reactions or interactions in the complex food system and it can diffuse rapidly from the surface into the food mass (Appendini and Hotchkiss 2002; Quintavalla and Vicini 2002). Furthermore, direct addition can lead to outgrowth of the surviving microorganisms as a result of mutating and adapting cells (Zhang *et al.* 2004). Antimicrobial packaging is an alternative method to overcome these limitations since the AM agents are slowly released from the

package onto the food surface during storage and therefore maintains a critical concentration necessary for inhibiting the microbial growth (Buonocore *et al.* 2004; Quintavalla and Vicini 2002; Vermeiren *et al.* 2002). Thus, AM packaging is of great value to the food industry as a post-processing safety measure (Joerger 2007). For consumers it seems safer when active agents are indirectly integrated in the food package and released onto the food product thereafter (Han 2000; Ozdemir and Floros 2004).

1.2.2 AM Packaging Systems

In 1945, a patent was obtained for food wrappers incorporating sorbic acid that were developed to inhibit mould growth (Kim *et al.* 2008). Since then, a variety of AM packaging systems have been developed and these systems have been reviewed by various researchers (Appendini and Hotchkiss 2002; Cooksey 2001; Han 2000; Han 2003; Han 2005a; Joerger 2007; Kruijf *et al.* 2002; López-Rubio *et al.* 2004; Ozdemir and Floros 2004; Quintavalla and Vicini 2002; Suppakul *et al.* 2003; Vermeiren *et al.* 2002). In general, AM packaging can be categorised into two types: non-migratory and migratory. Non-migratory AM packages may include polymers with inherent AM properties (e.g. chitosan) or structures that contain immobilized AM agents (López-Rubio *et al.* 2004). This type of AM packaging usually requires direct food contact for maximum effectiveness (Cooksey 2001; Vermeiren *et al.* 2002) and normally the application is limited to liquid foods (Han 2005a).

Migratory AM packages are designed to release AM agents into the headspace of the package or directly onto the food product. Migratory AM packaging may be incorporated with non-volatile or volatile AM substances (Appendini and Hotchkiss 2002; Han 2005a). In non-volatile systems, close contact between the packaging material and the food surface is required (Appendini and Hotchkiss 2002; Han 2005a; Suppakul *et al.* 2003; Vermeiren *et al.* 2002). Thus, non-volatile migration systems are applicable in one-piece solid, semi-solid and liquid foods (Han 2005a). In volatile AM systems, the AM agents can evaporate into the headspace of the packaged product (Han 2003). Once in the headspace, AM substances reach the surface of the food where they

are sorbed and then dispersed or diffused throughout the food product (López-Rubio *et al.* 2004). In volatile migration, it is not necessary to have close contact between the AM material and the food surface and this offers many advantages (Appendini and Hotchkiss 2002). Such systems can be used effectively for highly porous foods, powdered, shredded irregularly shaped and particulate foods (Han 2005a).

The first designs in AM packaging were made use in the form of sachets or pads containing the active ingredient that were enclosed or attached to the interior of the package (Appendini and Hotchkiss 2002). Three forms of AM packaging have predominated: oxygen absorbers, moisture absorbers and ethanol vapour generators (Appendini and Hotchkiss 2002). These forms were used primarily in packaging applications for bakery, pasta, meat and poultry products (Appendini and Hotchkiss 2002; Han 2005a; Suppakul *et al.* 2003). Alternatives which are being extensively studied include the incorporation of an AM substance within the packaging material and the coating or adsorption of the AM compound on the surface of the packaging film (Vermeiren *et al.* 2002). Coatings, in the form of edible waxes, wraps and casings (Labuza and Breene 1989) that contain AM compounds and are applied onto the inner surface of the packaging material have also been investigated (Miltz *et al.* 2006). Additional advantages of incorporating AM agents in the packaging film over their use in sachets are: package size reduction, potentially higher efficacy and higher output in packaging production (Appendini and Hotchkiss 2002). Currently, various plastic- or biopolymer-based AM packaging films and containers have been developed and successfully applied in many commercial food products especially in the US and Japanese markets (Kim *et al.* 2008). This is an area of great interest due to their potential to provide quality and safety benefits and many research efforts are focused on their development and implementation (Appendini and Hotchkiss 2002; Joerger 2007; Kim *et al.* 2008; Quintavalla and Vicini 2002).

The synthetic polymers used in food packaging include low-density polyethylene (LDPE), high density polyethylene (HDPE), polypropylene (PP), polystyrene (PS), polyethylene-terephthalate (PET), ethylene vinyl alcohol copolymers (EVOH), Surlyn ionomers, polyvinyl chloride (PVC), ethylene vinyl acetate (EVA) and more. These

polymers are convenient materials and readily processed (Brody 2008; Joerger 2007) into packages (López-Rubio *et al.* 2004). Advantages of plastics include their low cost, low density, ability to be thermo-sealed, relatively easy to print on. Furthermore, plastics can be incorporated in integrated production processes making the process quick and cheap thereby avoiding the transport and storage of empty packages and can be selected with adequate chemical inertness to suit almost every food product (López-Rubio *et al.* 2004). The availability of a variety of materials and compositions makes it possible to adopt the most convenient packaging structure and design to the very specific needs of each product. Different additives can be incorporated into the polymers in order to achieve specific and/or desired properties. The use of synthetic polymers as food packaging materials has increased significantly in recent decades due to their advantages over other traditional packaging materials (Han 2000; López-Rubio *et al.* 2004; Rooney 1995a).

1.2.3 AM Agents

The use of preservatives including AM agents is one of the oldest and most traditional food preservation techniques (López-Malo *et al.* 2000). Antimicrobial agents may be either synthetic or naturally occurring, biologically derived substances (or so-called "natural antimicrobials") that may be used as additives in the food for food preservation (López-Malo *et al.* 2006). Various AM agents impart AM activity when bound to food packaging materials (Han 2005a; Kim *et al.* 2008) and many of them are incorporated into the packaging material at concentrations of 0.1 to 5% (w/w) (Appendini and Hotchkiss 2002). The AM properties, spectrum of activity, mode of action, possible packaging materials, current uses and new applications of most of these AM agents have been reviewed in recent publications (Ahvenainen 2003; Appendini and Hotchkiss 2002; Brody 2008; Brody *et al.* 2001; Han 2000; Han 2003; Han 2005a; Joerger 2007; López-Malo *et al.* 2000; Roller and Board 2003; Suppakul *et al.* 2003; Vermeiren *et al.* 2002; Vermeiren *et al.* 1999).

Until now, primarily synthetic additives have been used commercially for the preservation of food products using AM packaging (Han 2003; Miltz *et al.* 2006).

However, allergenic reactions in sensitive individuals and potential toxicological problems (e.g. formation of hazardous by-products) have led to consumer mistrust in the use of synthetic additives and to numerous legislation reviews (Roller 2003). Consequently, there has been a renewed interest in natural AM compounds. In an effort to meet this demand, a wide range of natural systems from animals, plants and microorganisms have been studied (Han 2005a; Lopez-Malo *et al.* 2005; Roller 2003; Roller and Board 2003).

The use of appropriate packaging materials and methods to minimize food losses and provide safe and wholesome food products has always been the focus of AM food packaging (Ozdemir and Floros 2004). Forecasts suggest that, in due course, many packages will contain AM agents. Current applications of AM packaging for food products are deficient due to the limited availability of suitable antimicrobials, appropriate polymer materials, regulatory concerns, and because of doubts about their effectiveness (Cha and Chinnan 2004; Joerger 2007; Kerry and Butler 2008). New AM packaging materials are continually being developed with a greater emphasis on safety issues associated with the addition of AM agents.

1.3 Aims

Reflecting the current demands and research needs in the area of AM food packaging, this study is aimed at developing AM food packaging films containing natural AM agents with the following specific aims:

- To investigate the potential use of thymol and carvacrol as AM agents in packaging films using LDPE as the polymeric substrate and using different film processing methods;
- To investigate the ability of additive polymers EVA and polyethylene glycol (PEG) to enhance the retention of AM agents during film processing;
- To investigate the optimum concentrations of AM agents required in the films to impart AM activity on laboratory media and to specify the range of microorganisms for which these films are effective;

- To evaluate the AM efficacy of films containing combinations of AM agents;
- To determine the physical and mechanical properties and the AM retention during storage.
- To investigate the migration of AM agents from films to the atmosphere and into food simulants to postulate the subsequent AM activity and safety effects;
- To assess the feasibility of AM films in food packaging applications using Cheddar cheese as a model food system.

Chapter 2. Literature Review

This chapter provides a detailed discussion on the potential of thyme and oregano as natural antimicrobials in AM packaging. The sources of these natural agents, their AM activity and possible future applications in food packaging as well other benefits are reviewed. The microbiology, quality and shelf life of Cheddar cheese used as a substrate in the present study for evaluation of the possible application of AM films in food packaging are also reviewed.

2.1 Thyme and Oregano as a Natural Source of AM Agents

Spices and herbal plant species are known to contain a wide range of compounds capable of exhibiting AM activity. These compounds are produced as secondary metabolites associated with the volatile essential oil (EO) fraction of these plants. Essential oils are generally extracted from non-woody plant materials and comprise of variable mixtures, primarily terpenoids and a variety of esters, aldehydes, ketones, acids and alcohols (Dorman and Deans 2000; Meyer *et al.* 2002). The species thyme and oregano are popular culinary herbs belonging to the *Lamiaceae* (*Labiatae*) family. They contain EOs that are highly enriched with terpenoids, particularly monoterpenoid phenols (thymol and carvacrol), that are reported to possess numerous functional properties (Davidson and Naidu 2000; Dorman and Deans 2000; Meyer *et al.* 2002).

Attributed to the properties of their EOs and EO constituents, thyme and oregano provide foods with flavor and preserving capabilities. They also demonstrate potential health benefits, antioxidant activity, and AM effects. The use of thyme as an AM preservative and oregano as a culinary and medicinal herb can be traced back many generations (Hirasa and Takemasa 1998). However, as with most other botanical preservatives, scientific evidence on the preservative effects of thyme and oregano was documented only recently. The potential of EOs of thyme and oregano and/or their major EO components as AM agents in food preservation has been reviewed in several scientific publications (Burt 2004; Conner 1994; Davidson and Naidu 2000; Kintzios

2004; Stahl-Biskup 2004). It was reported that these are amongst the best broad-spectrum candidates for inhibition of food-borne pathogens and spoilage organisms (Suhr and Nielsen 2003).

Consumer demands directed towards the use of natural rather than synthetic ingredients has led to resurgence in the use of natural AM agents, particularly those derived from plants (Hammer *et al.* 1999). Attention has been paid to the exploitation of plant-derived AM agents in the fields of pharmacology, medical and clinical microbiology, phytopathology and food preservation. The emergence and success of synthetic preservatives, particularly in the Western world, have dramatically reduced, for a long period, the use of traditional "natural" food preservatives (Meyer *et al.* 2002) and only in recent years AM plant compounds have regained momentum. The future of naturally occurring AM systems seems positive, since novel preservation techniques based on such systems are being rapidly developed and used in a variety of foods (Draughon 2004).

2.1.1 Synonyms and Botanical Classification

Among the aromatic plants belonging to the *Lamiaceae* (*Labiatae*) family, the genus *Thymus* is noteworthy for the numerous species and varieties (Burdock 1997; Burdock 2005). Estimates of legitimate species of thyme distributed world wide range from 100 to 400 and several species of *Thymus* are identified as a source of thyme (Leung and Foster 1996). For example, *T. vulgaris* is the most important species and is widely used as a flavoring agent, a culinary herb and an herbal medicine. However, *T. zygis*, *T. pulegiodes* and *T. serpyllum* are often used for similar purposes or as a substitute for *T. vulgaris* (Stahl-Biskup 2004). Wild thyme is derived from *T. serpyllum* L. (syn. *Thymus praecox* ssp. *arcticus*) while other thyme species used include**:** *T.* x *citriodorus* (Pers.) Schreb. (syn. *T. serpyllum* L. var. vulgaris Benth.), a lemon scented thyme; and *T. mastichina* (Leung and Foster 1996). The most important processed products obtained from thyme are EOs, herb oleoresins and solvent extracts (Burdock 2005; Stahl-Biskup 2004)**.** The species *T. vulgaris* L. and *T. zygis* L. are commonly used for the distillation of the red (crude) and white (redistilled) thyme oil (Burdock 1997). The steam distilled

EO from *T. capitatus* is richer in carvacrol (about 50%) than that from *T. vulgaris* and *T. zygis*, and resembles the odor of oregano (which contains carvacrol), thus giving the characteristic pungent, warm smell (Burdock 1997).

In popular and scientific texts, confusion exists as to the identity of oregano. This is partly because a variety of plant species have been commonly called 'oregano' or 'origanum' and marjoram and these plants are often interchanged (Burdock 1997). Most of the oregano plants bear a unifying chemical signature**:** carvacrol and, to a lesser extent, thymol. Thus, oregano is mostly considered as a flavour and not as a particular species of plant (Tucker and Maciarello 1994), but it usually refers to the genus *Origanum*, namely, the European Oregano (Kintzios 2004).

Oregano is derived from leaves and flowering tops of several genera of plants, mainly from two families: Lamiaceae and Verbenaceae. In Lamiaceae, the genus *Origanum* is the main source of oregano, although, the name 'oregano' has been applied to many other species in this family (Tucker and Maciarello 1994). The European commercial oregano is commonly *Origanum vulgare* L., particularly; *O. vulgare* ssp. *hirtum*. Commercial oregano may contain many other species, and sub species of *O. vulgare* like *O. onites* (known as Turkish oregano), and *O. syriacum*, known in the USA as 'Lebanese oregano'. South American or Mexican oregano belongs to the genus *Lippia* in the family Verbernaceae. It is usually derived from *Lippa graveolens*, and occasionally from *L. palmeri*. Both are aromatic shrubs native to Mexico and are termed "Mexican wild sage" while *Lantana involucrate* from the same family has earned the name "Mexican Oregano" (Tucker and Maciarello 1994; Vernin *et al.* 2001).

Origanum species that are used for the production of origanum oil are the ones that yield EOs with carvacrol as the major phenolic component (Leung and Foster 1996). The major source of Spanish origanum oil is, however, not an *Origanum* species but *T. capitatus* (Burdock 1997; Tucker and Maciarello 1994). The botanical source of sweet marjoram is *O. majorana* L., which is also known as *Majorana hortensis* Monech without any discrepancy. The source of pot marjoram is attributed to a single species, *O. onites* (syn. *M. onites*) (Leung and Foster 1996).

2.1.2 Chemical Composition

The composition of EOs of thyme and oregano has been reported in several scientific publications (Kintzios 2004; Leung and Foster 1996; Stahl-Biskup 2004; Tucker and Maciarello 1994). Members of the genus *Origanum* and genus *Thymus* are often characterized by differences in the EO content and composition (Sivropoulou *et al.* 1996). The prevalence of thymol or carvacrol in their EOs is responsible for their commercial classification as thyme or oregano oil respectively (Sivropoulou *et al.* 1996). Thymol is normally the major phenolic component in thyme and causes its typically strong and spicy smell. Thymol is accompanied by some monoterpenes such as carvacrol, *p*-cymene and γ-terpene (Stahl-Biskup 2004). *Thymus* EOs exhibit a widespread chemical polymorphism (Pina-Vaz *et al.* 2004; Stahl-Biskup 2004). Depending on the EO profiles of populations of various *Thymus* species, several chemotypes have been identified and are named according to their dominant monoterpene in the EO. The predominant chemotypes of most *Thymus* species are the thymol types, followed by the carvacrol and thymol-carvacrol types and other chemotypes (Pina-Vaz *et al.* 2004; Stahl-Biskup 2004; Usai *et al.* 2003).

The EOs of different oregano species are known to exhibit highly variable compositions (Kokkini *et al.* 2004; Sivropoulou *et al.* 1996; Veres *et al.* 2003). Chemical polymorphism exists among the oregano populations and remarkable chemical variations have been observed not only between but also within populations and accessions. The differences in EO composition often enables these to be separated into chemotypes (Kintzios 2004).

Quantitative and qualitative variations of plant extracts including EOs can result in marked differences in AM properties. Hence, correct identification of the botanical source and standardization of extraction methods are paramount in utilizing their AM properties (Burt and Reinders 2003; Cosentino *et al.* 1999; Draughon 2004; Sivropoulou *et al.* 1996; Vigil 2005). In many publications on the AM activity of thyme and oregano, the commercial source has been mentioned but the botanical source has not been defined, except for a few studies, thus creating serious doubts and implications

on their effective utilization. Identification of the active AM compounds from these plant extracts/EOs can enable a more thorough determination of their effectiveness (Vigil 2005). The use of identified active constituents may give more predictability and help in expanding the knowledge on their potential use in industrial applications.

2.1.3 Applications and Maximum Use Levels

Thyme, oregano and their processed products are primarily used as flavor ingredients and seasonings in food preparations as outlined in Table 2.1. They are also used as crude drugs and in folk (traditional) medicine and aromatherapy because of their antiseptic and purported healing and therapeutic ingredients. Additionally, thyme and oregano and their EOs are used in perfumery, in pharmaceuticals and cosmetics (Burt 2004; Cosentino *et al.* 1999; Kintzios 2004; Leung *et al.* 2003; Manou *et al.* 1998; Stahl-Biskup 2004).

Table 2.1 Maximum levels (ppm) of thyme, oregano and their major constituents as direct additives.

Additive (FEMA[a] No.)	Thyme (3063)	Thyme oil (3065)	Oregano (2827)	Thymol (3066)	Carvacrol (2245)
Foods[b]	200-1716	2.9-28.5	151-3167	15.6-78	9-22.4
Beverages[c]	-	5.0	450	5-5.9	1-28.5

Reference: (Burdock 2005).
[a]Flavor and Extract Manufacturers Association (FEMA) identification number.
[b]Foods including: baked goods, condiments, relishes, fats, oils, gravies, meat products, processed vegetables, soups, frozen dairy, gelatin, pudding, candy, snack food, milk products.
[c]Beverages including: alcoholic and non alcoholic.

The concentration of oregano oil in food applications varies from about 320 to 3,200 ppm (Tucker and Maciarello 1994). The highest level of oregano reported is about 0.3% in condiments and relishes (3170 ppm) and milk products (2900 ppm), whereas for thyme it is 1720 ppm (Burdock 2005; Leung and Foster 1996). The highest reported levels of origanum oil (Spanish) and marjoram (sweet, pot and wild) are usually below

40 ppm (Leung and Foster 1996). Thymol is generally used for flavoring purposes in food and beverages at levels of 5-78 ppm (Burdock 2005; Jerry 1996)

2.1.4 Regulatory Status

Thyme, oregano, origanum or marjoram and their EOs are considered to be Generally Recognized as Safe (GRAS) by the US Food and Drug Administration (FDA) under the conditions of intended use (FDA 2005). Thymol and carvacrol are approved food additives, particularly as flavoring agents, in Europe (EC 2005) and USA (FDA 2005) and are included in the European Union (EU) list of flavoring substances. The regulatory status of thyme, oregano and their phenolic constituents suggest minimal safety concerns when being used as food antimicrobials.

When AM agents are added to food *via* its packaging, they are considered in the USA as food additives (FDA 1999). According to the new European Union framework directive (EC 2004), substances authorized as food additives or flavors can be released by active materials and articles either into or onto the packaged food, or released into the environment surrounding the food. In the case where an active component is to be released, the total quantity of the substance shall not exceed the specific migration limits (EC 2004). The principal function of EOs and EO constituents are to impart desirable flavors and aromas and not necessarily to act as AM agents. Therefore, when used as AM agents, additional safety and toxicological data may be required before regulatory approval for their use as novel food preservatives can be granted (Nychas and Skandamis 2003). However, the amount of data required would be significantly less for AM agents derived from food flavorings than for other substances.

2.1.5 Toxicology and Safety

Most of the botanical biopreservatives that could be used in foods have been consumed safely by humans for thousands of years (Draughon 2004). Presently however, the use of spices, herbs and products derived from them is the subject of extensive toxicological scrutiny (Nychas and Skandamis 2003). Different authorities and/or expert advisory

groups have evaluated the human safety of EOs of thyme and oregano, thymol and carvacrol. Among these are the Committee of Experts on Flavoring Substances of the Council of Europe, an expert panel sponsored by the Flavor and Extract Manufacturers Association (see Table 2.1), and the Joint Food and Agriculture Organization of the United Nations/World Health Organization (WHO) Expert Committee on Food Additives (JECFA).

As part of the assessment of an additive, JECFA examines the available toxicological data and chemical specifications of the additive and establishes an acceptable daily intake (ADI) (Ottaway 2003). For effective product development with optimized functionality and flavor, it is essential to calculate the ADI to ensure that no negative nutritional or health consequences would occur (Draughon 2004). According to the results of the safety evaluations of phenol and phenol derivatives used as flavoring agents (JECFA Monographs and Evaluations), thymol and carvacrol are included in structural class I that: (i) poses no safety concern at current levels of intake when used as a flavoring agent and (ii) has a threshold for human intake of 1.8 mg/day (WHO 2000). The estimated daily per capita intake of thymol and carvacrol in Europe and in the USA are below the threshold for human intake for class I. Furthermore, quantitative data on natural occurrence and consumption ratios (annual consumption in food (kg)/most recently reported amount as a flavoring agent (kg)) indicate that they are consumed primarily as natural components of food (i.e. consumption ratios > 1). The combined intake of thymol and carvacrol would preferably not exceed that threshold for class I (WHO 2000).

Although little has been reported on the toxic effects of thyme and oregano, data on Oral LD_{50} values (the dose lethal to 50% of animals in mg/kg of body weight) tested in rats can be used as an indication of their acute toxicities. The Oral LD_{50} values of herbal parts or EOs of thyme and oregano are in the range of 1000-5000 while those for thymol and carvacrol are 980 and 810, respectively (Jerry 1996; Nychas and Skandamis 2003; Stahl-Biskup 2004). The acute toxicities of phenolic constituents are higher than the EOs of thyme and oregano and carvacrol is somewhat more toxic than thymol. Toxic effects of thyme and oregano are attributed to the terpene phenols (Stahl-Biskup 2004).

Phenols may become toxic if natural barriers or detoxification mechanisms are overloaded by the amount of ingested phenols (Singleton and Kratzer 1969). However, there is a general agreement that phenolics from common foodstuffs at the regular intake levels have very low toxicities, attributed to their rapid metabolism as well as the efficient defense mechanisms in mammals (Shahidi and Naczk 2004). Thymol and carvacrol, ingested as natural or added components of food, are readily absorbed from the gastrointestinal tract (Huges and Hall 1995) and are essentially excreted in the urine *via* the kidney within 24 h either unconjugated or as sulfate or glucuronide conjugates (Jerry 1996; WHO 2000).

An upper limit for inclusion of thymol established by the WHO is 50 mg/kg in food and 10 mg/L in beverages (Delgado *et al.* 2004; Jerry 1996). Under Council of Europe (COE) regulations, the upper limit for inclusion of carvacrol is 5 mg/kg and 2 mg/L in food and beverages respectively (Burdock 2005). So, the inclusion of these compounds in foods and beverages is of no danger as long as the residue of these compounds does not exceed the recommended levels.

2.1.6 Antimicrobial Activity

Antimicrobial compounds are primarily located in the EO fractions of plants (Beuchat and Golden 1989). The inhibitory efficacy of EOs is mainly due to the most abundant components (Cosentino *et al.* 1999; Farag *et al.* 1989) and there is a relationship between the chemical structure of these components in the EOs, their concentration and the AM efficacy (Bagamboula *et al.* 2004; Davidson and Naidu 2000; Farag *et al.* 1989; Friedman *et al.* 2002; Vigil 2005). Generally, the extent to which the growth of microorganisms is inhibited by EOs can be attributed to the presence of an aromatic nucleus containing a polar functional group (Cosentino *et al.* 1999; Deans and Ritchie 1987; Dorman and Deans 2000; Farag *et al.* 1989), but other factors such as the hydrophilic/lipophilic balance are also likely to play a role (Farag *et al.* 1989).

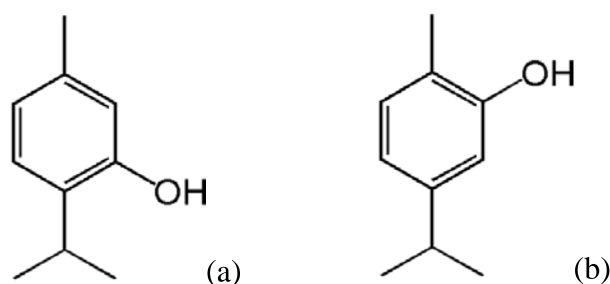

Figure 2.1 Structure of (a) thymol and (b) carvacrol.

Numerous studies have demonstrated that thyme and oregano are among the most potent herbs with regards to AM properties (see Table 2.2). The AM activity of thyme and oregano is apparently related to their phenolic constituents in the EO fraction, the predominant ones being thymol and carvacrol (Arras and Usai 2001; Bagamboula *et al.* 2004; Cosentino *et al.* 1999; Davidson and Naidu 2000; Dorman and Deans 2000; Farag *et al.* 1989; Lambert *et al.* 2001; Paster *et al.* 1990; Sagdic *et al.* 2002; Sivropoulou *et al.* 1996; Stahl-Biskup 2004) (see Figure 2.1). However, there is evidence that EOs exert a stronger AM efficacy than can be attributed to additive effects of their major AM components (Burt and Reinders 2003; Friedman *et al.* 2004). For instance, 96% of the growth inhibition of *Ps. aeruginosa* observed with the essential oil of oregano can be attributed to the additive effect of thymol and carvacrol with the remaining 4% attributed to the other components (Lambert *et al.* 2001). Thus, there is a possibility that the minor components possess some AM power (Burt and Reinders 2003; Cosentino *et al.* 1999; Friedman *et al.* 2004).

Table 2.2 Relative antimicrobial effectiveness of thyme, oregano, thymol and carvacrol.

Organism	Type of AM agent	Degree of inhibition[a]	Reference(s)
Gram-negative bacteria	EOs	thyme, oregano > clove, bay	(Burt and Reinders 2003; Mejlholm and Dalgaard 2002)
		thyme, oregano > basil, rosemary	(Elgayyar *et al.* 2001; Mejlholm and Dalgaard 2002)
		oregano > > coriander > basil > anise	(Zivanovic *et al.* 2005)
	active constituents	thymol, carvacrol > *p*-cymene	(Cosentino *et al.* 1999; Juven *et al.* 1994)
		thymol, carvacrol > linalool	(Cosentino *et al.* 1999)
		thymol, carvacrol > eugenol	(Friedman *et al.* 2002; Olasupo *et al.* 2003)
		thymol, carvacrol > eugenol, geraniol, citral	(Nazer *et al.* 2005)
Gram-positive bacteria	EOs	thyme > oregano > clove, black pepper	(Burt and Reinders 2003)
		oregano, thyme > basil	(Bagamboula *et al.* 2004; Elgayyar *et al.* 2001)
		oregano, thyme > mint, angelica	(Nevas *et al.* 2004)
	active constituents	thymol, carvacrol > *p*-cymene, linalool	(Bagamboula *et al.* 2004; Cosentino *et al.* 1999; Delgado *et al.* 2004; Yamazaki *et al.* 2004)
		thymol, carvacrol > cinnamaldehyde, eugenol> linalool, allyisothiosyanate	(Yamazaki *et al.* 2004)
Yeast and moulds	EOs	thyme > clove, rosemary, sage, bay	(Farag *et al.* 1989; Suhr and Nielsen 2003)
		thyme ≈ mustard ≈ lemon grass	(Suhr and Nielsen 2003)
	active constituents	thymol, carvacrol > eugenol	(Bennis *et al.* 2004; Farag *et al.* 1989; Scora and Scora 1998)
		thymol, carvacrol > *p*-cymene, linalool	(Arras and Usai 2001; Cosentino *et al.* 1999; Scora and Scora 1998)

[a]Comparisons based on inhibition results from the same study.

Antimicrobial Activity of Thymol and Carvacrol

Thymol and carvacrol each possess a phenolic structure as shown in Figure 2.1 and are highly active AM agents despite their relatively low solubility in water (Dorman and Deans 2000; Griffin *et al.* 1999; Helander *et al.* 1998). This activity may be attributed to the aromatic phenol ring and the hydroxyl group in the phenolic structure (Dorman and Deans 2000; Farag *et al.* 1989; Scora and Scora 1998). It is known that the -OH group is very reactive and can easily form hydrogen bonds with active sites of enzymes (Farag *et al.* 1989; Mason and Wasserman 1987) possibly through a reaction with sulfhydryl groups or through more non-specific interactions with the proteins (Mason and Wasserman 1987). The importance of the hydroxyl group in the phenolic structure is confirmed in terms of AM activity where carvacrol shows a wider spectrum of antibacterial activity than its methyl ether (Arfa *et al.* 2006; Dorman and Deans 2000). Furthermore, the position and the number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with growing evidence that increased hydroxylation results in increased toxicity (Geissman 1963). It has been reported that the relative position of the hydroxyl group also affects the AM effectiveness of thymol and carvacrol, thymol being the more effective inhibitor against both Gram-negative and Gram-positive bacteria (Dorman and Deans 2000). The high activity of these components may be further explained in terms of alkyl substitution in the phenolic nucleus. The introduction of alkylation has been proposed to alter the distribution ratio between the aqueous and the non aqueous phases (including bacterial phases) by reducing the surface tension or altering the species selectivity (Dorman and Deans 2000). The hydrophilic–lipophilic balance (HLB) number for carvacrol of 4.15 suggests that the compound predominantly dissolves in oil (Griffin *et al.* 1999). With slightly higher hydophobicity (measured by its partition coefficient in octanol/water ($P_{o/w}$)) of carvacrol ($P_{o/w}$ 3.64) than that of thymol ($P_{o/w}$ 3.30) (Griffin *et al.* 1999). In contrast, carvacrol and thymol have been reported to possess a relative hydrophilicity, having a water solubility of 830±10 and 846±9 ppm, respectively (Griffin *et al.* 1999; Nostro *et al.* 2007). Hence, these compounds could diffuse through the film of liquid on the food surface where the bacterial growth occurs (Broklehurst 2004).

Antimicrobial Activity In-Vitro

Although different results have been reported to assess AM activity, differences in EO composition and in the microorganism chosen, TEO, OEO, carvacrol and thymol have consistently been shown to have outstanding potentials as AM agents (Cosentino *et al.* 1999; Dorman and Deans 2000; Nevas *et al.* 2004; Olasupo *et al.* 2003; Stahl-Biskup 2004). These substances are reported to have bacteriostatic and bactericidal activity against both Gram-positive and Gram-negative bacteria (Bagamboula *et al.* 2004; Burt and Reinders 2003; Cosentino *et al.* 1999; Elgayyar *et al.* 2001; Helander *et al.* 1998; Nevas *et al.* 2004; Walsh *et al.* 2003). However, these compounds appear preferentially more active against Gram-positive than Gram-negative bacteria, with lactic acid bacteria being the most resistant among the Gram-positive bacteria (Cosentino *et al.* 1999; Dorman and Deans 2000; Jay 1996; Nevas *et al.* 2004; Smith-Palmer *et al.* 1998; Zaika 1988). In contrast, Elgayyar and others (2001) stated that the generalization that Gram-positives are more sensitive than Gram-negative bacteria is difficult to sustain because each EO is unique in its characteristics and Gram-positive bacteria vary widely in structure and functionality. The antibacterial effects of different derivatives of thyme and oregano have been reported in the literature (see Table 2.2, Table 2.3 and Table 2.4).

Thyme and oregano are strongly active against spoilage and pathogenic moulds and yeasts (Arras and Usai 2001; Conner 1994; Farag *et al.* 1989; Paster *et al.* 1990; Pina-Vaz *et al.* 2004; Salmeron *et al.* 1990; Vázquez *et al.* 2001). These compounds have been reported to inhibit the growth of toxigenic fungal species (Table 2.3 and Table 2.4), including the species of *Aspergillius*, *Fusarium* and *Penicillium,* and other non-toxigenic species (Arras and Usai 2001; Farag *et al.* 1989; Paster *et al.* 1990; Salmeron *et al.* 1990; Scora and Scora 1998; Thompson 1996; Zambonelli *et al.* 1996). Among the many EOs tested *in vitro* and *in vivo* against post-harvest fungi, those from plants of the genus *Thymus* are particularly active (Ippolito and Nigro 2003). These compounds effectively inhibit mycelium growth (Arras and Usai 2001; Farag *et al.* 1989; Scora and Scora 1998; Thompson 1996), spore germination (Paster *et al.* 1990) and/or the aflatoxin production of the toxigenic species (Farag *et al.* 1989; Salmeron *et al.* 1990; Vázquez *et al.* 2001).

Table 2.3 Inhibitory concentrations (in vitro) of oregano and thyme EOs against food spoilage and pathogenic microorganisms.

Type of organism		AM agent/s	Inhibitory concentration / type of inhibition	Reference
Gram-negative bacteria	*S. flexneri, S. sonnei*	TEO	< 10% (v/v) / inhibition zone	(Bagamboula *et al.* 2004)
	E. coli	TEO	450-900 µg/mL / MIC[a]	(Cosentino *et al.* 1999)
	E. coli O157:H7	TEO	450-900 µg/mL / MIC	(Cosentino *et al.* 1999)
		TEO, OEO	625-2500 µL/L / MBC[b]	(Burt and Reinders 2003)
	S. typhimurium	TEO	450-900 µg/mL / MIC	(Cosentino *et al.* 1999)
	Y. enterocolitica	TEO	450-900 µg/mL / MIC	(Cosentino *et al.* 1999)
		TEO, OEO	15 µL / inhibition zone	(Dorman and Deans 2000)
	V. parahaemolyticus	TEO, OEO	100 µg/mL / growth	(Beuchat 1976)
Gram-positive bacteria	*S. epidermidis*	TEO	450-900 µg/mL / MIC	(Cosentino *et al.* 1999)
	L. monocytogenes	TEO	225-900 µg/mL / MIC	(Cosentino *et al.* 1999)
	B. cereus	TEO	225-900 µg/mL / MIC	(Cosentino *et al.* 1999)
	S. aureus	OEO	575 mg/L / MIC	(Lambert *et al.* 2001)
		TEO	225-900 µg/mL / MIC	(Cosentino *et al.* 1999)
	C. botulinum	TEO, OEO	150-200 µg/mL / germination,vegetative growth	(Ismaiel and Pierson 1990a)
Fungi	*Aspergillus* spp., *Eurotium* spp., *Penicillium* spp.	TEO	50 µL, vapour phase / colony diameter	(Guynot *et al.* 2003)
			100-200 µL/L / colony diameter	(Suhr and Nielsen 2003)
Yeasts	*C. albicans*	TEO	225-450 µg/mL / MIC	(Cosentino *et al.* 1999)
	S. cerevisiae	TEO	225-450 µg/mL / MIC	(Cosentino *et al.* 1999)

[a]Minimum bactericidal concentration (MBC); [b]Minimum inhibitory concentration (MIC).

Table 2.4 Inhibitory concentrations (in vitro) of thymol and carvacrol against spoilage and pathogenic microorganisms.

Type of organism		AM agent/s[a]	Inhibitory concentration / type of inhibition	Reference(s)
Gram-negative bacteria	*P. aeruginosa*	T, C	T, 385 mg/L; C,450 mg/L / MIC[b]	(Lambert *et al.* 2001)
	E. coli	T	500 μg/mL / MIC	(Walsh *et al.* 2003)
		T, C	225 μg/mL / MIC	(Cosentino *et al.* 1999)
	E. coli O157:H7	T, C	C, 225 μg/mL; T, 450 μg/mL / MIC	(Cosentino *et al.* 1999)
	S. typhimurium	T, C	T, 56.25 μg/mL; C, 225 μg/mL / MIC	(Cosentino *et al.* 1999)
		T, C	1 mM/ MIC	(Nazer *et al.* 2005)
	Y. enterocolitica	T, C	225 μg/mL / MIC	(Cosentino *et al.* 1999)
Gram-positive bacteria	*S. flexneri, S. sonnei*	T	< 10% (v/v) / growth inhibition	(Bagamboula *et al.* 2004)
	B. cereus	T	0.4-0.8 mmol/L / growth inhibition	(Delgado *et al.* 2004; Ultee *et al.* 1998)
		T, C	T, 450 μg/mL; C, 900 μg/mL / MIC	(Cosentino *et al.* 1999)
	S. aureus	T, C	T, 140 mg/L; C, 175 mg/L / MIC	(Lambert *et al.* 2001)
		T	500 μg/mL / MIC	(Walsh *et al.* 2003)
		T, C	T, 225 μg/mL; C, 450 μg/mL / MIC	(Cosentino *et al.* 1999)
	L. monocytogenes	T, C	450 μg/mL / MIC	(Cosentino *et al.* 1999)
Fungi	*Fusarium sp.*	C	50-200 μg/mL / mycelia weight	(Thompson 1996)
Yeasts	*S. crevisiae*	T	1.5 mM / MIC; 1.8 mM / MFC[c]	(Bennis *et al.* 2004)
		T, C	112.5 μg/mL / MIC	(Cosentino *et al.* 1999)
	C. albicans	T, C	112.5 μg/mL / MIC	(Cosentino *et al.* 1999)

[a]Thymol (T), Carvacrol (C).
[b]Minimum inhibitory concentration (MIC).
[c]Minimum fungicidal concentration (MFC).

Cosentino and others (1999) found that yeasts are the most sensitive among the different types of microorganisms in the presence of TEOs as well as their main components, thymol and carvacrol. Germ tube formation of *Candida sp.* is inhibited by TEO, thymol and carvacrol (Pina-Vaz *et al.* 2004) while thymol is found to have a more pronounced effect on yeast cells than eugenol, a major constituent of clove oil (Bennis *et al.* 2004). For thymol, the MIC is 1.5 mM and it is fungicidal at 1.8 mM whereas eugenol at 1.8 mM (MIC) is able to completely inhibit the growth of *S. cerevisiae*, while 3 mM induces fungicidal activity (Bennis *et al.* 2004).

In general, *in vitro* experiments do not necessarily provide a good indication of the potential value in food preservation. However the results may suggest possible applications of these compounds in foods and may partly explain the observed reductions in AM activity of some EOs in food systems compared with *in vitro* performance.

2.1.7 Mechanism of Action

Impairment of Cell Membranes

Studies on the mechanism of action of EOs have employed a common approach of attempting to illustrate deleterious effects on cellular membranes (Becerril *et al.* 2007; Gill and Holley 2006a; Pina-Vaz *et al.* 2004; Skandamis and Nychas 2001; Ultee *et al.* 2002; Ultee *et al.* 1999). This approach was taken due to the presence of compounds such as thymol and carvacrol, that are lipophilic in nature and are known to cause structural and functional damages to plasma membranes followed by cell death (Burt and Reinders 2003; Conner 1994; Lambert *et al.* 2001; Paster *et al.* 1990; Pol and Smid 1999; Sikkema *et al.* 1995; Thompson 1996; Ultee *et al.* 2002). These hydrophobic compounds are likely to dissolve and accumulate in the lipid bi-layer of the cytoplasmic membrane of bacterial cells according to the partition coefficient that is specific to the compound applied, leading to a disruption of the membrane structure and function (Helander and others 1998). A critical concentration of the EO is needed to cause leakage of cellular constituents (Paster *et al.* 1990; Ultee *et al.* 1998). Ultee and others (1998) observed that *B. cereus* partially recovers from the inhibition action of carvacrol

when applied below the MIC value. Thyme oil, thymol and carvacrol can cause a sudden significant reduction in viable bacteria counts once a certain (critical) concentration is applied. This occurs rather than a progressive increase in the AM effect as the concentration of AM agent is increased. The authors hypothesized that the phenolic compounds sensitize the membrane and, when saturation of the site(s) of action occurs, there is gross damage with a sudden collapse of the bacterial membrane integrity and subsequent loss of cytoplasmic constituents (Paster *et al.* 1990; Vigil 2005).

The permeability of cell membranes depends on the hydrophobicity of the solutes as well as the composition of the membrane (Sikkema *et al.* 1995). Given the latter, the partition coefficient (or the partition of the microbial cell out of the water phase and in the lipid membrane) is a crucial determinant for the effectiveness of these AM compounds. Thus, quantitative variations in the efficacy against different bacteria are expected, primarily when bacteria with different Gram-staining responses are examined (Bagamboula *et al.* 2004; Lambert *et al.* 2001). The general reduction in activity for these compounds towards Gram-negative bacteria may be a result of an impediment to the lipophilic phenolic compounds to diffuse across the outer cell membrane (Nychas and Tassou 2000) which serves as an effective penetration barrier to these hydrophobic compounds (Helander *et al.* 1998). However, unlike many antibiotics, the hydrophobic constituents of EOs, including thymol and carvacrol, are capable of gaining access to the periplasm of Gram-negative bacteria through the porin proteins of the outer membrane (Helander *et al.* 1998).

After penetration into the lipid-rich interior of the cytoplasmic membrane of bacteria, these AM agents may interact with membrane proteins *via* hydrogen bonding and hydrophobic interaction and cause a back-flow of protons across the membrane thus affecting the cellular activities powered by the proton motive force (Paster *et al.* 1990; Ultee *et al.* 2002). The hypothesized impairments in membranes are reflected by metabolic disturbances, mainly on the dissipation of the two components of the proton motive force, the pH gradient and the electrical potential (Dorman and Deans 2000; Lambert *et al.* 2001; Sikkema *et al.* 1995; Ultee *et al.* 2002; Ultee *et al.* 1999; Ultee *et*

al. 2000; Ultee and Smid 2001). Carvacrol has been shown to destabilize the cytoplasm and the outer membrane of bacteria and act as a 'proton exchanger', resulting in a reduction of the pH gradient across the cytoplasm membrane (Helander *et al.* 1998; Lambert *et al.* 2001; Ultee *et al.* 2002). Other studies report on the impairment of a variety of enzyme systems, including those involved in energy production and structural component synthesis (Farag *et al.* 1989; Ismaiel and Pierson 1990a; Ultee and Smid 2001) and inhibition of membrane bound enzyme activity of bacterial cells (Gill and Holley 2006a; Gill and Holley 2006b) as secondary rather than a primary causes of cell death (Gill and Holley 2006b).

Inhibition of Toxin Production

Oregano EO, thymol and carvacrol have also been reported as inhibitors to toxin production by bacteria and aflatoxigenic fungi (Farag *et al.* 1989; Ultee and Smid 2001). Farag and others (1989) noted a reduction in the production of aflatoxin, metabolites of *A. parasiticus*, by TEO, similar to the action of carvacrol on toxin production by *B. cereus* (Ultee and Smid 2001). As hypothesized by Ultee and Smid (2001), the mechanism of inhibition of toxin production by *B. cereus* may be a gene regulation, transcription or translation, or a mechanism affecting the excretion and transportation of the toxin. Excretion may be an active process and an insufficient driving force (adenosine triphosphate or proton motive), due to membrane damage, and would result in the accumulation of the toxin inside the cell. Consequently, intracellular toxin might inhibit its own synthesis (feedback inhibition). Inhibition of toxin synthesis may also be a result of a lower specific growth rate due to the presence of AM agents. The cell may use the metabolic energy for maintaining its viability rather than for toxin production that also requires energy (Ultee and Smid 2001).

2.2 Thyme and Oregano as Natural Food Preservatives

The efficacy of thyme and oregano has been extensively tested in a range of food products and a summary of these tests is presented in Table 2.5. However, compared to *in vitro* studies, only a relatively small number of studies have investigated the AM

potential of EOs and their components of thyme and oregano in real foods (Chiasson *et al.* 2004; Mejlholm and Dalgaard 2002; Plotto *et al.* 2003; Roller and Seedhar 2002; Seaberg *et al.* 2003; Skandamis and Nychas 2001; Smith-Palmer *et al.* 2001; Suhr and Nielsen 2003; Tsigarida *et al.* 2000; Ultee and Smid 2001). It has been established that microorganisms present in food are less susceptible to EOs rather than in vitro or there is an effect of foodstuffs on microbial resistance to EOs. A review of the possible causes of this microbial resistance to EOs has found that direct application of AM substances onto food has limited benefits due to factors such as neutralization or rapid diffusion into the bulk of the food (Burt 2004). There is clearly a need to investigate the most appropriate mode of application of these compounds as food preservatives.

Fruits and Vegetables

Consumer demand for minimally processed, ready-to-eat fruits and vegetables has led to a significant growth in the freshly-cut products. However, during minimal processing spoilage and pathogenic microorganisms can gain access to the nutrients inside fruits and vegetables and multiply. As it is impossible to completely prevent contamination, washing and treatment with chemical disinfectants are necessary to decontaminate the surface of fresh produce (Roller and Seedhar 2002). These procedures are only partially effective in reducing the microbial load and therefore, there is a need to develop new treatments for decontaminating fruits and vegetables (Beuchat 1998). It has been shown that TEO, thymol and carvacrol have a potential for use in the decontamination of minimally processed vegetables (Bagamboula *et al.* 2004; Hernández-Herrero *et al.* 2008; Molinos *et al.* 2008; Valero and Giner 2006; Weissinger *et al.* 2001) and fruits (Guillen *et al.* 2007; Roller and Seedhar 2002). Vegetable-based food products are the primary candidates for use in the evaluation of the potential antibacterial properties of herbal AM agents because they are low in protein and lipid-food components that can interact with the active compounds in spices and herbs and therefore decrease the AM action of these compounds (Bagamboula *et al.* 2004).

A decrease of the *Shigellae* and *Enterobacteriaceae* populations is observed in fresh lettuce after washing with 0.05% and 1.0% (v/v) TEO, thymol and carvacrol. However, the AM effect on a subsequent lettuce sample exposed to the same decontamination solution is significantly lower. The low to moderate solubility of TEO and its

compounds in water may result in the adsorption and reaction of the EO and its compounds with the first sample subjected to decontamination and a subsequent reduction in the AM potential (Bagamboula *et al.* 2004). The application of TEO or thymol or carvacrol for decontamination may be hampered due to changes in the sensoric properties of lettuce (e.g. browning, strong odor). However, in this study, the inoculum of *Shigellae* was higher than would be encountered in naturally contaminated lettuce, suggesting that lower concentrations of TEO or thymol or carvacrol may suffice for naturally contaminated products (Bagamboula *et al.* 2004). Similar undesirable color, odor and taste changes (browning, pungent and unpleasant aroma, bad aftertaste) are observed in fresh-cut kiwifruit that was dipped in carvacrol solutions at 5-15 mM (Roller and Seedhar 2002) and minimally processed carrot broth dosed with 2.5-10 µL/100mL of carvacrol and 20-35 mg/100mL of thymol (Valero and Giner 2006). Treatment of fresh-cut honeydew melon with 1 mM carvacrol or cinnamic acid extend the lag phase of the microbial flora from less than 1 day in the untreated controls to 3 days at 8°C and 5 days at 4°C. Although the aroma of both compounds is detectable in the treated fruits after storage, it was not considered unpleasant. The reported spicy odor of carvacrol or cinnamic acid is less readily detected on melon than on kiwifruit (Roller and Seedhar 2002).

Apart from decontamination, there is a renewed interest in the application of these AM substances for reducing the post-harvest microbiological alterations in fruits and vegetables (Martínez-Romero *et al.* 2007; Valverde *et al.* 2005). Thyme essential oil and carvacrol are effective in inhibiting *P. digitatum* growth on oranges (Arras and Usai 2001) and TEO and OEO exhibit fungicidal activities in tomato (Plotto *et al.* 2003).

Dairy and Bakery Products

In these products, the composition of cheese is an important factor in determining the AM effectiveness of EOs. The effect of the plant EOs from bay, clove, cinnamon and thyme at concentrations of 0.1, 0.5 and 1% w/w was studied in low-fat (protein 8.5%, fat 16%, (w/w)) and full-fat soft cheese (protein 6.4%, fat 30%, (w/w)) against *L. monocytogenes* and *S. enteritidis* at 4°C and 10°C respectively, over a 14 day period. In the low fat cheese, all four oils at 1%, reduced *L. monocytogenes* and *S. enteritidis* to ≤ 1.0 log10 CFU m/L. In contrast, oil of clove was the only one to achieve such reduction

in the full fat cheese (Smith-Palmer *et al.* 2001). The volatile fraction of the TEO exerts considerable antifungal activity against several fungi, commonly causing deterioration of bakery products, e.g. sponge cake analogues (Guynot *et al.* 2003) and rye bread (Suhr and Nielsen 2003). However, it has also found that the activity of the TEO volatile fraction is limited compared to lemongrass oil or mustard, both containing smaller and non-phenolic volatile compounds (Suhr and Nielsen 2003).

Fish

The compounds of thyme and oregano, particulary OEO, are found to be effective in extending the shelf-life of fish or fish dishes without any negative effect on sensorial qualities. Oregano essential oil (0.05%, v/w) extended the shelf-life of modified atmosphere packaged (MAP), naturally contaminated cod fillets from 11-12 days to 21-26 days at 2°C. A distinctive but pleasant herbal flavor of OEO was detected initially, but decreased gradually during storage. Neither texture nor appearance of cod fillets were affected by the addition of oregano oil (Mejlholm and Dalgaard 2002). The effectiveness of OEOs against *Photobacterium phosphoreum* on salmon, a fatty fish, was less than that on cod fillets (Mejlholm and Dalgaard 2002) suggesting that a high fat content appears to reduce the effectiveness of OEOs. Dipping common carp (*C. carpio*) fillets into a solution containing both carvacrol and thymol lead to a remarkable reduction in the growth and numbers of bacteria, consequently extending the shelf-life of the fish. The treated samples extended the shelf-life by 8 days at 5°C and by 4 days at 10°C, depending on sensory qualities (Mahmoud *et al.* 2004). Among five treatments examined, including storage on ice (usual commercial method of preservation), a combination of 0.1% (v/w) OEO and MAP was the most effective in inhibiting the microbial and sensory spoilage of fresh Mediterranean swordfish fillets. Using this combination enabled a shelf life extension by 8 to 9 days (Giatrakou *et al.* 2008).

Meat and Meat Products

Thyme, oregano, thymol and carvacrol are superior antimicrobials for many meat applications and are effective against pathogenic or spoilage microoraginsms in meat at lower levels than many other compounds (Burt 2004). The AM potential of carvacrol, thymol and thyme in ground (minced) meat was evaluated by determining the MIC in the presence of *E. coli* and *Salmonella Typhi*. For both organisms the order of MICs was

found to be: carvacrol < thymol < thyme (Chiasson *et al.* 2004). Moreover, the addition of OEO delayed the microbial growth and suppressed the final counts of microbial numbers in minced meat stored under MAP without affecting the color and the odor of the product (Skandamis and Nychas 2001). The OEO also caused a pronounced alteration in the physico-chemical properties of minced meat by delaying glucose and lactate consumption under aerobic as well as under MAP conditions (Skandamis and Nychas 2001). It was also demonstrated that the addition of OEO resulted in a reduction of the initial bacterial population, including lactic acid bacteria and *L. monocytogenes*, in beef fillets stored in different gaseous environments (Tsigarida *et al.* 2000). The effectiveness of OEO against *C. botulinum* spores was studied in a vacuum packed and pasteurised minced (ground) pork product. There was no significant effect by OEO at a concentration of 0.4 μL/g. However, in combination with low levels of sodium nitrite, the same concentration of OEO was able to delay the spore growth while the activity depended on the number of inoculated spores (Ismaiel and Pierson 1990a). Thymol was tested as a bactericide solution effective in controlling biofilms formed by pathogenic or spoilage bacteria on traditional dry sausages. Regardless of the time/temperature conditions applied, 1-10 mM thymol had only a slight lowering effect (Lebert *et al.* 2007). This may be due to the higher resistance of bacterial biofilms to antimicrobial treatments than the individual cells grown in suspension (Lebert *et al.* 2007).

Ready-to-eat Foods

Alcohol extracts of thyme were applied to the surface of cooked, ready-to-eat chicken meat to determine the AM activity against low (10 CFU/g) or high (10^5 CFU/g) populations of *A. hydrophila* and *L. monocytogenes*. Thyme extract suppressed somewhat the growth of *Aeromonas* but was not effective against *L. monocytogenes* (Hao *et al.* 1998). Oregano essential oil and carvacrol inhibited *L. monocytogenes* in ready-to-eat-beef slices with the efficacy of the OEO being greater than that of carvacrol (Seaberg *et al.* 2003). Ultee and others (Ultee *et al.* 2000; Ultee and Smid 2001) suggested that the main components of thyme and oregano can be used in cooked products, preferably those that are low in fat and protein (such as vegetable purees, pasta, rice and soup), to control the microbial growth. Carvacrol was investigated for its effect on toxin production of *B. cereus* in different types of soups. It was found that at 3.0 mg/mL, the amount of toxin production by *B. cereus* in mushroom soup was below the detection threshold (Ultee and Smid 2001).

Table 2.5 Antimicrobial effects of essential oil of thyme, oregano, thymol and carvacrol in different types of food products.

Food product	AM agent/s[a]	Target microorganism/s	Mode of application	Effective concentration	Effect	Reference
Fresh-cut kiwifruit	C	residential flora	dipping	1 mM	decontamination	(Roller and Seedhar 2002)
Table grapes	T, C	molds, yeasts and mesophilic aerobes	added in thermosealed baskets + MAP[b]		lowered berry decay, improve quality	(Guillen *et al.* 2007)
	T	molds, yeasts and mesophilic aerobes	vapor contact + MAP	0.5 mL/bag	significant inhibition, improve quality	(Valverde *et al.* 2005)
	C	*B. cinerea*	vapor contact	0.01-1.0 mL/L of package	reduce berry decay	(Martínez-Romero *et al.* 2007)
Fresh-cut lettuce	TEO, T, C	*S. sonnei*	washing	0.5% & 1.0% (v/v)	decontamination	(Bagamboula *et al.* 2004)
Alfalfa sprouts	C	*B. cereus*	washing	0.3% (v/v)	significant reduction of viable cells	(Molinos *et al.* 2008)
Minimally processed carrot	T, C	*B. cereus*	direct incorporation	2.5-10 µL/100mL of C and 20-35 mg/100mL of T	increase lag phase, decrease growth rate	(Hernández-Herrero *et al.* 2008; Valero and Giner 2006)
Egg plant salad	OEO	E. coli O157:H7	direct incorporation	7-21 µL/g	effective inhibition, reduce final populations	(Skandamis and Nychas 2000)
Raw cod fillets	TEO, OEO	*P. phosphoreum*	surface application + MAP	0.5% (w/v)	extension of lag phase, reduction of cell numbers	(Mejlholm and Dalgaard 2002)
Raw salmon fillets	TEO, OEO	*P. phosphoreum*	surface application + MAP	0.5% (w/v)	no significant inhibition	(Mejlholm and Dalgaard 2002)
Fresh fish fillets	OEO	*S. aureus*, *S. enteritidis*, residential flora	dressing + MAP		bactero-static and -cidal effect	(Tassou *et al.* 1996)

Food product	AM agent/s[a]	Target microorganism/s	Mode of application	Effective concentration	Effect	Reference
Common carp fillets	T, C	residential flora	dipping	0.5% (v/v)	extend the shelf-life from 4-12 d	(Mahmoud *et al.* 2004)
Swordfish fillets	OEO	residential flora	combination of OEO + MAP	0.1% (v/w)	extend the shelf-life from 8-9 d	(Giatrakou *et al.* 2008)
Carp fillets (dried)	T, C	aerobic bacteria	combined treatment with electrolyzed NaCl solutions	0.5% (v/v)	strong antimicrobial and antioxidant effects	(Mahmoud *et al.* 2006)
Shrimp	TEO	natural spoilage flora	coating, coating + irradiation	0.9% and 1.8% (v/w)	inhibition of growth, reduced acceptability	(Ouattara *et al.* 2001)
Ground beef	TEO, T, C	*E. coli* *Salmonella* Typhi	direct incorporation	0.8-3% (w/w); MIC[d]	reduce cell numbers	(Chiasson *et al.* 2004)
Beef meat fillets	OEO	*L. monocytogenes,* autochthonous flora	surface dipping + high- and low-O_2 permeable films + air, MAP and VP[c]	0.8% (v/w)	2-3 $\log_{10}$ reduction under all conditions	(Tsigarida *et al.* 2000)
Minced beef	OEO	spoilage microbiota	meat-EO mixture + air, MAP; + permeable polythene bags	0.05-1% (v/w)	reduction in microbial loads	(Skandamis and Nychas 2001)
Minced pork	OEO	*C. botulinum* spores	direct addition + MAP	100-200 ppm	no inhibition	(Ismaiel and Pierson 1990b)
	TEO	*L. monocytogenes*	direct addition	0.02 mL/25 g of meat	extended lag phase	(Aureli *et al.* 1992)
Chicken	C	*S. enterica*	vapour contact	20% (v/v), MIC	significant reduction of viable cell numbers	(Burt *et al.* 2007)
Sponge cake analogues	TEO	*Eurotium* spp., *Aspergillus* spp., *Pencillium* sp.	vapour contact	50 µL	significant growth reduction at 0.8 a_w	(Guynot *et al.* 2003)
Rye bread	TEO	*Pencillium* spp., *E. repens*, *A. flavus*	vapour contact	135 or 270 µL/L	inhibition of growth	(Suhr and Nielsen 2003)

Food product	AM agent/s[a]	Target microorganism/s	Mode of application	Effective concentration	Effect	Reference
Soft cheese	TEO	*L. monocytogenes* *S. enteritidis*	cheese-EO-mixture	1% (v/v)	low-fat cheese: inhibition of growth full-fat cheese: no inhibition	(Smith-Palmer *et al.* 2001)
Semi skimmed milk	C	*L. monocytogenes*	direct addition + high hydrostatic pressure	2-3 mmol/L	reduction in final population	(Karatzas *et al.* 2001)
Spanish cheese (Arzúa-Ulloa)	T	*P. citrinum*	surface application	100-150 µg/mL	inhibition of toxin production up to 5-6 d	(Vázquez *et al.* 2001)
Spanish cheese (Cebreiro)	T	*P. citrinum*	surface application	100-150 µg/mL	no effect on toxin production	(Vázquez *et al.* 2001)
Cooked rice	C	*B. cereus*	direct incorporation	>0.15 mg/g	inhibition of growth	(Ultee *et al.* 2000)
Ready-to-eat soup	C	*B. cereus*	direct incorporation	3 mg/mL	reduce toxin production	(Ultee and Smid 2001)
Cooked poultry	TEO	*A. hydrophila, L. monocytogenes*	surface application	1:5 dilutions	inhibition of only *A. hydrophila*	(Hao *et al.* 1998)
Caprese salad	T	natural microflora	dipping + MAP	400-ppm	inhibition of growth, extended the shelf life from 3-12 d	(Bevilacqua *et al.* 2007)
Thin-sliced beef	OEO, C	*L. monocytogenes*	surface spreading + sealed containers	800 ppm	inhibition of growth	(Seaberg *et al.* 2003)
Apple juice	C	*E. coli* O157:H7	direct addition	1.25 mM	reduced cell numbers to undetectable levels within 1-2 d	(Kisko and Roller 2005)

[a]EO of oregano(OEO), EO of thyme (TEO), Thymol (T), Carvacrol (C).
[b]Modified atmosphere packaging (MAP).
[c]Vaccum packaging (VP).
[d]Minimum inhibitory concentration (MIC).

2.2.1 Limitations in Food Applications

Achieving Organoleptically Acceptable Levels

Effective doses required to gain AM benefits in food products may exceed organoleptically acceptable levels. The required levels for EOs (or their constituents) to instigate an AM effect in food products are usually higher than the amount used in flavoring applications and may therefore result in adverse sensorial effects (Bagamboula *et al.* 2004; Smith-Palmer *et al.* 2001). According to Ultee and others (2000), carvacrol is capable of inhibiting *B. cereus* in soup, but at an approximately 50-fold higher concentration than needed to reach the same effect as in broth. Therefore, the usefulness of EOs for food preservation purposes depends greatly on their AM efficiency in the particular type of food and/or may be limited to foods where a flavor effect would be desirable or acceptable. For instance, the aroma of OEO is pleasant when added at 0.05% (v/w) to cod fillets (Mejlholm and Dalgaard 2002), and acceptable up to 1% in minced meat (Skandamis and Nychas 2001) and ready-to-eat soups (Ultee and Smid 2001). Similar concentrations, however, may be less acceptable in other foods (Burt and Reinders 2003). The incorporation of thyme oil in an AM coating for pre-cooked shrimps, for example, reduces the acceptability scores for taste and odor (Ouattara *et al.* 2001).

To overcome this application barrier, a number of options can be considered: (i) View the EOs not only as preservatives, but also as flavor components (Smith-Palmer *et al.* 2001). Existing uses of these AM compounds as flavoring agents are already applied in a wide range of food products. (ii) Understand the relationship between MIC and sensory acceptability to enable a balance between acceptability and AM efficacy (Lambert *et al.* 2001). (iii) Reduce the strong aroma associated with EOs and their compounds by the use of de-aromarization methods (Bagamboula *et al.* 2004). (iv) Substitution of a particular EO with its principal constituents that may be equally AM effective but with milder flavoring attributes (Lambert *et al.* 2001; Smith-Palmer *et al.* 2001). (v) Addition of a flavorless additive to the EO and thus increase the AM efficacy and enable a lower EO concentration to be used (Burt and Reinders 2003; Mejlholm and Dalgaard 2002). (vi) Use of a microencapsulation process whereby the active AM

compounds can be trapped, masking odor and flavor, until they are slowly released into the atmosphere at constant low doses. This can protect the product from microbial growth without affecting its sensory attributes (Ayala-Zavala *et al.* 2008). (vii) Application of EOs or their constituents as part of a hurdle system and to use them as a component in a series of preservation techniques, or to use a synergistic combination of essential oils and their compounds, thus enabling to decrease the individual concentrations and minimize adverse sensorial effects (Bagamboula *et al.* 2004; Gutierrez *et al.* 2009; López *et al.* 2005; Smith-Palmer *et al.* 2001; Ultee *et al.* 2000).

Volatility and Lipophilicity Effects

The application of spices and herb-based AM agents in food systems is often discouraged due to their volatility and lipophilicity (Bagamboula *et al.* 2004). Due to their lipophilic nature, these compounds partition into the lipid phase and thereby lose some of their AM potency. However, thymol and carvacrol, being phenolic AM agents with their mode of action believed to be on cell membranes, could benefit from lipophilicity due to their affinity to the hydrophobic domain of the cytoplasmic membrane of bacterial cells. Furthermore, by using the AM compound *via* the vapor phase, the problem of lipophilicity could be partly compensated (Manou *et al.* 1998) and the volatility could be a distinct advantage in lowering microbial contamination in air and surfaces that are difficult to reach (Dorman and Deans 2000). The EOs from thyme, oregano and their major constituents have shown to act *via* the vapor phase (Guynot *et al.* 2003; López *et al.* 2007; Suhr and Nielsen 2003). For instance, thymol being a smaller and more volatile molecule than the ether-containing eugenol is more effective in the vapor phase. Hence, thyme is more effective than clove and cinnamon in experiments containing volatile compounds (Suhr and Nielsen 2003). With improved formulation and packaging techniques, both characteristics can be potentially employed in the favour of the final product in terms of marketing and consumer safety (Manou *et al.* 1998). For example, the volatility characteristic would be a distinct advantage for reducing microbial contamination in package-headspace systems. Compared to a non-volatile substance, a volatile substance can migrate through the headspace and air gaps between the package and the food (Han 2000).

Potential Loss of AM Action in the Food Matrix

The AM activity of EOs and their compounds diminishes, in most cases, when added to more complex materials such as food products (Bagamboula *et al.* 2004), because of the interactions between phenolic compounds and the components in the food (Nevas *et al.* 2004; Nychas and Tassou 2000; Vigil 2005). As demonstrated by Mejlholm and Dalgaard (2002) and Smith-Palmer and others (2001), the concentration and type of oil or fat present in a food can affect the AM efficacy of EOs or their components. Partitioning of the hydrophobic antibacterial EO components in the fat content of the food may prevent them from coming in contact with the target bacterial cells growing in the hydrophilic regions (Mejlholm and Dalgaard 2002). Farbood and others (1976) suggested that fatty coatings could form on the surface of bacterial cells and thereby prevent the penetration of the inhibitory compound. In contrast to a significant AM effect of oregano on the inhibition of *P. phosphoreum* present in MAP cod fillets, there is no detectable effect in MAP fillets of salmon (Farbood *et al.* 1976). This finding corresponds with the difference in fat content in the two fish types which are low and high in lipid content respectively (Farbood *et al.* 1976). The effect of fat content in the food is demonstrated with TEO which shows limited inhibition against *S. enteritidis* in full-fat cheese (Smith-Palmer *et al.* 2001). Carvacrol, however, shows a significant reduction in AM activity when applied to meat containing 93% fat (Seaberg *et al.* 2003). Furthermore, it is believed that reduced water, protein and/or salt content in foods could impede the AM activity (Smith-Palmer *et al.* 2001). Ting and Diebel (1992) studied the effect of cloves and oregano on the growth and survival of *L. monocytogenes* in meat slurry at 4°C and 24°C and found that these compounds at 1.0% have little effect on the growth of the microorganism in meat slurry. This is in agreement with previous findings that foods with high protein, fat and water contents require increased concentration of preservatives. Moreover, Ultee and others (2000) found that a solution of 1.25 g/L sodium chloride antagonizes the effect of carvacrol against *B. cereus* on rice. The interactions between EOs and their components with other food ingredients require more thorough investigation to fully understand their complexity (Burt and Reinders 2003). A much less complex and alternative application route would be to consider potential applications that do not require the direct incorporation of the EO in the food matrix, e.g. a water rinse decontamination process

for fresh cut vegetables (Wan *et al.* 1998) or the slow release of AM compounds onto the food surface from an active AM packaging system.

Development of Resistance and/or Bacterial Recovery

Antimicrobial resistant microorganisms often arise from pre-exposure to sub-lethal/sub-inhibitory levels of AM agents (Friedman *et al.* 2004). The use of AM agent concentrations that are too low in food products might allow bacterial recovery as was evident for TEO including three other EOs at 0.5% (v/v) against *S. enteritidis* in low-fat cheese (Smith-Palmer *et al.* 2001). Conversely, OEO, TEO and carvacrol showed antibacterial activity against strains of *B. cereus*, *E. coli*, and *S. aurues* that had previously developed resistance to antibiotics (Friedman *et al.* 2004).

2.2.2 Enhanced AM Efficacy of Essential Oils

The use of EO based AM compounds at low concentrations in combination with other microbial stress factors cannot only enhance their AM activity but may also contribute to the overall food preservation system (Bagamboula *et al.* 2004; Vigil 2005). Vigil (2005) evaluated the potential of these AM agents in combination with traditional (e.g. salting, heating, acidification) and modern (e.g. vacuum packaging, MAP) preservation techniques. It was established that the inclusion of thyme and oregano or their components as part of a hurdle technology might assist in the preservation of foods and enhance food safety by inhibiting the growth of specific microorganisms. For instance, a test involving different combinations of inhibitory growth or stress factors (temperature, pH, NaCl, inclusion of 1% (w/v) thyme or basil) showed that thyme can inhibit the growth of *Shigella* spp. as part of a hurdle technology (Bagamboula *et al.* 2003).

Temperature

Microorganisms are more susceptible to AM compounds close to their optimum growth temperature because of their increased metabolic activity under these conditions. Die-off of *Shigella* sp. and indigenous flora on lettuce is enhanced at room temperature in

the presence of solutions in water of TEO, thymol and carvacrol compared to exposure to 7°C (Bagamboula *et al.* 2004). Ultee and others (1998) found that the exposure temperatures (8°C and 30°C) have a significant effect on the survival of vegetative cells of *B. cereus*, with a higher death rate at 30°C. It is believed that carvacrol migrates more easily into the membrane of *B. cereus* because of its higher membrane fluidity at 30°C compared to 8°C. It was also observed that the *Shigella* die-off in fruit juices is higher at room temperature compared to refrigeration (Bagamboula *et al.* 2002). According to Hao and others (1998) an increased storage temperature enhances the inhibitory effect of some plant extracts, including thyme, against *L. monocytogenes* and *A. hydrophilla*.

Thus the application of extracts of herbs or spices on food may provide protection against temperature abuse during storage and distribution. Despite this possibility, it is believed that it would be imprudent to recommend the use of AM plant extracts for food preservation in the absence of proper refrigeration (Hao *et al.* 1998). There is little evidence of synergism between AM compounds and the physical hurdle of chilling, although it was shown that carvacrol and cinamic acid inhibit microbial growth at chilling temperatures of 4°C and 8°C (Roller and Seedhar 2002). The retention of the AM activity of TEO and OEO within a broad temperature range (Burt and Reinders 2003) and the reported bacteriostatic or bactericidal effect at low (chilled) temperatures (Smith-Palmer *et al.* 1998) is very encouraging considering the wide application of low temperature storage for food preservation (Burt and Reinders 2003).

Effect of pH

The pH of the medium is believed to have a strong effect on the AM activity of EOs. A stronger antibacterial effect of TEO and thymol is observed against *S. Ttyphimurium* at pH 5.5 compared to pH 6.5. At low pH values, the thymol molecule is mostly un-dissociated, more hydrophobic, may bind better to hydrophobic regions of membrane proteins and dissolve better in the lipid phase of bacterial membrane (Paster *et al.* 1990). An increased biocidal action of carvacrol has also been reported in *B. cereus* at acidic pH 5.5 compared to neutral pH 7.0 (Ultee *et al.* 1998). Roller and Seedhar (2002) observed a similar behavior of carvacrol in acidic fruits like kiwifruit (pH 3.2-3.6) and melon (pH 5.4-5.5). In contrast, Ultee and others (1998) observed a four-fold increased

sensitivity of *B. cereus* at pH 8.0 compared to that under acidic conditions (pH 5.5). At higher pH levels, carvacrol dissociates partially, resulting in a decrease in its hydrophobicity. Consequently, the inhibitory activity should decrease at high pH levels (Ultee *et al.* 1998). The pK_a of phenols is approximately 10 and this, in combination with the observed increased death rates at higher pH values, led to the conclusion that the pH effect on bactericidal activity cannot be explained by dissociation of the phenolic proton from the hydroxyl group in carvacrol (Ultee *et al.* 1998).

Water Activity and Oxygen Availability

Guynot and others (2003) found that the activity of TEO in cake dough samples with varying water activity levels (a_w 0.80-0.90) is substrate dependent. Both factors, EO and a_w, and their interaction had a significant impact on the growth of all tested fungi. In general, AM activity favors low a_w values (0.80 and below). As a_w was increased, fungal growth was favored, in some cases, leading to the conclusion that these EOs are most effective when applied to foods with low water activity (Guynot *et al.* 2003).

The inhibitory effect of the EOs of oregano and thyme, as well as of thymol and carvacrol against *Stapylococcus* spp. and *Salmonella* sp. was significantly enhanced under microaerobic (where oxygen is present in sub-saturating amounts) or anaerobic conditions (Juven *et al.* 1994; Paster *et al.* 1990; Tassou *et al.* 1996). One explanation could be that fewer oxidative changes occur in the EOs at low oxygen concentrations (Paster *et al.* 1990; Tassou *et al.* 1996). It has also been hypothesized that this behavior might be related to the lower bacterial metabolism at low oxygen concentrations and consequent increased sensitivity of the microorganisms to the AM agents (Paster *et al.* 1990).

Packaging Conditions and Irradiation

Studies using fish, meat and minimally processed foods showed that MAP acts synergistically with EOs, since only a selected proportion of microbiota is allowed to develop compared to aerobic storage (Bevilacqua *et al.* 2007; Giatrakou *et al.* 2008; Paster *et al.* 1990; Skandamis and Nychas 2001; Tassou *et al.* 1996; Tsigarida *et al.*

2000). It was noted that the inhibition of *S. aureues* and *Salmonella enteritidis* by a dressing consisting of olive oil, lemon juice and oregano added to Mediterranean gilt-head seabream fish fillet was more pronounced under MAP compared to aerobic packaging (Tassou *et al.* 1996). It is well established that microorganisms are not equally susceptible to the different hurdles applied for food preservation purposes. For example, Gram-negative organisms are more susceptible to CO_2 than Gram-positive ones. Gram-positives like *B. thermosphacta* and lactic acid bacteria thrive better under MAP (i.e. increased CO_2) than under aerobic conditions (Skandamis and Nychas 2001; Tassou *et al.* 1996; Tsigarida *et al.* 2000) while Gram-negative ones like *Pseudomonads* seem to predominate under aerobic conditions (Tsigarida and others 2000). Incorporation of OEO reduced the numbers of Gram-positive lactic acid bacteria under vacuum packaging and MAP (Tsigarida *et al.* 2000). The variability of packaging film properties also affects the AM efficacy of EOs. The use of low oxygen permeability films enhanced the inhibitory effect of OEO on the microbial association on beef fillets compared to films of high O_2 permeability (Tsigarida *et al.* 2000).

Ouattara and others (2001) evaluated the combined effect of gamma irradiation and AM coatings containing various concentrations of thyme oil and trans-cinnamaldehyde, on the shelf-life of pre-cooked shrimps. They found that the combination of gamma irradiation and AM treated coatings produced synergistic effects in reducing the microbial counts, resulting in at least 12 days extension of shelf-life (Ouattara *et al.* 2001). The addition of carvacrol to ground beef increased the sensitivity of *E. coli* and *Salmonella Typhi* to irradiation. Radiosensitization of these bacteria by carvacrol was found to be significantly higher compared to tetrasodium pyrophosphate (Chiasson *et al.* 2004). An additive effect of carvacrol and MAP conditions on the bacterial radiosensitization at 4°C in ground beef was also observed (Chiasson *et al.* 2004).

2.2.3 Combinations of Antimicrobial Compounds

Combination of two or more antimicrobially effective compounds can offer many advantages over using a single AM agent. These include enhanced AM activity (Goñi *et al.* 2009; Lin *et al.* 2004; Nevas *et al.* 2004; Ultee *et al.* 2000; Yamazaki *et al.* 2004); a reduced effect on nutritional and sensoric attributes (Ultee *et al.* 2000; Walsh *et al.*

2003; Yamazaki *et al.* 2004); expansion of the range of applications; a reduced need for severe processing treatments (Delgado *et al.* 2004) and overcoming the restrictions on the use of certain preservatives. For example, the practical application of nisin, a bacteriocin, is limited because of its low stability and activity at high pH, limited efficacy in certain food matrices and emergence of resistant or tolerance bacteria (Pol and Smid 1999)..However, the use of combinations of nisin and carvacrol or thymol has overcome these limitations (Delgado *et al.* 2004; Ultee *et al.* 2000)

The combination of TEO and OEO at a ratio of 1:1 revealed no apparent additive effects in the agar disc diffusion method against *E. coli* O157:H7 (Burt and Reinders 2003). It was also found that the presence of both phenolic constituents in the EOs is preferred for its AM action, rather than a higher content of thymol or carvacrol alone, indicating a synergistic or cumulative effect between these two components (Lambert *et al.* 2001; Manou *et al.* 1998; Paster *et al.* 1990; Paster *et al.* 1995; Pina-Vaz *et al.* 2004). Since both compounds act on the cytoplasmic membrane, an additive or synergistic effect could be expected, and a lower total dosage of the compounds would be required to cause an inhibitory effect when in combination (Pol and Smid 1999; Yamazaki *et al.* 2004). Although, the actual mechanism of synergy is not clear (Pol and Smid 1999), the synergism between the two hydrophobic compounds, both of which are expected to partition preferentially in the membranes of cells, could possibly be explained by the action of one compound to facilitate the uptake of the other one in the lipid bilayer of the cytoplasmic membrane (Delgado *et al.* 2004).

Both carvacrol and thymol are known to have prominent outer membrane disintegrating properties. Combining these two compounds with other types of AM agents such as nisin (a bacteriocin reported to be inactive against Gram-negative bacteria) would allow the latter compounds to pass through the outer membrane of target microorganisms (Olasupo *et al.* 2003). Therefore, pronounced synergies between nisin and carvacrol or thymol against *B. cereus* and *L. monocytogenes* (Pol and Smid 1999), and between cymene and carvacrol or thymol against *B. cereus* (Delgado *et al.* 2004; Ultee *et al.* 2000) have been reported (see Table 2.6). In contrast, Olasupo and others (2003) did not observe any enhancement of AM activity when nisin was combined with thymol or

carvacrol. However, results of studies with synergistic mixtures (see Table 2.6) postulate that combinations of naturally occurring AM agents could expand the possibilities of using these AM agents in food preservation.

Table 2.6 Antimicrobial activity of combinations of EOs and active constituents.

Combination	Microorganism(s)	Outcome	Reference(s)
Oregano / cranberry	*L. monocytogenes*	synergy	(Lin *et al.* 2004)
Thymol / carvacrol	*P. aeruginosa*, *S. aureus, Candia spp.*	additive	(Lambert *et al.* 2001; Pina-Vaz *et al.* 2004)
Thymol / *p*-cymene	*Candia spp.*	synergy	(Pina-Vaz *et al.* 2004)
Carvacrol or thymol / cymene	*B. cereus*	synergy	(Delgado *et al.* 2004)
Carvacrol / cymene	*B. cereus*	synergy	(Ultee *et al.* 2000)
Carvacrol / cymene	*E. coli* O157:H7	synergy	(Kisko and Roller 2005)
Thymol, carvacrol / eugenol, geraniol, citral	*S. typhimurium*	additive	(Nazer *et al.* 2005)
Thymol or carvacrol / nisin	*B. cereus*, *L. monocytogenes*	synergy	(Pol and Smid 1999; Yamazaki *et al.* 2004)
Carvacrol / nisin	*B. cereus*	synergy	(Periago and Moezelaar 2001)
Carvacrol / enterocin AS-48	*Bacillus spp.*	synergy	(Molinos *et al.* 2008)

2.2.4 Applications of AM Agents in Food Preservation

Novel Applications

Further to traditional modes of application (such as dipping, surface spraying and direct incorporation) novel techniques of applying EOs and their constituents for food preservation purposes are emerging. Antimicrobial packaging is one such novel technique with increasing attention by the food and packaging industries. In this sense, innovative packaging materials containing either plant extracts (Becerril *et al.* 2007; Bentayeb *et al.* 2009; Ha *et al.* 2001; Lee *et al.* 1998; Rodríguez *et al.* 2007) or EO components have been developed (Suppakul *et al.* 2006). These are preferred by consumers as they are perceived as relatively safe. It was found that LDPE-based films

containing the EO components of basil, linalool and methylchavicol at a concentration of 0.34% (w/w) were effective in packaging of Cheddar cheese (Suppakul *et al.* 2004; Suppakul *et al.* 2008).

The use of thyme, oregano or their extracts as active, protective AM agents in food packaging is of particular interest. For example, chitosan films and biopolymer films (based on starch and pectin) enriched with OEO have been tested for their feasibility as AM films in food packaging (Robles-Simental *et al.* 2004; Zivanovic *et al.* 2005). The addition of OEO resulted in increased film thickness (Robles-Simental *et al.* 2004; Zivanovic *et al.* 2005) and the mechanical properties of the films were altered significantly by the OEO (Zivanovic *et al.* 2005). Chitosan films and chitosan-oregano EO films were applied on inoculated bologna samples and stored for 5 d at 10°C. Pure chitosan films reduced *L. monocytogenes* by 2 logs, whereas the films with 1% and 2% oregano EO decreased *L. monocytogenes* by 3.6 to 4 logs (Zivanovic *et al.* 2005). The biopolymer made from pectin and starch as carriers of OEO, exhibited microbial activity against *E. coli* and *S. aureus* at high concentrations of OEO (Robles-Simental *et al.* 2004). Flexible AM films consisting of either polypropylene (PP) or poly-ethylene/ethylene-vinyl-alcohol copolymer (PE/EVOH), with various concentrations of OEOs (1-12% (w/w)) were developed and evaluated against a wide range of microorganisms. Films with a nominal concentration of 4% (w/w) of OEO completely inhibited the growth of the fungi while higher concentrations (8 and 10%) were required for the inhibition of Gram-positive and -negative bacteria. The PP films were found to be more effective than PE/EVOH films. The analysis of atmospheres generated by the OEO-PP films revealed that these films released higher levels of carvacrol and thymol than the corresponding PE/EVOH films. This may be due to the greater ability of the PE/EVOH polymer matrix to retain the OEO constituents than the PP films. Interestingly, the antifungal activities of both films persisted for more than two months after their manufacturing (Lopez *et al.* 2007).

Carvacrol and thymol along with cinnamaldehyde and rosemary oleoresin, added into a polyamide solution (1% of final solution weight), were coated on one side of a LDPE film to develop a packaging material with self-sterilizing ability and thereby increasing

pathogen radiation sensitivity (Han *et al.* 2005). All films showed inhibition zones in an agar diffusion test against *L. innocua* and *E. coli*. In the liquid culture test, the active compounds reduced significantly the specific growth rate and final cell concentration of *L. innocua*. Low-density polyethylene films containing thymol or carvacrol as active AM agents, were found to be effective in controlling the growth of *E. coli, S. aureus, A. niger* and *S. cerevisiae* in *in vitro* assays. It was found that the type of AM agent and the post-processing concentration affected the AM activity (Rupika *et al.* 2008; Rupika *et al.* 2005). These are encouraging results in the development of active packaging (AP) systems for food safety applications.

Several types of industrial plastic packaging films containing *ca.* 0.012 g of EOs of clove, oregano, rosemary, citronella and propolis were evaluated for the antioxidant capacity by *in situ* gas-phase hydroxyl radical generation and high-performance liquid chromatography. Polymers containing clove and oregano were the most efficient ones (up to 7.2 and 4.7 times, respectively, more antioxidant effective than blanks), compared to polymers containing rosemary, citronella and propolis (Pezo *et al.* 2008). In the same study, EVOH copolymer and metallocene polyethylene containing thymol and many other antioxidant compounds were also evaluated. However, these films were able to show only limited or none antioxidant protection compared to EO-active films (Pezo *et al.* 2008).

Food matrices are more complex and in AP systems some reactions are likely to occur between active compounds and food constituents. Some of these reactions could potentially reduce the film's AM effectiveness or affect the safety and quality of food products. Therefore, in the application of these compounds in AM food packaging, different parameters must be considered and a controlled release of active compounds is essential to enhance the quality and safety of food (LaCoste *et al.* 2005; Rardniyom *et al.* 2008). The encapsulation of active compounds in chemically and physically stable host molecules like cyclodextrins (CDs) (Ayala-Zavala *et al.* 2008) or the use of multilayer (Rardniyom *et al.* 2008) to control the release of active agents, are potentially good candidates for the food industry. For example, inhibition by hydrophobic AM agents is preferred for fatty foods although an immediate release may cause undesirable

effects. Therefore, a multi-layer film containing one hydrophobic polymer layer (the reservoir) can be designed for slow controlled release by using an additional hydrophobic layer (the controlling layer) between the reservoir layer and the fatty food (Rardniyom *et al.* 2008).

Encapsulation can provide the necessary protection against evaporation, reaction with, or migration into food substances. These molecules can also act as a flavor carrier while allowing them to be used in the formulation of fortified and functional foods (Ayala-Zavala *et al.* 2008; Mourtzinos *et al.* 2008). Attempts have been made to stabilize and to use thymol and carvacrol by their inclusion in host matrices like β-CD (Locci *et al.* 2004). It was found that the encapsulation efficiency of a mixture of geraniol and thymol in β-CDs and in modified starch was greater for thymol and the encapsulation protected thymol from oxidation (Mourtzinos *et al.* 2008). The potential for developing AM active packages containing microencapsulated EO compounds to preserve the quality of highly perishable fresh-cut produce has recently been reported (Ayala-Zavala *et al.* 2008).

Other Functional Properties

Lipid peroxidation can cause various types of damage in living organisms and in foods (Nakatani 1994). This is a major deterioration process affecting both the sensory and nutritional quality of foods (Yanishlieva *et al.* 1999). Antioxidants markedly delay or prevent oxidation of the substrate when present at low concentrations in foods compared to that of an oxidizable substrate (Shahidi and Naczk 2004). Thyme and oregano as well as their extracts exhibit high antioxidative activity (Antoun and Tsimidou 1997; Lagouri *et al.* 1993; Nakatani 1994). Oregano and thyme have shown antioxidant activity in a range of fats (Miguel *et al.* 2004; Pezo *et al.* 2008; Shahidi and Naczk 2004). The use of thyme and oregano or their extracts as stabilizers in edible oils and foods has also been reported in the literature (Bhale *et al.* 2007; Lolos *et al.* 1999; Tsimidou and Dimitrios 1994; Yanishlieva *et al.* 1999). The antioxidant effect of EOs of oregano and thyme are related to their constituents: thymol and carvacrol (Kulisic *et al.* 2004; Lagouri *et al.* 1993; Miguel *et al.* 2004; Nakatani 1994; Puertas *et al.* 2002;

Tsimidou and Dimitrios 1994; Yanishlieva *et al.* 1999). This effect may be influenced by the synergy among minor compounds (Kulisic *et al.* 2004).

Further to their dual protective functionality as preservatives and antioxidants, thyme and oregano are valued for their nutritional and medicinal properties. The dietary value, health and therapeutic effects of these two spices and their extracts are well documented in the literature (Davidson and Naidu 2000; Kintzios 2004; Leung and Foster 1996; Stahl-Biskup 2004).

2.2.5 Future Applications

Recent studies suggest that thyme, oregano and their extracts are well suited to be used as dual-purpose additives in foods and potential alternatives for synthetic food additives. As natural food additives it is anticipated that growing consumer interest in natural products will further increase their demand. At present, knowledge of the applications of these compounds is limited to tests *in vitro* with few trials on real food products. Therefore, further research is needed to advance the understanding of their activity in real foods, alone and in combination with other preservatives. Food safety issues as well as sensory effects also need to be addressed. Such research would enable the identification of effective preservation strategies in relation to varying food product characteristics. The potential of thyme and oregano EOs in novel food preservation techniques such as active and AM packaging has not yet been extensively investigated.

2.3 Cheddar Cheese: Microbiology, Quality and Shelf life

Cheeses are fermented milk-based food products, produced in a wide range of flavours and forms throughout the world (Fox *et al.* 2004). Cheese is a rich source of essential nutrients including proteins, bioactive peptides, amino acids, fat, fatty acids, vitamins and minerals (Walther *et al.* 2008). There are more than 500 varieties of cheeses. The hard class of cheese is characterized by a moisture content of 26-50% and includes varieties such as Cheddar cheese. Cheddar cheese was first developed by cheese makers in the Cheddar Gorge in England. Cheddar is by far the most important variety of hard

cheese which is produced on an extremely large scale and ranks as the most popular cheese in the world (Drake *et al.* 2005; Drake *et al.* 2008; Fox *et al.* 2004; Varnam and Sutherland 1994). The process of ripening Cheddar cheese depends on the variety and can take from a few weeks to more than 2 years (Singh *et al.* 2003). The “mild” Cheddar is aged 60-90 days before packaging while “sharp” Cheddar is aged at least one year before packing (Drake *et al.* 2008).

2.3.1 Microorganisms and Cheddar Cheese

Microorganisms Associated with Ripening

Microorganisms, including bacteria, yeast and mould, are present in cheese throughout ripening and positively contribute to the maturation process. The growth of these microorganisms is therefore actively encouraged during cheese manufacturing. The microflora associated with cheese ripening may be conveniently divided into two groups: the starter lactic acid bacteria and the secondary microflora. Starter bacteria are primarily responsible for acid production during manufacture while secondary microflora do not play an active role in cheese manufacture, but impact on the organoleptic and biochemical changes in the ripening of cheese (Fox *et al.* 2004).

Generally, two or more strains of *Lactococcus lactis*, which are mesophilic anaerobes, are used as starter cultures for the manufacturing of Cheddar (Varnam and Sutherland 1994). The secondary microflora in Cheddar cheese include mainly non-starter lactic acid bacteria (NSLAB) consisting of lactobacilli (Banks and Williams 2004). The NSLAB occurring in Cheddar cheese produced in various countries is dominated by *Lactobacillus paracasei* and *Lactobacillus plantarum*. Other non-starter flora include Pediococci which has been used as an adjunct culture to improve the flavour of Cheddar cheese. (Fox *et al.* 2004). The application of yeasts *D. hansenii* and *Y. lipolytica* as part of the starter culture for the production of matured Cheddar cheese is recommended because of the good strong flavour achieved after a ripening period of 4 months (Ferreira and Viljoen 2003).

Spoilage and Pathogenic Microorganisms

Spoilage and pathogenic microorganisms have a negative impact on cheese quality and are a risk to human health with the pathogens *L. monocytogenes*, *S. aureus*, enteropathogenic and *E. coli* posing the greatest risk to the safety of dairy products (Fox *et al.* 2004; Rodriguez *et al.* 2005; Virto *et al.* 2006). Recent outbreaks involving raw and pasteurized Cheddar cheese include mainly *S. typhimurium*, *E. coli* O157:H7 and *L monocytogenes* (Fox *et al.* 2004).

In hard cheeses like Cheddar, *S. aureus* is considered to be the organism of primary concern (Varnam and Sutherland 1994). *S. aureus* is capable of producing enterotoxins responsible for staphylococcal food poisoning, one of the most prevalent causes of gastroenteritis worldwide. *S. aureus* may pose a significant risk for toxin production in cheese if numbers are sufficiently high. Cheddar cheese was found to contain *S. aureus* at high levels (>10^5 CFU g^{-1}) especially when active starter cultures are not used (Fox *et al.* 2004). Cheese production from raw milk, particularly in cases of slow or insufficient acidification of the curd, has led to staphylococcal outbreaks associated with this product. In the production of cheese from pasteurized milk under inadequate hygiene conditions, *S. aureus* may also contaminate heat-treated milk or curd (Arqués *et al.* 2005).

Escherichia coli O157:H7 has emerged as a foodborne pathogen of major concern for the food industry because of its ability to cause severe illnesses. Multiple outbreaks of *E. coli* O157:H7 linked to the ingestion of raw milk and dairy products have been reported (De Lamo-Castellvi *et al.* 2006). It was found that *E. coli* survive both in pasteurized or unpasteurized Cheddar cheese whey for up to 28 days (Marek *et al.* 2004) and is able to grow during Cheddar cheese manufacture, even with an initial inoculum in milk of 1 CFU mL^{-1} (Reitsma and Henning 1996). *L. monocytogenes*, being a facultative anaerobe, can survive more than 1 year in Cheddar cheese (Ryser and Marth 1987) and can remain viable during storage at refrigeration temperatures (Peccin and Shelef 1995).

Microbial spoilage of mature Cheddar cheese is rare, provided that suitable starter cultures are used which produce sufficient acidity. However, bacteria, yeasts and mould can grow on the surface of hard cheese especially where the surface is moist. Spoilage involves the production of slimes, discoloration, 'rots' due to proteolysis and may also be accompanied by off-flavours. *Candida*, for instance, has been associated with spoilage of Cheddar cheese due to the evolution of high levels of taints which imparted a 'fermented yeasty' flavour. Spoilage of Cheddar cheeses by internal gas production caused by *Clostridium* sp. (or in some cases *Enterobacteriaceae*, yeasts and species of *Bacillus*) can occur in young cheese or during maturation (Varnam and Sutherland 1994).

Spoilage can arise due to surface growth of moulds which is a common occurrence during ageing and storage at low temperatures. Moulds can produce discoloration on the surface although there is little attack or penetration into the cheese (Hockinga and Faedo 1992). Moulds and yeasts implicated in such spoilage include species of *Penicillium* (green discolouration), *Cladosporium* (green to black) and *Candida* (black) (Hayes 1992). Despite the protection provided by packaging, cheese blocks can develop mould on their surfaces (Blank *et al.* 1992). In the case of refrigerated Cheddar cheese, moulds belonging to the genus *Penicillium* and *Aspergillus* have been isolated (Bullerman and Olivigni 1974) and mycotoxigenic fungi have also been reported in cheese (Taniwaki *et al.* 2001).

Thread mould is a defect which occurs sporadically in maturing vacuum packaged Cheddar cheese. It is caused by the growth of fungi (possibly in association with yeasts) in folds and wrinkles of the plastic film in which the cheese is packaged. This results in black, dark brown or green spots, or threads and is associated with formation of free whey release from the cheese (Varnam and Sutherland 1994). The major causative species of thread mould defects were found to be *Cladosporium cladosporioides*, *Penicillium commune, C. herbarum, P. glabrum* and a *Phoma* species (Hockinga and Faedo 1992).

Factors that influence the safety of cheeses with respect to pathogenic bacteria include milk quality, starter culture, pH, salt, a_w, control of aging conditions (including chemical changes), temperature abuse and natural inhibitory substances (bacteriosins) in the raw milk (Fox *et al.* 2004). Recent research has shown that *S. typhimurium*, *E. coli* O157:H7 and *L monocytogenes* can survive well beyond the 60 day mandatory holding time of Cheddar cheese prepared from pasturised milk (Reitsma and Henning 1996; Schlesser *et al.* 2006) suggesting that aging alone may not be a sufficient pathogen control step to eliminate these microorganisms in Cheddar cheeses (Altekruse *et al.* 1998; Reitsma and Henning 1996; Schlesser *et al.* 2006).

2.3.2 Sensorial Qualities of Cheddar Cheese

Most people consume cheese primarily for its organoleptic properties. Cheddar has a rich, creamy flavor that becomes increasingly sharp and complex with a smooth, firm texture that becomes more granular and crumbly as it ages (Fox *et al.* 2004). Cheddar varies in colour from pale to deep yellow (Robinson 1995). The volatile flavour compounds in cheese originate from degradation of the major milk constituents; namely lactose, citrate, milk lipids, and milk proteins (collectively called caseins) during ripening (Singh *et al.* 2003). At 6-8 months of age, Cheddar cheese develops most of its flavor, and a full Cheddar cheese flavor is generally achieved after 1 year of ripening (Caspia *et al.* 2006; Drake *et al.* 2008). Cheddar cheeses aged for 7 and 9 months are classified as having young or undeveloped flavours such as "cooked", "buttery" and "creamy" and are found to posses volatiles that are responsible for the creamy flavour. A 12 month old cheese is characterized by aged or developed flavours and is found to include volatile compounds responsible for fruity aromas and sulfurous, earthy and free fatty acid flavours (Caspia *et al.* 2006; Drake *et al.* 2005). The aroma and texture of Cheddar cheese also change over ripening time with aroma attributes such as "fruity," "sulfur," "free fatty acid" and "pungent" noticeably higher in the 12 month ripened cheese. The 7 and 9 month ripened cheeses are clearly characterized by "cooked", "whey", "buttery" and "creamy" aromas (Caspia *et al.* 2006; McEwan *et al.* 1989).

2.3.3 Packaging and Shelf life of Cheddar Cheese

Traditionally, Cheddar was a so-called ‘table cheese’ and was purchased by the consumer shortly before consumption. In the current food supply chain, rindless cheese, including Cheddar, is mass produced and typically subjected to long-term storage (Robertson 2006). Cheeses, including Cheddar in particular, are commonly purchased from the manufacturer, repackaged, often in vacuum packs, and sold to supermarkets or wholesalers (Fox *et al.* 2004). Various flexible and barrier films are used to package cheese for aging (Agarwal *et al.* 2005). As a hard cheese with a low moisture content, Cheddar has a longer shelf-life than softer cheeses (Varnam and Sutherland 1994). The stability of cheeses greatly depends on the pH and a_w and while the packaging will not affect the pH of the cheese, the a_w of the surface (and ultimately the interior) of the cheese may be affected by the water vapour permeability of the packaging material. Packaging film for Cheddar cheese must be sufficiently impermeable to oxygen to prevent fat oxidation and mould growth (Robertson 2006). The type of packaging (e.g. gas-flush or vacuum), the light barrier properties and the intimacy between the packaging material and the cheese surface are of primary importance in preventing or delaying lipid oxidation and the appearance of calcium lactate crystals on Cheddar cheeses (Agarwal *et al.* 2005; Robertson 2006). Adequate retention of flavour and textural characteristics is also important during packaging and storage (Fox *et al.* 2004).

The internal cheese environment is not particularly conducive to the growth of many microorganisms and this is especially the case with hard cheeses. However, cheese can become contaminated with pathogens as a result of their presence in raw milk and subsequent survival during the cheese making process. Alternatively, pathogens can contaminate cheese via post processing contamination or in the packaging and distribution stages and/or during repeated opening of re-closable packaging by the consumer (Fox *et al.* 2004; Varnam and Sutherland 1994). The packaging films of cheese can provide a good physical barrier to future contamination but they may not be effective in retarding incipient spoilage, especially if loss of vacuum, improper handling, or both have occurred (Blank *et al.* 1992). Therefore, technologies like AM

packaging that maintain the initial microbiological quality and can control microbial growth upon re-contamination are required.

2.3.4 AM Packaging for Cheese

Cheeses have been identified as one of the main potential applications of AM packaging (Han 2005a). Among the many types of AM agents available, organic acids and their salts, fungicides and essential oils or their main components have been applied in AM packaging films for cheeses (Han 1996; Suppakul 2004; Weng and Hotchkiss 1992). Sorbate-releasing film is a good example of a successful application of AM packaging in cheeses. When processed American cheeses were packaged in 40 µm thick HDPE containing 10% (w/w) potassium sorbate, the AM package enabled the cheese to be microbe free for five months at room temperature (Han 1996). Low-density polyethylene films containing the principal constituents of the EO of basil, linalool and methylchavicol at 0.34% (w/w) are one such an example in which the shelf life of Cheddar cheese is extended while maintaining the sensorial quality (Suppakul 2004). However, with the demand for application of natural AM agents in packaging, the possibility of developing AM packaging materials for cheese based on plant antimicrobials are extensive and relatively unexplored at present.

Chapter 3. Materials and Methods

3.1 Materials

3.1.1 Polymers

The polymers that were used to prepare the films for the present study were low-density polyethylene (LDPE, XJF143/1700 Qenos, Australia), ethylene vinyl acetate copolymer (EVA, ELVAX® 3120, Dupont, Australia) and polyethylene glycol (PEG, A1683 PEG 4000 Ajax Finechem, Australia). Typical properties of the polymers are presented in Appendix A.

3.1.2 Antimicrobial Additives

Purified versions of thymol (W306606, ≥ 99%) and carvacrol (W224502, ≥ 99%), purchased from Sigma-Aldrich Pty. Ltd., Australia, were used as the AM additives. The product characteristics and additional properties of the AM additives are given in Appendix A.

3.1.3 Solvents

Ethanol (absolute, AR grade) supplied by Merck Pty. Ltd., Australia, and iso-octane (2,2,4–Trimethylpentane, OmniSolv®, TX 1389-1) supplied by EMD™ Chemicals Inc., USA, were used as the solvents.

3.1.4 Media, Culture Plates and Diluents

The media and media supplements used in the present study, brain heart infusion broth (AM 11); baird parker agar base (AM 14-500); blood agar base C (AM 24); egg yolk-tellurite emulsion (SP 420); malt agar (AM 109); malt extract broth (AM 110); nutrient agar (AM 130); nutrient broth (AM 131); plate count agar (AM 144); potato dextrose

agar (AM 149) and bacteriological agar (RM 250) were obtained from Amyl, Australia. The MRS agar (De Man, Rogosa, Sharpe) (CM0361) and peptone bacteriological (LP0034) were purchased from Oxoid, Australia. The count plates, 3M Petrifilm™ aerobic count plates; 3M Petrifilm™ *E. coli* count plates and 3M Petrifilm™ yeast and mould count plates, were purchased from 3M Microbiology Products, USA. For the preparation of diluents in microbial assay, potassium dihydrogen phosphate (KH_2PO_4) (104871), sodium chloride (567440) and sodium hydroxide (NaOH) (410203) were purchased from Merk™ Chemicals, Australia.

3.1.5 Microorganisms

The films containing AM agents were assessed for their AM activity against the reference microorganisms: *E. coli* (UNSW 080300); *P. aeruginosa* (UNSW 080400); *S. aureus* (UNSW 051300); *L. innocua* (ACM 4984); *S. cerevisiae* (UNSW 703100) and *A. niger* (UNSW 80900). The details of the test strains are presented in Appendix B.

3.1.6 Cheddar Cheese

Cheddar cheese was purchased from a retail outlet. According to the manufacturer (Murray Goulburn Cooperative Co. Ltd., Australia), a 100 g sample of cheese contains the main components: fat 35.2 g; protein 24.3 g; carbohydrates 0.1 g; calcium 735 mg and sodium 635 mg.

3.2 Compression Moulded Film Production

3.2.1 Compression Moulding

To investigate the ability of the LDPE/EVA blend to retain and release the AM agent, films were prepared by compression moulding of the AM-polymer formulations. A hard-chrome plated steel frame of 2 mm in thickness was placed between the two platens of the compression moulding press (Laboratory press 15T, L0003, IDM Instrument Pty. Ltd., Australia) with temperatures of the upper and lower plates set to 120°C. A predetermined amount of AM-polymer formulation was placed at the centre

of the lower frame and was sandwiched between the two platens. As the polymer formulation melted, the compression force was gradually increased up to 30 kPa and maintained for 10 min. The platens were then quench cooled by running water to 30-35°C and the compression force was released. The films were removed from the press and immediately wrapped in aluminium foil to prevent loss of the AM agent. The film thickness was measured using a hand-held micrometer with an accuracy of ±1 μm (Mitutoyo Corporation, Japan) using an average of five readings from the sample centre and the perimeter.

3.2.2 Effect of AM Agent Concentration

Films containing AM agents thymol or carvacrol at different formulation concentrations were prepared in order to find the optimum concentration of the AM agent remaining after processing. Master batches were prepared by doping powdered EVA with solutions of ethanol containing AM agent to achieve the final formulation concentrations listed in Table 3.1. In each case a ratio of *ca.* 1:4 (w/v) of AM agent to ethanol (70% (v/v)) was used. Master batches were then dried at room temperature (23 ± 2°C) in a fume hood for 24 - 48 h to evaporate any remaining solvent. Master batches were then mixed with powdered LDPE, and processed as described in Section 3.2.1. Control films containing no AM agent were prepared and processed using the same procedure.

Table 3.1 Film formulations with different concentrations of AM agents.

Formulation	**Composition/% (w/w)***	
	AM agent	**LDPE**
Control	0	90
TF1 or CF1	1	89
TF2 or CF2	2	88
TF3 or CF3	3	87
TF4 or CF4	4	86
TF5 or CF5	5	85

Note: * The balance of each formulation is 10% (w/w) EVA
TF = thymol, CF = carvacrol

Relationship between Concentration and AM Activity

The relationships between the inhibition zone on agar media (see Appendix C) and the concentrations of the TF1 to TF5 or CF1 to CF5 films were derived using Gompertz equation altered by Lambert and Pearson (2000). In the present study, the altered Gompertz equation relates the zone of inhibition (z) to the % (w/w) concentration (c), Equation 3.1.

$$z = A + C\exp^{(-\exp^{(-S(c-M))})} \tag{3.1}$$

where, A is the lower asymptote of z (approximately zero), S is the slope parameter, C is the difference between upper and lower asymptote and M is the concentration at the absolute maximum rate of inhibition (see Figure 3.1). The minimum inhibitory concentration (MIC) was taken as the intercept of the tangent at the inflection point with the horizontal tangent at $z = A$. The maximum effective concentration (MEC) was taken as the intercept of tangent to the growth curve at its inflection point. Curve fitting and optimum values of S and M were obtained using the non-linear regression procedure, Solver (Anonymous 2003).

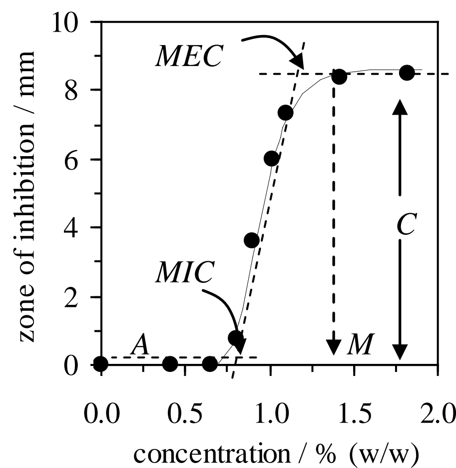

Figure 3.1 Relationship between the zone of inhibition and the AM concentration given by the modified Gompertz equation (Lambert and Pearson 2000).

3.2.3 Effect of PEG and EVA on AM Agent Retention

In order to enhance the retention of the AM agents, films were prepared with PEG and EVA additives. The film formulations containing 5% (w/w) AM agent in blends with PEG and EVA were prepared in accordance with Table 3.2. Except for the formulation P/E3, master batches were prepared by mixing powdered PEG and EVA with AM agent dissolved in 70% (v/v) ethanol. In the formulation P/E3, PEG was mixed with the master batch at a later stage along with LDPE. Films were prepared using the procedures described in Sections 3.2.1 and 3.2.2.

Table 3.2 Film formulations used to study the effect of PEG and EVA.

Formulation	Composition / % (w/w)*		
	EVA	**PEG**	**LDPE**
P/E0	-	2.5	92.5
P/E1	5	2.5	87.5
P/E2	10	2.5	82.5
P/E3	10	2.5#	82.5
P/E4	10	-	85.0

Notes: * Formulation balance is 5% (w/w) AM agent.
PEG was incorporated at a later stage.

3.2.4 Quantification of AM Agent Concentration

The post-processing concentrations of AM agent in the film samples were determined using Soxhlet extraction and gas chromatography (GC). The AM agent in the film was extracted for 18 h using 150 mL of isooctane. A sample from the extract was then taken for GC analysis. An auto-sampler (Varian 8200 C_x) attached to a Varian Star 3400-C_x GC system equipped with a fused silica capillary column (DB-5: 30 m × 0.25 mm i.d., film thickness 0.25 μm, J & W Scientific, USA) was used. The conditions applied in the GC were as follows: injected volume: 1.0 μL; initial column temperature: 80°C; heating

rate: 5°C min^{-1} up to 120°C, held at this temperature for an additional 10 min; injector temperature: 250°C; FID detector temperature: 300°C; column flow rate: 2 mL min^{-1}; carrier gas: nitrogen. The AM agent concentration was determined from standard curves (see Appendix D).

3.2.5 Microbial Culture Maintenance and Preparation of Inocula

The bacterial and yeast cultures were maintained at -80°C in nutrient broth, brain heart infusion broth or malt extract broth containing 30% glycerol. The cultures were sub-cultured in liquid medium twice before being used in accordance with Table 3.3. A spore suspension of *A. niger* in malt extract broth and 30% glycerol was used for long-term maintenance.

Table 3.3 Conditions used for the growth and maintenance of test cultures.

Reference organism*	Liquid Medium#	Solid Medium≠	Incubation Conditions	
			Time	Temperature
E. coli (G/-)	NB	NA	24 h	37°C
P. aeruginosa (G/-)	NB	NA	24 h	37°C
L. innocua (G/+)	BHI	BA	24 h	37°C
S. aureus (G/+)	NB	NA	24 h	37°C
A. niger (F)	MEB	MA	1 week	25°C
S. cerevisiae (Y)	MEB	MA	48 h	25°C

Notes: * (G/-), Gram-negative bacteria; (G/+), Gram-positive bacteria; (F), fungi; (Y), yeast.
NB, Nutrient broth; BHI, Brain heart infusion broth; MEB, Malt extract broth.
≠ NA, Nutrient agar; BA, Blood agar; MA, Malt agar

For the bacteria and yeast cultures, cell densities of *ca.* 10^6 CFU mL^{-1} were prepared in 0.1% (w/v) sterile peptone solution from early stationary phase cells and confirmed by spread plate count on plate count agar and potato dextrose agar for bacteria and yeast respectively. The fungal species *A. niger* inoculum were prepared by growing spores for 1 week on malt agar at 25°C prior to harvesting and suspension in 5 mL of 0.85% (v/v) saline. Spores were then counted in a Neubauer counting chamber and the spore density

was adjusted to *ca*. 10^6 spores mL^{-1} by serial dilution in 0.85% (v/v) saline. The spore density of the final inoculum was confirmed by colony counts on potato dextrose agar.

3.2.6 Antimicrobial Activity Assay on Solid Media

Efficacy experiments on bacteria and yeast were carried out on solid media in accordance with the method described by Suppakul (2004). Disks (*ca*. 5 mm diameter) were taken from the prepared AM films and aseptically placed on the agar plates (6 disks/plate) seeded with 0.1 mL of inoculum from Section 3.1.5. Following incubation (see Table 3.3), the inhibition zones around the discs (see Appendix C) were measured as an average of four diameter measurements, taken 45° apart from each other.

The modified micro-atmosphere method described by Guynot et al. (2003) was used for the inhibitory efficacy experiments on *A. niger*. Malt agar plates were prepared and centrally inoculated with 20 μL of the inoculum from Section 3.2.5. The inoculated plates were maintained in an inverted position and samples of the prepared AM films, cut into squares of *ca*. 2.5 × 2.5 cm, were placed in the centre of the lid. The lids were then tightly sealed to the plates with Parafilm™ and incubated for 1 week at 25°C. The efficacy was determined by measuring the diameter of the growing fungus colonies after 2 days and 1 week of incubation respectively.

3.2.7 Data Analysis

Three replicates of bacterial and yeast plates (n = 15) and five replicates of *A. niger* plates (n = 10) for each film arranged in a completely randomised design (CRD) were tested. Data analysis was performed using a one-way ANOVA procedure. The treatment differences ($p < 0.05$) were identified using Tukey's test. The statistical software package MINITAB™ (Anonymous 2000) was used.

3.3 Films Containing AM Combinations

3.3.1 Film Preparation

The films containing individual AM agents (AMI-films) were prepared by incorporating thymol or carvacrol at a concentration of 5% (w/w) (see Table 3.4). For the films containing combinations (AMC-films), thymol and carvacrol were mixed at the ratios: 1:1, 1:2 and 2:1 in the formulation to give a total AM concentration of 5% (w/w). Thus, the individual concentrations of thymol or carvacrol in the AMC-films were targeted to be less than their concentrations in the AMI films. The AM agents, either as a mixture or as a single component were blended into a master batch. The polymer blends consisting of LDPE, EVA and AM agent(s) were subsequently compression moulded (see Section 3.2.1) into films, with an average thickness of approximately 110 μm. The concentrations of the AM agent(s) in the films, after processing, were determined using the procedure in Section 3.2.4.

Table 3.4 Formulations of films containing individual or combined AM agents.

Film Type	**Formulation Ratio (Thymol:Carvacrol)**	**Composition / % (w/w)***	
		Thymol	**Carvacrol**
Thymol		5	
Carvacrol			5
Thymol/Carvacrol	1:2	1.7	3.3
Thymol/Carvacrol	1:1	2.5	2.5
Thymol/Carvacrol	2:1	3.3	1.7

Note: * The balance of each formulation is 10% (w/w) EVA and 85% (w/w) LDPE.

3.3.2 Antimicrobial Activity on Solid Media

The AMI and AMC films were assessed for AM activity against *E. coli*, *S. aureus* and *S. cerevisiae* on solid media using the procedure described in Section 3.2.6. Individual experiments on solid media were replicated twice (n = 10). Data were analysed using a one-way ANOVA procedure. Significant differences ($p < 0.05$) among the AMI and AMC films were analysed using Tukey's test in MINITAB™ (Anonymous 2000).

3.3.3 Analysis of Interaction Effects

Dose Additivity: Fractional Inhibitory Concentrations

In a mixture, the ratio of the concentration of an individual inhibitor to its own MIC is termed its fractional inhibitory concentration (FIC):

$$\mathrm{FIC_A} = \frac{a}{\mathrm{MIC_A}} \tag{3.2a}$$

$$\mathrm{FIC_B} = \frac{b}{\mathrm{MIC_B}} \tag{3.2b}$$

where $\mathrm{FIC_A}$ and $\mathrm{FIC_B}$ are the FIC values and $\mathrm{MIC_A}$ and $\mathrm{MIC_B}$ are the MIC values of preservatives A and B, respectively; *a* and *b* are concentrations of the preservatives A and B respectively present in a mixture of these preservatives. The FIC of two compounds in a mixture are added to give the summed fractional inhibitory concentrations (ΣFIC) or $\mathrm{FIC_{index}}$:

$$\sum \mathrm{FIC} = \mathrm{FIC_A} + \mathrm{FIC_B} \tag{3.3}$$

For combinations, in general, simple addition is defined when the observed MIC for a mixture is equivalent to the sum of FIC of the individual components, i.e. ΣFIC = 1. If the combined system performs better than that predicted by the ΣFIC, i.e. the observed MIC of a mixture has a ΣFIC < 1, then antimicrobial synergy is reported. If the observed MIC of a mixture has a ΣFIC > 1, then the combination is reported as antagonistic (Lambert and Lambert 2003; López-Malo *et al.* 2006).

The MICs of AMC films were not tested in the present study. Therefore a modified equation of ΣFIC was introduced (see Equation 3.4).

$$\sum \mathrm{FIC} = \frac{c_{\mathrm{thy}}}{\mathrm{C_{thyl}}} + \frac{c_{\mathrm{car}}}{\mathrm{C_{carl}}} \tag{3.4}$$

where, c_{thy} and c_{car} are the concentrations of the two AM agents present in a combination of thymol/carvacrol in AMC films that produced a specified effect; C_{thy} and C_{car} are concentrations of thymol and carvacrol in AMI films which on their own produces the same effect as the combination. The inhibitory curve from Section 3.2.2

was used to obtain the estimated values of C_{thy} and C_{car}. Statistical analysis was performed using student's two-tailed *t*-test to identify the significant deviations ($p < 0.05$) from the additivity value, ΣFIC = 1.

Effect Additivity

In this model, the combined effect of two agents in a mixture is considered to be equal to the sum of the effects of the single compounds; thus the AM activity of any mixture can be predicted (Lau *et al.* 2006). In the absence of interactions, it would be expected that two compounds in combination at fixed concentrations would have the same effect as the individual compounds at those same concentrations; this was taken as the "theoretically expected values" (i.e., zero interaction). Deviations from this expected constant inhibition are either synergistic (above expected zero effect) or antagonistic (below expected zero effect) (Lau *et al.* 2006).

The inhibitory effects given by AMI films were used to calculate the theoretically expected inhibitory values of different AMC films against each microorganism. Observed values and the expected values were presented with the respective 95% confidence limits. Statistical analysis was performed using student's two-tailed *t*-test to identify the significant deviations ($p < 0.05$).

3.4 Production of Film by Extrusion

3.4.1 Blown Film Extrusion

Films containing two different levels (L1 and L2) of AM agents were prepared by blown film extrusion. Master batches containing AM agents at 2% and 4% (w/w) and EVA were compounded with LDPE (see Table 3.5) in a twin screw extruder. The extrudate was immediately cooled in a water bath, dried and pelletized. Each compounded blend was blown into film of 40 - 50 µm in thickness in a single screw extruder using an operating speed of 30 rpm. The temperature profile in the extruder was maintained at approximately 150°C (all zones). An LDPE-EVA-control film

without AM agent was manufactured in a similar manner. The extruded film was immediately wrapped in aluminium foil to prevent loss of the AM agent by evaporation. The determination of the film thickness and post-processing concentration of AM agents in the extruded films followed the procedure of Sections 3.2.1 and 3.2.4, respectively.

Table 3.5 Polymer formulations for blown film extrusion.

Formulation	**Composition / % (w/w)***	
	AM agent	**LDPE**
Control	0	90
TL1 or CL1#	2	88
TL2 or CL2	4	86

Note: *The balance of each formulation is 10% (w/w) EVA
TL = thymol, CL = carvacrol

3.4.2 Antimicrobial Activity on Solid Media

The AM efficacies of the films, film cutting at 0/90° of extrusion direction and 45/45° of extrusion direction, on solid media were determined against *E. coli* and *S. aureus* on solid media as described in Section 3.2.6. Film cuttings at different extrusion directions were tested to determine whether there is an intrinsic characteristic of AM activity resulting from the extrusion direction. Film pieces cut into 1 × 1 cm were aseptically placed on nutrient agar and Baird parker agar seeded with 0.1 mL of *E. coli* and *S. aureus* cell suspensions, respectively. Following incubation, plates were examined for clear zones formed around the film samples that were measured as the zone of inhibition.

3.4.3 Antimicrobial Activity in Liquid Media

Measurement of Bacterial Growth

The AM activity of films in the liquid medium was assessed using *E. coli* and *S. aureus*. Bacterial cells were activated on nutrient agar plates at 37°C for 24 h. Cells of several

well-grown colonies on the plates were sub-cultured twice in a nutrient broth. Cultured cells were harvested by centrifugation (Sorvall®, Kendro Laboratory Products, U.S.A.) at 4000 × *g* for 10 min at 4°C and washed twice with a sterile 1% (w/v) peptone solution. Cells were re-harvested and suspended in fresh nutrient broth. This was used to prepare 400 mL of nutrient broth in a 1 L flask containing two different concentrations of bacteria. The area per unit volume of each film was maintained at 1 cm^2 mL^{-1} (Suppakul 2004). The film samples, cut into small pieces (1 × 10 cm), were added to the bacterial cell suspension and incubated in an incubation shaker (Innova™ 4230, New Brunswick Scientific, U.S.A.), maintained at a constant temperature of 37°C with a continuous rotation speed of 30 rpm. A bacterial solution added with control film served as a negative control.

During the growth period, samples were taken at equal time intervals to measure bacterial growth in the presence of AM films. A series of decimal dilutions of samples was carried out with sterilized peptone solution. To enumerate *E. coli*, 1 mL of each of the diluted samples was plated in duplicate on 3M Petrifilm™ *E. coli*/coliform count plates and incubated aerobically at 35°C for 48 h. The 3M Petrifilm™ aerobic count plates spread with 1 mL of *S. aureus* samples were incubated aerobically at 35°C for 24 h. Colonies were counted after the incubation and experiments were performed in duplicate.

Modeling Growth Kinetics

The plate counts were transformed into $\log_{10}$ CFU mL^{-1} and modeled as a function of time using the modified Gompertz model (Zwietering *et al.* 1991; Zwietering *et al.* 1990) and Baranyi and Roberts model (Baranyi and Roberts 1994) (see Appendix E). Individual growth curves were fitted to the models using the Microsoft Excel™ add-in program DMFit 2.1 available from the Institute of Food Research, Norwich Research Park, Norwich, UK (www.ifr.bbsrc.ac.uk). Kinetic parameters including the specific growth rate, μ_{max} (h^{-1}); the lag time, t_{lag} (h); and the final cell numbers, y_{max} expressed as $\log_{10}$ (CFU mL^{-1}) were estimated for each fitted curve. The parameter y_{max} represents

the upper asymptote of the fitted curve. When a curve does not reach this asymptote, i.e. the stationary phase, the maximum predicted data point is reported.

The statistical indicators root mean square error (RMSE) and the correlation coefficients (R^2) were used to compare the performance of the models. The lower the RMSE value, the better is the goodness of fit of each model. The AM effect of the packaging films on the kinetic parameters of bacterial growth was analysed statistically by subjecting model parameters from individual experiments to the general linear model (GLM) procedure and the Tukey's test at the 0.05 significance level using the statistical software MINITAB™ (Anonymous 2000).

3.5 Characterisation of AM Films

3.5.1 Mechanical Properties

The effect of AM agents on the mechanical properties of the extruded films was investigated by measuring the tensile properties. Tensile test was performed in both the machine direction (MD) and transverse direction (TD) for each film. The peak load of the film section 2 × 10 cm was determined using an Instron 4465 (USA) tensile tester in accordance with ASTM Method D 882-97. At least five replicates from each film type were tested. Results were analysed using one-way ANOVA procedure and Tukey's test in MINITAB™ (Anonymous 2000).

3.5.2 Thermal Characterisation by DSC

The melting behaviour of each LDPE/EVA/AM film and pure LDPE was determined using a Perkin-Elmer DSC-7 differential scanning calorimeter in accordance with ASTM Method D3417. For all DSC measurements, nitrogen was used as the purge gas and an empty aluminium pan was used as a reference. The melting thermogram of a sample of the film (*ca.* 10 mg) was sealed in an aluminium pan and heated in the instrument at a rate of 10°C min^{-1} over the temperature range of 50 to 150°C. The

crystalline melting temperature (T_m) of each film was determined from the temperature axis on its thermogram.

3.5.3 Retention of AM Agent during Storage

The retention of AM agent in the extruded films under three different storage conditions: (i) exposed to the air at room temperature; (ii) covered in foil and stored at room temperature, and (iii) covered in foil and stored in a refrigerator during short-term (up to 28 d) and long-term storage (up to 102 d) was examined. The AM films were analysed for the retained AM agent at regular intervals using a Fourier Transform Infrared (FT-IR) spectrophotometer (Bruker model Vector 22). The IR absorbance spectra were measured in the wave-number range of 4000 to 370 cm^{-1} (32 scans at a resolution of 4 cm^{-1}). A wave-number of the spectra that was representative of the AM agent was selected and its peak height was recorded. The peak height of AM agent was normalised to the peak height representative of EVA, in each run. The concentration of the AM agent that was retained in the films was calculated as a proportion of the initial concentration.

3.6 Release Experiments

3.6.1 Release of AM Agent to the Atmosphere

The data from section 3.5.3 was used in the determination of release kinetics of AM agent in the extruded films to the atmosphere. The release was represented as a plot of the mass fraction of retained AM agent (m_t/m_∞) versus time (t). To describe the release kinetics, the logarithm of the retained mass fraction (ln (1- m_t/m_∞)) was plotted as a function of time (t), which was tested for linearity by calculating the regression coefficients (R^2). The rate constant (k) was obtained from the slope of the regression line (see equation 3.7).

3.6.2 Antimicrobial Agent Release in Food Simulants

The release of AM agents from the extruded films into the food simulants was assessed using the double-sided, total immersion migration tests (both sides of films immersed into the liquid stimulant). Tests were performed using the food simulants: isooctane and 95% (v/v) ethanol/water as a fatty-food simulants and 10% (v/v) ethanol/water as an aqueous and/or acidic foods simulant (EC 1997; FDA 2007). Film samples weighing *ca.* 0.5 g (4 pieces, 5×5 cm) (Mistry, 2006) were immersed into 100 mL of food simulant in a tightly sealed vessel which was mildly agitated (50 rpm) in an incubator shaker (InnovaTM 4230, New Brunswick Scientific, U.S.A.). The migration test for each film in each simulant was performed at three temperatures 10, 15 and 20°C. The amount of AM agent released was monitored until equilibrium was attained. An aliquot of the solution was analysed by GC analysis at different time intervals. The AM release in food simulants was quantified by the GC conditions as described in the Section 3.2.4. For the quantification of AM agent release in ethanol the following modifications were made on method in Section 3.2.4. The injected volume was increased to 5.0 µL with a column flow rate of 2 mL min^{-1}. The initial column temperature was 80°C and held at this temperature for 2 min. Then the column was heated at a heating rate: 20°C min^{-1} up to 120°C, held at this temperature for an additional 4 min.

3.6.3 Data Analysis

The release of AM agent from the polymer matrix were analysed in two data analysis treatments: diffusion and kinetic process (Mistry 2006). In the diffusion process, the release of the AM agent from the film into a food simulant was considered in two stages: short-term and long-term (Crank 1975; Miltz 1987). For short-term migration (m_t/m_∞) < 0.6:

$$\frac{m_t}{m_\infty} = 4\left[\frac{Dt}{\pi l^2}\right]^{1/2} \tag{3.5}$$

where m_t is the amount of AM agent released from the film, m_∞ is the equilibrium amount of AM agent released from the film, D is the diffusion coefficient and l is the

film thickness. A plot of (m_t/m_∞) versus $t^{½}$ should yield a straight line from which the diffusion coefficient can be obtained.

For long-term migration $(m_t/m_\infty) > 0.6$, equation 3.6 was used:

$$\frac{m_t}{m_\infty} = 1 - \frac{8}{\pi^2} \exp\left[\frac{-\pi^2 Dt}{l^2}\right] \qquad (3.6)$$

Rearranging equation 3.6 becomes:

$$\ln\left[1 - \frac{m_t}{m_\infty}\right] = \ln\left[\frac{8}{\pi^2}\right] - kt \qquad (3.7)$$

where k_1 is the rate constant. From equation 3.7, a plot of $\ln(1 - m_t/m_\infty)$ versus time should yield a straight line with slope, $-k_1$. For the diffusion process, the diffusion coefficients were calculated using equation 3.5 for short-term migration and the rate constants were calculated using equation 3.7 for long-term migration.

In addition to the diffusion analysis, the release of AM agent into the food simulant was further analysed for the fit to first-order kinetics. For a first-order system, equation 3.8 was used:

$$\ln(1 - \frac{m_t}{m_\infty}) = -k_2 t \qquad (3.8)$$

where m_t is the amount of AM agent released into the food simulant and k_2 is the rate constant. It is assumed that the total amount of AM agent incorporated (m_0) will be released given enough time (i.e. $m_0 \approx m_\infty$). From equation 3.8, a plot of $\ln(1 - m_t/m_\infty)$ versus time should yield a straight line with slope, $-k_2$ (Mistry 2006).

The initial rate of release of the AM agent, v_0, at time, $t = 0$, is given by:

$$v_0 = m_\infty k_2 \qquad (3.9)$$

For the kinetic approach to data analysis, the rate constants were calculated using equation 3.8 and the initial release rates of AM agent were calculated using equation 3.9.

In order to determine the temperature dependence of AM release, an Arrhenius activation energy equation was used (Rardniyom 2008; Suppakul 2004). The activation energy of diffusion (E_a) was obtained from k or D using equation 3.10 and equation 3.11.

$$D = D_0 \exp\left(\frac{-E_\mathrm{a}}{RT}\right) \tag{3.10}$$

$$k = k_0 \exp\left(\frac{-E_\mathrm{a}}{RT}\right) \tag{3.11}$$

where k_0 and D_0 are pre-exponential factors, R is the ideal gas constant, and T is the absolute temperature.

3.7 Application of AM Films on Cheddar Cheese

3.7.1 Challenge Test

Sample Preparation, Packaging and Storage

Cheddar cheese was purchased from a local retail outlet a few days prior to commencing the experiments and stored at 4°C until use. For the AM packaging experiments, this cheese was cut into *ca.* 25 g slices measuring approximately 6 × 4 × 0.5 cm. The cheese was randomly divided into 2 sets for different bacterial inoculations, *E. coli* and *S. aureus*, and each set was divided into five lots for different packaging treatments (control-, carvacrol L1-, carvacrol L2-, thymol L1- and thymol L2- films). *E. coli* or *S. aureus* was inoculated onto the top and bottom surfaces of the Cheddar cheese slices and then spread using a sterile glass rod to obtain *ca.* 10^4 CFU g^{-1}. Samples were then placed between folded films (8 × 10 cm) and the three open sides were heat-sealed. The packaged cheese samples were stored at 12°C for 14 days to mimic temperature abuse conditions (Suppakul 2004). Bacteriological analyses of the Cheese samples were undertaken immediately after inoculation and periodically afterwards.

Bacterial Enumeration

Slices of Cheddar cheese were tested at appropriate intervals for numbers of *E. coli* or *S. aureus*. Two packages from each treatment were opened aseptically on the sampling days, 1:10 dilutions were prepared from samples of 11 g of cheese dispersed in 99 mL of slightly warm (40-45°C) sterile peptone saline diluent (pH 7.0 ± 0.1 at 25°C) (Eliot *et al.* 1998; Limjaroen *et al.* 2005). To improve homogeneity, the cheese samples were grated prior to weighing. Samples were homogenized in a laboratory blender (Seward Stomacher™ 400, Seward Medical, UK) for 3 min and serial decimal dilutions of this solution were prepared. In order to examine *E. coli* and *S. aureus* populations, 1 mL of each serially diluted sample was plated in duplicate on 3M Petrifilm™ *E. coli* count plates and Baired-Parker agar plates, respectively. Plates containing *E. coli* and *S. aureus* were incubated aerobically at 35°C for 48 h and 24 h, respectively (Horwitz 2006). Colonies were counted and the results were expressed as CFU g^{-1}. The experiments on each bacterium and on each AM film were replicated twice (n = 4).

Curve Fitting and Inactivation Kinetics

The Weibull model (Geeraerd *et al.* 2005; Virto *et al.* 2006) and the first-order biphasic model (Geeraerd *et al.* 2005) (see Appendix E) were used to describe the inactivation kinetics of bacteria on Cheddar cheese wrapped with various AM films.

The Weibull model is described by the following equation:

$$\log_{10}(N) = \log_{10}(N_0) - \left(\frac{t}{\delta}\right)^p \qquad (3.12)$$

where, N represents the microbial cell density (CFU g^{-1}), N_0 the initial microbial cell density (CFU g^{-1}), δ is a scale parameter and denotes the time for the first decimal reduction and p is the shape parameter. The survival curve is linear if p = 1. For p> 1, convex curves are obtained, while for p < 1, concave curves are described.

The mathematical expression of the first-order biphasic inactivation is:

$$\log_{10} S = \log_{10}\left[(P)e^{-s_1 t} + (1-P)e^{-s_2 t}\right] \qquad (3.13)$$

where, S is the survival fraction (N_t/N_0), N_t (log CFU g^{-1}) is the number of micro-organisms at time t, N_0 (log CFU g^{-1}) is the initial number of microorganisms, P is the fraction of survivors in subpopulation 1, s_1 the specific death rate of subpopulation 1, (1 - P) is the initial fraction of survivors in subpopulation 2 and s_2 the specific death rate of subpopulation 2.

The curve fitting of the inactivation data and kinetic parameter values were obtained from the GInaFiT (http://cit.kuleuven.be/biotec/downloads.php), Version 1.4.2, a freeware add-in for Microsoft® Excel developed by Geeraerd et al. (2005). The goodness of fit of the models was assessed using RMSE and R^2 between the observed and predicted values. The significant differences between the inactivation parameters were identified as in Section 3.4.3.

3.7.2 Effect of AM Films under Storage Conditions

Sample Preparation, Packaging and Storage

Cheese samples for various AM packaging experiments were prepared and samples were randomly divided into five lots for different packaging treatments as described in Section 3.7.1. Samples from each packaging treatment were stored at refrigeration temperatures (*ca*. 4°C) for about 40 days and periodically analysed for their microbiological and physio-chemical qualities during storage.

Microbial Analysis

Cheese samples were examined for the evolution of the microbial groups: total aerobic bacteria (TAB), lactic acid bacteria (LB), *E. coli*/coliform (EC/C) bacteria and yeasts and moulds (YM) to evaluate the AM effectiveness of the films. Two packages drawn randomly from each treatment were aseptically opened on the sampling days, and samples were dispersed in sterile Butterfields buffered phosphate diluents (Horwitz 2006) (pH 7.2 ± 0.2 at 25°C), then blended and serially diluted as in Section 3.7.1. The TAB, EC/C and YM counts were determined by plating 1 mL of appropriate dilutions on 3M Petrifilm™ aerobic count plates, 3M Petrifilm™ *E. coli*/coliform count plates, and 3M Petrifilm™ yeast and mould count plates, respectively. For LB, appropriate

dilutions were inoculated on MRS agar using the pour-plate method (Dinakari and Mistry 1994). The aerobic count plates and *E. coli*/coliform count plates were incubated aerobically for 24 - 48 h at 35°C, while yeast and mould count plates were incubated for 5 days at 25°C. MRS agar plates were incubated for 48 h at 37°C under gas pack anaerobic systems (Anaerocult®). Following incubation, colonies were counted and the results were expressed as CFU g^{-1}.

Evaluation of pH of Cheese Samples

The pH changes were evaluated using *ca.* 10 g of the cheese sample from each packaging treatment. Each sample was homogenized in a blender with 25 mL distilled water for 2 min (Whitley, Muir et al. 2000). The pH of the homogenate was measured with a pH meter (Model 8417, Hanna Instruments Pty. Ltd., Singapore) after calibrating with fresh pH 4.0 and 7.0 standard buffers.

3.7.3 Detection Odour Threshold of AM Agents

Sensory Panel

An untrained panel of 12 assessors (male and female) participated in a sensory analysis. All were students or staff members of Victoria University. Prior to assessment, the panellists were presented with an information sheet (Appendix G) and an orientation session was held to familiarize them with the test procedures applicable to the study.

Measurement of Detection Odour Threshold and Probable Threshold

The odour thresholds of AM agents were determined in order to assess the relative odour intensities of these compounds and to demonstrate the ability of the panellists to differentiate a very low level of odour of AM agent. Aqueous solutions of AM agents and blank samples were prepared according to the method of Ahmed et al. (1978). The sample solutions contained different dilutions of AM agent in water. The dilution series of thymol was in the range of 0.01 to 10 ppm and that of carvacrol was in the range of 0.1 to 100 ppm. Blank samples contained the same amount of water. A triangle test was performed (see Appendix G) according to the method of Ito and Kubota (2005). Three containers were presented to each panelist with one containing the sample solution and

the other two blank solutions, or one containing the blank and the other two the sample solutions. The panelists were told that two of the solutions had the same odor and one was different, and they were asked to smell each sample and indicate the odd sample. The minimum concentration at which a sample solution could be correctly distinguished from the blank sample was determined as the detection odour threshold (DOT) (Ito and Kubota 2005). The concentration at which a sample solution could be significantly distinguished from the blank sample by 50% of the panellists was also determined and is referred to as the "probable threshold" (PT).

Selection of Odour Terms

For the purpose of selecting the main relevant terms for odour of AM agents and Cheddar cheese, panellists were presented with a group of odour terms and their definitions (see Appendix G) as found in the literature (Burdock 2005; Kilcawley *et al.* 2007; Lawlor *et al.* 2003; Lee and Chambers 2007; Singh *et al.* 2003). Panellists were asked to select odour terms representative of the sensory note perceived. The odour terms with highest preference were used in the descriptive sensory analysis.

3.7.4 Effect of AM Films on Sensory Quality

Sensory Panel

The same panel as described in Section 3.7.3 participated in the sensory evaluation studies on Cheddar cheese.

Sample Preparation

Prior to the evaluations, cheese samples were opened from randomly chosen packages removed from storage (*ca.* 4°C), cut into *ca.* 5 g slices (4 × 2 × 0.5 cm) and stored in closed containers until sensory analysis could be performed simultaneously for all samples. Each sample was allowed to equilibrate to room temperature and was presented to assessors for evaluation in coded cups sealed with lids.

Triangle Test

To determine whether a sensory difference, on the basis of odour, exists between the cheese samples packaged in AM films, a standard triangle test was used (Meilgaard *et*

al. 1999; Suppakul 2004; Suppakul *et al.* 2008). In one session, six sets of triangle tests were carried out. Four sets were used to compare the cheese samples packaged in AM films with the cheese samples packaged in the control film. In the other two sets, cheese samples packaged in AM films with different concentrations were evaluated. Equal numbers of six possible combinations of cheese were prepared (AAB, ABA, BAA, BBA, BAB and ABB where A is control film and B is the AM film). To prevent potentially biasing effects of order of sample presentation, sample carry-over effects and exhaustion effects, the order in which samples were presented to assessors was balanced and randomised (Muir and Hunter 1991, 1992). Panellists were asked to examine the samples in the order from left to right and to choose the odd sample in each set. Cheese samples were tested for odour differences during 1 - 5 weeks of storage. Significant differences were determined using the probability tables of Roessler (1978).

Descriptive Sensory Analysis

To determine whether the addition of AM agent in the packaging film influences the acceptability and/or the shelf-life of the packaged cheese, the cheese samples were evaluated on a nine-point hedonic scale (9 = highest intensity and 0 = lowest intensity) for odour attributes and acceptance. For simplicity, a total of only five odour descriptors for Cheddar cheese, thymol and carvacrol, were selected. These terms were selected from the preferences given by the assessors to describe their perceptions of the samples during the DOT determinations (see Section 3.7.3). To facilitate assessors as to the exact meaning of each attribute, each panellist was provided with a full list of definitions for each of the attributes (Appendix G). The intensity of each of the descriptive terms was evaluated for each cheese. For acceptance rating, panellists were instructed to evaluate their perception of the overall "like" of the cheese samples. All cheese samples were analysed for likeliness and odour attributes by each panellist over a period of 1 - 5 weeks of storage. One-way analysis of variance (ANOVA) was used to determine differences between means, with a significant level at p = 0.05. When significant differences were found among treatments, means were compared using Tukey's test. Simple linear correlation analysis was used to determine a relationship between the average scores (from week 1 to 5) of sensory attributes and that of acceptability.

Chapter 4. Results and Discussion

4.1 Film Preparation by Compression Moulding

Prior to the preparation of extruded films, the effect of different concentrations of AM agents in the films, the effect of compounding the additive polymer (PEG) and/or copolymer (EVA) with LDPE and the effect of mixtures of AM agents in the films were investigated in compression moulded films.

4.1.1 Properties of Compression Moulded Films

The point thickness of the compression moulded films varied in the range of 70 - 140 μm (Sections 4.1.1 and 4.1.2). The calculated average thickness was approximately 110 μm which is twice the thickness of the extruded LDPE-based AM films used by Suppakul (2004) for the determination of AM activity. In the present study, the sample films prepared using compression moulding with the intention of comparing results and screening, were considered acceptable.

4.1.2 Effect of AM Agent Concentration

Post Processing Retention of AM Agents

Films containing thymol or carvacrol at formulation concentrations between 1 - 5% (w/w) were prepared and the post-processing retention at each formulation concentration was determined. The post-processing retention of AM agents in the formulations is presented in Table 4.1.

Proportional to the formulation concentrations, films with five different concentrations of AM agent in an increasing order were obtained. However, the retention of both thymol and carvacrol was found to be *ca.* 20 - 40% (w/w) of the original composition in the compression-moulded films. The low retention may be due to the high volatility of the AM agents (see Appendix A).

Table 4.1 Post processing retentions of AM agents in compression moulded films.

Formulation	Post processing retention#/% (w/w)	
	TF (Thymol)	**CF (Carvacrol)**
1	0.2	0.4
2	0.4	0.7
3	0.5	1.0
4	0.7	1.4
5	1.1	1.8

Note #: Refer to Table 3.1 for film formulations.

The volatility of thymol is much lower than that of carvacrol (see Appendix A) and a higher retention of thymol in the films was anticipated after processing. However, the retention of thymol in the compression moulded films was found to be significantly lower than that of carvacrol. During processing of the AM film, part of the AM agent is chemically bonded to the polymer backbone (Buonocore *et al.* 2005; Lopez *et al.* 2007). Carvacrol and thymol have polar functional groups (mainly hydroxyl groups), which may interact with the LDPE/EVA matrix and with EVA in particular (Lopez *et al.* 2007). Thymol is more reactive than carvacrol (Dorman and Deans 2000), and therefore may be more strongly retained in the LDPE/EVA matrix, thus reducing its extractability.

AM Activity and Optimum Concentrations

The TF1 to TF5 or CF1 to CF5 films were tested on solid media inoculated with bacteria (*ca.* 10^6 CFU/mL) to determine the optimum concentration of AM agent that is required in the film for microbial inhibition. The relationship between the AM activity, measured in terms of inhibition zone (see Appendix C), and AM concentrations of the TF1 to TF5 or CF1 to CF5 films was obtained using Gompertz function (see Figure 4.1). The RMSE values of the inhibitory data (see Table 4.2) showed acceptable goodness of fit (Fujikawa *et al.* 2004) to the Gompertz function.

In general, plotting the zone of inhibition against concentration using the Gompertz function gives a sigmoid shape curve with three principal regions: a region where the AM agent has no effect on the growth of the micro-organisms, a region where the AM effect significantly increased almost linearly with the increase in the concentration of AM agent and a region where the AM activity appears to reach an equilibrium. These three phases of behaviour are supported by the findings on the inhibitory effect of carvacrol vapour on agar media (Burt *et al.* 2007).

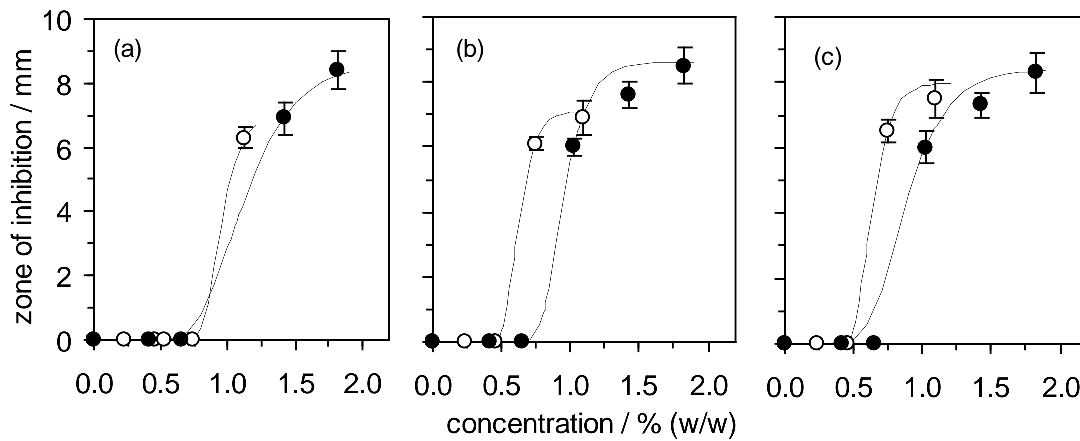

Figure 4.1 The inhibition profiles of thymol-films and carvacrol-films against (a) E. coli, (b) S. aureus and (c) S. cerevisiae: (○) observed values of thymol-films; (•) observed values of carvacrol-films; (—) fitted Gompertz curve.

Terms have been assigned to two specific concentrations in the inhibitory curve. The MIC is the minimum AM agent concentration required for the AM films to have a negative effect on growth and the MEC is the concentration of AM agent where the inhibitory activity is at a maximum. The values of MIC and MEC against different microorganisms are presented in Table 4.2.

Table 4.2 Inhibitory effect of films containing different concentrations of AM agents

Microorganism*	**Film Type**	**MIC#**	**MEC≠**	**RMSE§**
E. coli	Thymol	0.7	1.2	0.0424
	Carvacrol	0.7	1.6	0.0974
S. aureus	Thymol	0.5	0.8	0.0830
	Carvacrol	0.6	1.4	0.1157
S. cerevisiae	Thymol	0.5	0.9	0.2076
	Carvacrol	0.6	1.8	0.3756

Notes: * (G/-), Gram-negative bacteria; (G/+), Gram-positive bacteria; (Y), yeast.
MIC = Minimum inhibitory concentration % (w/w).
≠ MEC = Maximum effective concentration % (w/w).
§ RMSE = Root mean square error.

For the inhibition of microbial growth to occur, a critical concentration of the AM agent in the film is needed (Paster *et al.* 1990; Ultee *et al.* 1998). Beyond this critical concentration, a dosage-dependent AM activity is observed. With an increasing concentration of AM agent in the film, more AM agent migrates from the film into the agar medium. However, the increase in AM activity occurs only up to the MEC after which there is no significant increase in the inhibition zone. This may be attributable to the maximum diffusion capacity of the AM agent under the given conditions. Thus, the optimum AM concentration is in the range between MIC and MEC. However, this may be only a theoretical or a "potential" value because the estimated values of MIC and MAE depend on the experimental conditions, such as test organism, temperature and time of incubation and the size of the test inoculum (Friedman *et al.* 2002; Lambert and Pearson 2000). According to the inhibitory values (Table 4.2), the Gram-positive bacterium *S. aurues*, was found to be the most sensitive followed by *S. cerevisiae* (yeast) and *E. coli* (Gram-negative). In general, Gram-positive bacteria are more sensitive to thymol and carvacrol than Gram-negative bacteria (Cosentino *et al.* 1999; Nevas *et al.* 2004).

The efficacy of AM films should improve with increasing temperature, presumably due to the increase in volatility of the AM agent. Therefore, a higher AM activity would be expected against bacteria due to a higher incubation temperature (37°C) than that of yeast or fungi (25°C). However, *E. coli*, which was incubated at 37°C, was found to be fairly resistant to the AM activity of films in the present study. This result is consistent with a study on carvacrol vapour that had no further significant improvement in AM efficacy at 37°C compared to that at 25°C (Burt *et al.* 2007).

None of the AM films showed any inhibition against *L. innocua* which is also a Gram-positive bacterium tested in this experiment (as above). As thymol and carvacrol have been reported to demonstrate AM activity against *L. monocytogenes*, an identical strain to *L. innocua* (Cosentino *et al.* 1999), some activity of the AM agent at the present levels in the films could be expected. In accordance with the studies using pure compounds (Cosentino *et al.* 1999; Walsh *et al.* 2003), none of the AM films were able to inhibit the growth of *P. aeruginosa*, a bacterium well known for its resistance and ability to metabolize EOs in an agar disc diffusion assay.

Films containing thymol were found to be more effective having lower MIC and MEC values in many cases. Mixed results on the relative activity of thymol and carvacrol have been reported in the literature with some studies concluding that thymol is more active than carvacrol (Dorman and Deans 2000) while others report an AM activity of carvacrol comparable to that of thymol (Lambert *et al.* 2001; Zhou *et al.* 2007). In the case of AM films, a higher AM activity for carvacrol films than that of thymol films would be expected due to high volatility and subsequently high availability of carvacrol on the agar medium. However, in the present study, thymol films were found to be more active than carvacrol. Since the solubility of thymol in water is greater than that of carvacrol (see Appendix A), thymol may be present at higher concentrations in an aqueous-based agar media. Therefore, the higher inhibition exhibited by thymol films could be due to both its greater inherent AM activity (Dorman and Deans 2000), and its higher rate of diffusion in the aqueous agar medium compared to that of carvacrol. Similar results were found by Suppakul (2004) for linear low-density polyethylene (LLDPE) films containing linalool and methylchavicol. Although methylchavicol

possesses greater AM activity than linalool, a higher level of inhibition was observed in the linalool films in the agar disc diffusion assay.

4.1.3 Effect of PEG and EVA

Effect of PEG and EVA on AM Retention

The post-processing concentration of the AM agent in the film is an important parameter as it relates directly to the AM activity. In order to enhance the retention of volatile AM agents during thermal processing, blends containing AM agents, LDPE and different combinations of PEG and EVA were prepared. In these formulations the concentration of the AM agent was constant at 5% (w/w) to ensure sufficient post-processing concentrations.

The post-processing retentions of AM agents in the film formulations are presented in Table 4.3. The formulation P/E0, which contains no EVA in the master batch, shows the lowest retention of AM agents suggesting that the presence of EVA can have a significant effect on the retention of AM agents during thermal processing. Moreover, with the increasing concentration of EVA in the formulation, the retention of AM agents increased. The formulations from P/E1 to P/E4, with either 5% or 10% (w/w) EVA, retained 3 - 4 times more thymol and *ca.* 2 - 2.5 times more carvacrol than that of the control (P/E0). This is consistent with the findings of Suppakul (2004), who observed an increased retention of AM agents in LDPE films containing 10% (w/w) EVA compared to that of LLDPE alone. Increasing the concentration of EVA from 0 to 10% (w/w) has a greater effect on the retention of thymol than that of carvacrol. Increasing the concentration of EVA from 5% (w/w) to 10% (w/w), however, becomes significant only when PEG is present in the master batch of the formulation (P/E2). This suggests that the increased retention is a combined effect of PEG and EVA.

Table 4.3 Effect of PEG and EVA on AM retention in compression moulded films.

Formulation	Post processing retention#/% (w/w)	
	Thymol	**Carvacrol**
P/E0	0.5	1.0
P/E1	1.4	1.7
P/E2	1.9	2.3
P/E3	1.6	2.0
P/E4	1.5	1.9

Note #: Refer to Table 3.2 for film formulations.

In the present study, PEG was used as a binding agent between the AM agent and the polymer to enhance the retention. However, PEG could not sufficiently retain the AM agents in the absence of EVA. Due to the differences in melting temperatures of PEG (*ca.* 55°C) and LDPE (*ca.* 110°C), there is a significant phase separation of LDPE and PEG during compression moulding in which the PEG melted first and separated out to the periphery of the solid LDPE (Mistry 2006). When present in the master batch alone (P/E0), the PEG interacts with thymol or carvacrol (Kabadi and Hammarlund 1966) which can facilitate the loss of a considerable amount of AM agent from the LDPE matrix thereby reducing the retention. This hypothesis is supported by the higher retentions of AM agents shown by P/E3 films, which was added with PEG at a later stage in the formulation process.

The effective retention of the AM agents by EVA is possibly due to the presence of the hydroxyl group in the EVA structure (Mistry 2006). The improved miscibility of LDPE/EVA blends, which is caused by the similarities between the backbone chains of LDPE and EVA, gives a more uniform dispersion of EVA in the LDPE matrix (Khonakdar *et al.* 2004). Therefore, when the AM agents are "attached" to EVA, there may be a greater dispersion of the agents in the polymer matrix. Nevertheless, EVA has a relatively bulky structure (Khonakdar *et al.* 2004) which may enhance the interactions between PEG and LDPE thus minimising phase separation that would otherwise occur in these immiscible polymers.

Effect of PEG and EVA on AM Activity

The film formulations were subsequently tested on solid media using either the agar disc diffusion assay or the microatmospheric method to investigate the effect of the different blends on AM activity. The inhibition of bacteria, yeast and fungi by the films prepared by the AM films and the control film are presented in Table 4.4. The results suggest that the AM activity of these films varies significantly. Since the AM retentions are well below the MICs for the P/E0 formulations, these films show seemingly no inhibition against bacteria and yeast as expected. In contrast to the dosage-dependent activity shown in Section 4.1.2, the activity of these films against bacteria or yeast did not necessarily increase with increased retention levels. For example, although the P/E1 films have a slightly higher AM retention than their respective MIC values, these films show no inhibition against either bacteria or yeast on the agar disc diffusion assay. The P/E4 films, however, have lower retention than P/E2 and P/E3 films and show a significantly higher antibacterial activity compared to any of the other films.

Compared to the control film, all AM films significantly reduced the colony diameter of the fungal species *A. niger* (see Appendix C). Moreover, films with very low concentrations of AM agents (e.g. P/E0) showed effective inhibition compared to the control film after 2 days and 1 week of incubation. This may be due to the lower MICs of thymol and carvacrol against *A. niger* and/or due to the higher activity of AM agents in their volatile state. It has been suggested that the best antifungal activity of volatile compounds is achieved through gaseous contact as opposed to aqueous or agar contact (Guynot *et al.* 2003).

Table 4.4 The effect of EVA and PEG on AM activity

Film Type	Formulation	Zone of inhibition* (mm)			Colony diameter* (mm)	
					A. niger (F)	
		E. coli (G-)#	***S. aureus*** (G+)	***S. cerevisiae*** (Y)	**After 2 d**	**After 1 wk**
Control		NI≠	NI	NI	$41.6^{b, y}$	$73.7^{b, y}$
Thymol	P/E0	NI	NI	NI	19.7^{a}	39.7^{a}
	P/E1	6.2 ± 0.5^{a}	6.2 ± 0.4^{a}	NI	16.6^{a}	28.2^{a}
	P/E2	6.1 ± 0.5^{a}	6.3 ± 0.7^{a}	7.0 ± 1.1^{b}	15.7^{a}	33.2^{a}
	P/E3	5.8 ± 0.2^{a}	6.1 ± 0.4^{a}	6.1 ± 0.2^{a}	13.6^{a}	32.5^{a}
	P/E4	7.2 ± 1.1^{b}	6.8 ± 0.8^{b}	6.6 ± 0.6^{ab}	10.3^{a}	26.5^{a}
Carvacrol	P/E0	NI	NI	NI	13.5^{x}	37.7^{x}
	P/E1	NI	5.9 ± 0.3^{x}	NI	18.3^{x}	35.7^{x}
	P/E2	6.2 ± 0.4^{x}	6.1 ± 0.3^{x}	6.5 ± 0.7^{x}	14.3^{x}	40.5^{x}
	P/E3	6.2 ± 0.4^{x}	6.2 ± 0.4^{x}	6.3 ± 0.5^{x}	13.2^{x}	38.0^{x}
	P/E4	7.5 ± 1.0^{y}	7.6 ± 1.5^{y}	7.4 ± 1.3^{x}	14.2^{x}	33.7^{x}

Notes: * Means in the same column followed by different letters are significantly ($p < 0.05$) different in the Tukey's test.
(G-): Gram-negative bacteria; (G+): Gram-positive bacteria; (Y): yeast; (F): fungi.
≠ NI = no inhibition.

The copolymer EVA has a higher amorphous content in its structure compared to that of LDPE (Khonakdar *et al.* 2004). Thus, the crystallinity is reduced, the amorphous regions and the free volume are enhanced with increasing EVA content in LDPE/EVA blends (Khonakdar *et al.* 2004). The increased mobility of the amorphous phase with increasing EVA content may have facilitated the migration of AM agents from the films (Khonakdar *et al.* 2004). In a study involving AM films made of LDPE/EVA blends containing 0%, 10% and 50% (w/w) EVA, it was found that the AM agent migrates at a faster rate from the film containing a higher EVA content (Mistry 2006). The blend containing 10% (w/w) EVA, however, was found to reduce the migration of AM agents compared with all of the other blends tested (Mistry 2006).

The interaction of a phenolic AM compound with a non-ionic hydrophilic polymer such as PEG can result in a loss of AM properties of the phenolic compound (Kabadi and Hammarlund 1966). The significantly lower bacterial inhibition by the films containing PEG compared to that of films with no PEG may be attributed either to this loss of AM activity or to the possibility that PEG acts as a binding agent slowing the release of AM agents. The AM activity against yeasts and fungi that have a longer incubation time, however, do not indicate a reduced activity by the films containing PEG. These findings are therefore in accordance with the findings of Mistry (2006) who found that PEG can retain volatile AM compounds containing hydroxyl groups such as thymol but only in the short term. Thus, films with no PEG or a higher release rate of AM agents would be beneficial against bacteria that have a shorter incubation time or a faster growth rate. Conversely, for the inhibition of yeasts or fungi, films containing a blend of PEG and EVA with a sustained release of AM agents would be desirable.

4.2 Films Containing Combinations of Antimicrobials

In this section, the effects of combined AM systems in LDPE/EVA-based films are described. The combined use of thymol and carvacrol in the polymeric substrate was aimed at obtaining a wide AM spectrum at relatively low concentrations of AM agents. The utilization of conventional approaches in the analysis of interaction effects between AM agents in packaging films was explored.

4.2.1 Effect on Post Processing Retentions

The amount of thymol and carvacrol in the films containing AM combinations (AMC-films) and films containing individual AM agents (AMI-films) after processing were measured and the retention of AM agents is given in Table 4.5.

Table 4.5 Effect of AM combinations on post processing retention

Film Type	Retention#/% (w/w)	
	Thymol	**Carvacrol**
Thymol	1.7	
Carvacrol		1.1
Thymol/Carvacrol 1:1	1.5	1.6
Thymol/Carvacrol 2:1	2.0	0.9
Thymol/Carvacrol 1:2	1.2	2.5

Note #: Refer to Table 3.4 for film formulations.

The retentions of thymol or carvacrol in the AMI-films were similar to the targeted concentration, i.e. 1 - 2% (w/w). The ratios between the post processing concentration of thymol and carvacrol in AMC films were quite similar to the formulation ratios. However, the post-processing retention of the AM agents in AMC films were substantially higher than that observed in AMI films. For instance, the carvacrol film contained only 1.12% (w/w) carvacrol after processing equalling *ca.* 22% (w/w) retention. With lower concentrations of carvacrol in the formulations the AMC films had 1.62% and 2.48% (w/w) of carvacrol retained in the thymol/carvacrol 1:1 and 1:2 films equalling to 65% and 74% (w/w) retention respectively. These results indicate a possible interaction between thymol and carvacrol resulting in improved retention. However, further studies are needed to identify and predict the interaction effects (e.g. synergistic) on the retention of combined antimicrobials in polymeric substrates.

4.2.2 Effect on Antimicrobial Activity

In order to evaluate the AM efficacy, the AMI and films were tested on solid agar media against different microorganisms. The zone of inhibition (see Appendix C) achieved with various films against bacteria and yeast on solid media are given in Table 4.6.

Table 4.6 Effect of AM combinations on AM activity

Film Type	**Zone of inhibition* (mm)**		
	E. coli$^{(G-)}$	***S. aureus***$^{(G+)}$	***S. cerevisiae***$^{(Y)}$
Thymol	6.4 ± 0.5^{a}	6.2 ± 0.4^{a}	6.5 ± 0.5^{a}
Carvacrol	6.3 ± 0.2^{a}	6.4 ± 0.1^{a}	6.1 ± 0.5^{a}
Thymol/Carvacrol 1:1	8.0 ± 0.8^{c}	8.4 ± 0.6^{b}	8.3 ± 0.7^{b}
Thymol/Carvacrol 2:1	7.4 ± 0.4^{b}	7.8 ± 0.2^{b}	9.2 ± 0.9^{c}
Thymol/Carvacrol 1:2	7.4 ± 0.3^{b}	7.8 ± 0.4^{b}	7.9 ± 0.8^{b}

Notes: * Means in the same column followed by different letters are significantly ($p < 0.05$) different in Tukey's test.
[#] (G-): Gram-negative bacteria; (G+): Gram-positive bacteria; (Y): yeast.

A significantly higher inhibition was observed for AMC films than the AMI films. However, the total (or in some cases the individual) concentrations of thymol or carvacrol retained in the AMC films were found to be much higher compared to that in the AMI films. Therefore, direct comparisons between AMI and AMC films are not possible. The comparable total AM concentration in thymol/carvacrol 1:1 and 2:1 films allows some degree of comparison between these two AMC-films. Although the total AM concentrations are comparable in both formulations, there is a significant difference in inhibitory effects of thymol/carvacrol 1:1 and 2:1 films against *E. coli* and *S. cerevisiae*. Similarly, thymol/carvacrol 1:1 and 2:1 films, with lower total concentrations than thymol/carvacrol 1:2, show either a greater than or equal inhibitory effect against bacteria and yeast. This indicates a possible synergistic or additive effect dependence on the ratio of thymol/carvacrol in AMC-films. However, a conclusion on interaction effects could not be made based on a simple statistical analysis.

4.2.3 Antimicrobial Interactions

Interaction effects between different combinations of thymol and carvacrol in AMC films were analysed using the conventional models, FICs and effect additivity. The applicability and limitations of each analytical model in the assessment of interaction effects of combined antimicrobials in AM films were identified.

Dose Additivity: Summed FIC Analysis

The results of the ΣFIC values against different microorganisms are summarised in Table 4.7. Based on the ΣFIC values, a synergistic effect was observed only for films with thymol:carvacrol 2:1 against *S. cerevisiae*. AMC films with any combination of thymol:carvacrol have an antagonistic effect in the inhibition of *E. coli*. Approximate additivity was detected for AMC films with 1:1 and 2:1 thymol:carvacrol against *S. aureus*, and for AMC films with 1:1 thymol:carvacrol against *S. cerevisiae*.

Table 4.7 ΣFIC values of AMI- and AMC-films against different microorganisms.

Film Type	ΣFIC		
	E. coli **(G-)**	***S. aureus*** **(G+)**	***S. cerevisiae*** **(Y)**
Thymol/Carvacrol 1:2	1.6#	1.7#	1.4#
Thymol/Carvacrol 1:1	1.2#	1.0	1.1
Thymol/Carvacrol 2:1	1.3#	1.1	0.8†

Notes: * (G-): Gram-negative bacteria; (G+): Gram-positive bacteria; (Y): yeast.
\# ΣFIC significantly ($p < 0.05$) higher than 1; indicates antagonism.
† ΣFIC significantly ($p < 0.05$) lower than 1; indicates synergy.

The ΣFIC method assumes that the dose-response profiles of all AM agents within a combination are identical (Lambert and Lambert 2003). However, this behaviour is not observed in films containing thymol or carvacrol (see Section 4.1.2). In fact, the ΣFIC method appears to suggest that half the concentration of an AM agent gives half the effect (Lambert *et al.* 2001). This assumption, based on there being a linear dose response to each inhibitor (Lambert and Pearson 2000), however, is known to be either incorrect, in many cases, or to exist only within a range of AM concentrations in films

(see Section 4.1.2). If these assumptions were shown to be false then the interpretation of the results using the ΣFIC method may be open to question (Lambert *et al.* 2001).

Effect Additivity

The results of the interaction effects of various combinations of thymol/carvacrol in AM films measured by the effect additivity model are presented in Figure 4.2. The combinations 1:2, 1:1 and 2:1 of thymol/carvacrol are given in the *x*-axis and marked by the percentage (w/w) of thymol in each film. The films containing thymol/carvacrol 1:1 and 2:1 show an additive effect against *S. cerevisiae*. In all other cases, AMC films show significantly lower inhibition zones than the expected additivity resulting in an antagonistic effect.

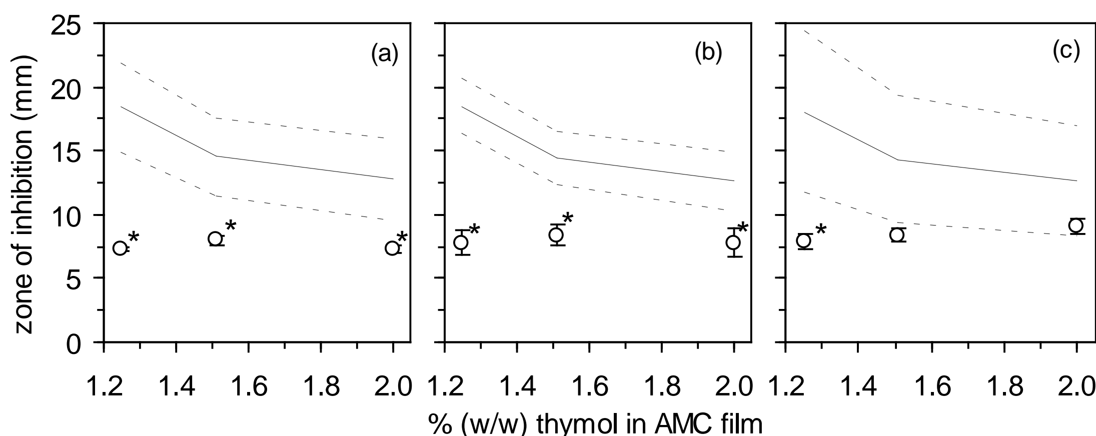

Figure 4.2 Interaction effects between thymol and carvacrol combinations in LDPE/EVA films on the growth of (a) E. coli, (b) S. aureus and (c) S. cerevisiae: (—) expected additivity line; (--) theoretically calculated 95% confidence limits (see Section 3.3.3); (○) observed inhibitory data with their respective 95% confidence limits as error bars; (*) data points significantly deviated (p < 0.05) from their expected values.

In the effect additivity model, the AM activity of a combination is predicted based on the assumption that the expected effect of two combined agents is equal to the sum of the effects of the single compounds. To determine if this assumption is appropriate, separate experiments on different formulations are needed. Conversely, this assumption should be supported by the fact that there is a linear relationship between the AM concentration and inhibitory activity. However, in the agar disc diffusion assay, a linear

relationship exists only within a certain range of AM concentrations in the film (see Section 4.1.2). Therefore, when attempting to predict the effect of a combination, the expected additivity cannot be calculated by simply adding the effects of the individual agents in the AMI films. For example, an increase in the concentration (e.g. doubling) could give different increases in the AM activity for each inhibitor.

4.2.4 Interactive Effects of Thymol and Carvacrol

Combinations of thymol and carvacrol are reported to have an additive or synergistic AM effect (Lambert *et al.* 2001; Paster *et al.* 1990; Pina-Vaz *et al.* 2004; Zhou *et al.* 2007). However, this is not the case in all literature data (Michiels *et al.* 2007). According to the conventional models, films containing various combinations of thymol and carvacrol show mainly an antagonistic effect. In the present study, interactive effects of AMC films show a more positive effect. In each case the effect is either antagonistic to additive or additive to synergistic with an increasing concentration of thymol in the combination and with an increasing time of incubation.

When AM agents are combined in an LDPE/EVA matrix, complex effects may result from the interactions between thymol and carvacrol. These interactions may results in greater resistance to their diffusion and consequently a reduction in the availability of AM agents in the agar media. This may prevent the AM agent from reaching lethal levels in cell membranes, especially against the rapidly growing bacteria. This assumption is supported by the AM efficacies shown against different microorganisms in the present study. Although *E. coli* is relatively resistant, *S. aureus* was found to be more sensitive to the inhibitory activity of films containing thymol and carvacrol than *S. cerevisiae*. Regardless of the microbial sensitivity, higher efficacies of AMC films were observed against *S. cerevisiae* that has a longer incubation time. Thus, AMC films require a longer period to release AM agents in order to enable effective interactions with microbial cells. However, these findings should be supported by release experiments on AMC films.

Although carvacrol can be released at a faster rate than thymol, an increasing concentration of carvacrol in the AM combination results in progressively negative interaction effects of AMC films. This may be due to the relative AM efficacies of AMI films (see Section 4.1.2) and aqueous solubility of the AM agents. Aqueous solubility is an important determinant of the AM efficacy since active antimicrobial compounds are more water soluble than inactive compounds (Cox *et al.* 2000). While thymol is slightly more soluble in aqueous media than carvacrol (Burdock 2005), increasing the level of insoluble carvacrol (presumably released first into the agar media) would decrease the solubility of thymol in the agar media. Hence, the cumulative AM effect would be reduced. A similar observation was made by Cox et al. (2000) for AM combinations containing soluble and insoluble components of tea tree oil. Therefore an appropriate ratio of carvacrol/thymol has to be chosen for maximizing synergism (Michiels *et al.* 2007).

4.3 Film Prepared by Extrusion Blowing

In this section, films containing thymol or carvacrol were prepared by extrusion film blowing, which is considered as a large-scale process for AM film production. The AM effect of extrusion on AM agent retention was investigated. The AM efficacy of the extruded films was verified on laboratory media.

4.3.1 Post Processing Retention of AM Agents

Blends of LDPE/EVA containing two different levels (2% and 4% (w/w)) of AM agents were extruded into *ca.* 50 μm films at an extrusion temperature of 150°C. The amount of AM agents retained in the films is summarised in Table 4.8.

Table 4.8 The post processing retentions of AM agents in extruded films

Formulation	Post processing retention[#]/% (w/w)	
	Thymol	Carvacrol
TL1	0.9	-
TL2	3.2	-
CL1	-	1.3
CL2	-	2.7

Note #: Refer to Table 3.1 for film formulations.

Carvacrol showed *ca.* 66% (w/w) retention in both CL1 and CL2 films. Thymol showed a higher retention (*ca.* 80% (w/w)) than that of carvacrol in TL2 film as expected. However, the retention of thymol in the TL1 film was unexpectedly low (*ca.* 42% (w/w)). Approximately 30% and 60% (w/w) retentions was reported for linalool in extruded LDPE/EVA films at an extrusion temperatures of 160°C (Suppakul 2004) and 150°C (Mistry 2006), respectively. The higher retentions reported in the present study suggest that thymol and carvacrol can withstand a temperature of 150°C during both compounding and extrusion. This might be attributed to the higher boiling points of thymol (232°C) and carvacrol (236 - 237°C) compared with that of linalool (194-197°C) (see Appendix A).

Regardless of the higher processing temperatures in extrusion, the retention of AM agents in the extruded films was found to be significantly higher than in the compression moulded films (see Section 4.1.2). The average thicknesses of compression moulded films were *ca*. twice the thickness of the extruded AM films (see Section 4.1.1), thus the extraction of AM agents from films may have been affected by the film thickness with thicker films resulting in lower retentions.

4.3.2 AM Activity on Solid Media

For a feasibility test, the AM activity of films was assessed against *E. coli* and *S. aureus* on agar media. Only the films with higher concentrations (TL2 and CL2) of thymol or carvacrol formed a clear zone around the film pieces against *E. coli* (see Appendix C).

The clear zones formed against *S. aureus* were confined to the area underneath the film. This was taken as an indication of growth inhibition compared to the complete bacterial growth underneath the control film. In contrast to the findings by Suppakul (2004) in LDPE/EVA films containing linalool, no clear difference for the inhibition zones between film cuttings at 0/90° and 45/45° to the extrusion directions was detected (see Appendix C).

4.3.3 AM Activity in Liquid Media

The AM films were subsequently tested in a liquid medium before being used in experiments involving real foodstuffs. Bacterial growth in the presence of different AM films was measured. Growth curves were derived for the evaluation of the inhibitory activity by AM films.

Availability of AM Agent

Depending on the amount of AM agents retained in the films after extrusion and the weight of the films required to give a film area to medium volume ratio of 1 cm^2 mL^{-1}, the corresponding theoretical concentrations of AM agents available in the liquid medium were calculated and are summarised in Table 4.9.

Table 4.9 Availability of AM agents in the liquid medium.

Film type and AM conc. / % (w/w)	**AM agent concentration in the liquid medium / mg mL^{-1}**	
	E. coli	***S. aureus***
Control	-	-
TL1 (0.9)	0.03	0.04
TL2 (3.2)	0.11	0.12
CL1 (1.3)	0.04	0.04
CL2 (2.7)	0.09	0.11

The concentration of carvacrol available per 1 mL of liquid medium by the CL2 film was *ca.* twice that of the CL1 film. The thymol concentration available from the TL2 film was almost four times higher than that of the TL1 film. Hence, the ratios between the AM availability in liquid medium were quite similar to the ratios between the post-processing retentions of AM agents in the films.

Effect of AM Films on E. coli Growth

The viable count data and curve fitting of *E. coli* growth at various initial concentrations are shown in Figures 4.3 and 4.4. With the exception of the TL2 film which showed a slight reduction in cell numbers during the first 2 h at 1.4 $\log_{10}$ CFU mL^{-1}, sigmoidal growth of *E. coli* was observed in the nutrient broth.

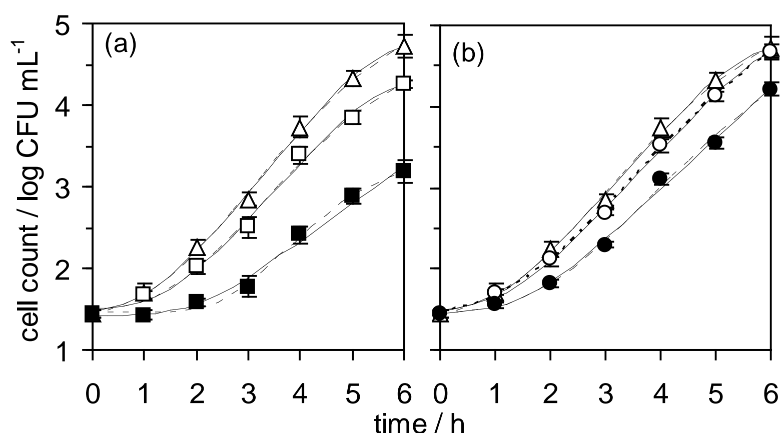

Figure 4.3 Growth of E. coli at the initial cell concentration of 1.4 $\log_{10}$ CFU mL^{-1} in nutrient broth at 37°C in the presence of: (a) thymol and (b) carvacrol films. Observed growth in: (∆) control film; (□) TL1 film; (■) TL2 film; (○) CL1 film and (•) CL2 film. Curve fitting of: (--) Gompertz model; and (—) Baranyi model.

A primary model for microbial growth aims to describe the kinetics of the growth process. Kinetic parameters of the primary models for the *E. coli* growth at various initial cell concentrations and the statistical index, RMSE, are shown in Tables 4.10 and 4.11. Both the Gompertz and the Baranyi models fitted well the growth profiles with small RMSE values (Fujikawa *et al.* 2004; Slongo *et al.* 2009) and correlation coefficients (R^2) values greater than 0.98 in all cases (values not shown). The predicted

curves for each model are observed to cross over each other several times during the growth period. However, Gompertz model presented a slight superiority in the curve fitting by having smaller RMSE values.

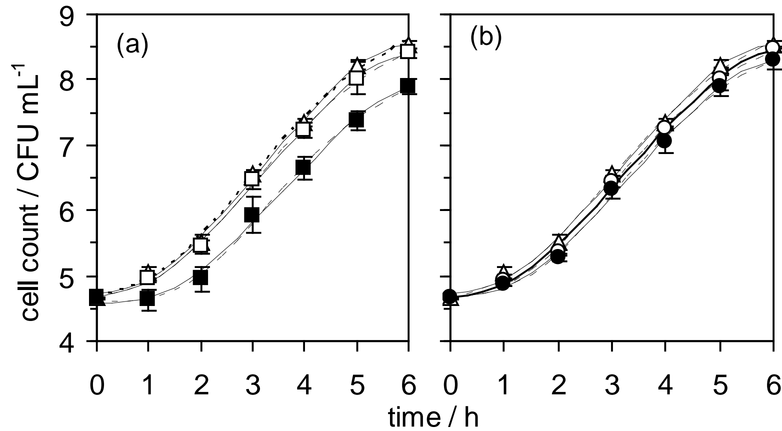

Figure 4.4 Growth of E. coli at the initial cell concentration of 4.6 log_{10} CFU mL^{-1} in nutrient broth at 37°C in the presence of: (a) thymol and (b) carvacrol films. Observed growth in: (Δ) control film; (□) TL1 film; (■) TL2 film; (○) CL1 film and (•) CL2 film. Curve fitting of: (--) Gompertz model; and (—) Baranyi model.

The main difference between the Gompertz curve and the Baranyi model is that the mid-phase in the Baranyi model is very close to linear, unlike the sigmoidal curve which has a pronounced curvature. Therefore, the Baranyi model did not give the upper asymptote parameter y_{max}, for the curves of CL2 and TL2 films at 1.4 log_{10} CFU mL^{-1} initial cell concentration of *E. coli* indicating that growth of *E.coli* is still in the mid exponential phase. The extended t_{lag} and comparatively low μ_{max} given by both films at 1.4 log_{10} CFU mL^{-1} of *E. coli* explains why the curves did not reach y_{max} during the time of measurements.

The Gompertz model overestimated the μ_{max} in many cases. For example at 1.4 log_{10} CFU mL^{-1} of *E. coli*, the Gompertz gives *ca.* 20% difference between the growth rates of the control and TL2 films compared to that of *ca.* 40% obtained by the Baranyi model. A similar overestimation of μ_{max} by the Gompertz model has been observed in a previous study (Fujikawa *et al.* 2004).

Table 4.10 Parameter estimates from Baranyi model for the growth of E. coli at various initial cell concentrations.

Cell conc. / log_{10} CFU mL^{-1}	**AM Film**	**Estimated parameters***			**RMSE**
		μ_{max} / h^{-1}	**t_{lag} / h**	**y_{max} / log_{10} CFU mL^{-1}**	
1.4	Control	0.76^{a}	1.1^{a}	4.8^{a}	0.0697
	TL1	0.69^{ab}	1.5^{b}	4.3^{b}	0.0789
	TL2	0.47^{c}	2.1^{c}	3.2^{c}	0.0922
	CL1	0.73^{a}	1.2^{a}	4.9^{a}	0.0961
	CL2	0.62^{b}	1.6^{b}	4.2^{b}	0.0728
4.6	Control	0.95^{x}	1.1^{a}	8.6^{x}	0.0937
	TL1	0.90^{x}	1.1^{a}	8.4^{x}	0.1074
	TL2	0.84^{y}	1.5^{a}	7.9^{y}	0.0902
	CL1	0.92^{x}	1.2^{a}	8.5^{x}	0.0879
	CL2	0.91^{x}	1.3^{a}	8.3^{x}	0.1050

Notes:* Means in the same column followed by different letters are significantly different in Tukey's test (i.e. $p < 0.05$).

Table 4.11 Parameter estimates from Gompertz model for the growth of E. coli at various initial cell concentrations.

Cell Conc. / log_{10} CFU mL^{-1}	**AM Film**	**Estimated parameters***			**RMSE**
		μ_{max} / h^{-1}	**t_{lag} / h**	**y_{max} / log_{10} CFU mL^{-1}**	
1.4	Control	0.77^{a}	1.0^{a}	5.6^{a}	0.0633
	TL1	0.69^{b}	1.3^{a}	5.0^{a}	0.0702
	TL2	0.59^{c}	2.4^{c}	3.5^{b}	0.0912
	CL1	0.72^{a}	1.1^{a}	6.1^{a}	0.0866
	CL2	0.67^{b}	1.7^{b}	5.4^{a}	0.0472
4.6	Control	0.96^{x}	1.1^{x}	9.4^{x}	0.0909
	TL1	0.93^{x}	1.1^{x}	9.1^{x}	0.0849
	TL2	0.92^{x}	1.7^{y}	8.4^{y}	0.0765
	CL1	0.98^{x}	1.3^{x}	9.1^{x}	0.0747
	CL2	0.95^{x}	1.4^{xy}	8.9^{xy}	0.0775

Notes:* Means in the same column followed by different letters are significantly different in Tukey's test (i.e. $p < 0.05$).

The initial cell concentration of *E. coli* affects the values of the kinetic parameters in both the Gompertz and Baranyi models. At 1.4 $\log_{10}$ CFU mL^{-1} of *E. coli* each of the AM films lowered significantly the μ_{max} and y_{max} and extended significantly the t_{lag} compared to the control film with the exception of the CL1 film. In most cases, the AM activity of the films was in the order of TL2 > CL2 > TL1 > CL1. The effect of AM films on inhibition was only marginal at high initial concentrations of *E. coli*. A rapid growth of *E. coli* occurred with higher μ_{max} and y_{max} and lower t_{lag} than that at low initial concentration. In many cases, the same order of AM effectiveness of films as that at lower cell concentration was observed.

Effect of AM Films on S. aureus Growth

The growth kinetics of *S. aureus* in liquid media in the presence of different AM films was also investigated. Following a preliminary experiment, the Gompertz model, which showed a slight superiority in fitting the experimental results, was selected for curve fitting of *S. aureus*. The growth of *S. aureus* in log viable counts and the fitted curves at various initial cell concentrations are presented in Figures 4.5 and 4.6. The values of the estimated kinetic parameters are given in Table 4.12.

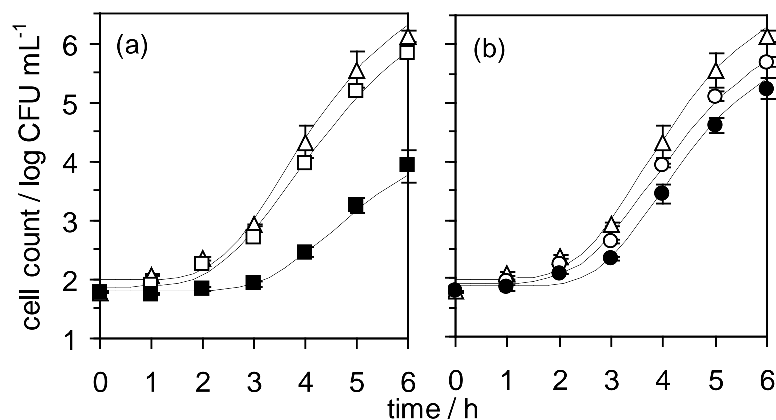

Figure 4.5 Growth of S. aureus at the initial cell concentration of 1.7 $\log_{10}$ CFU mL^{-1} in nutrient broth at 37°C in the presence of: (a) thymol and (b) carvacrol films. Observed growth in: (Δ) control film; (□) TL1 film; (■) TL2 film; (○) CL1 film and (•) CL2 film. Curve fitting of: (—) Gompertz model.

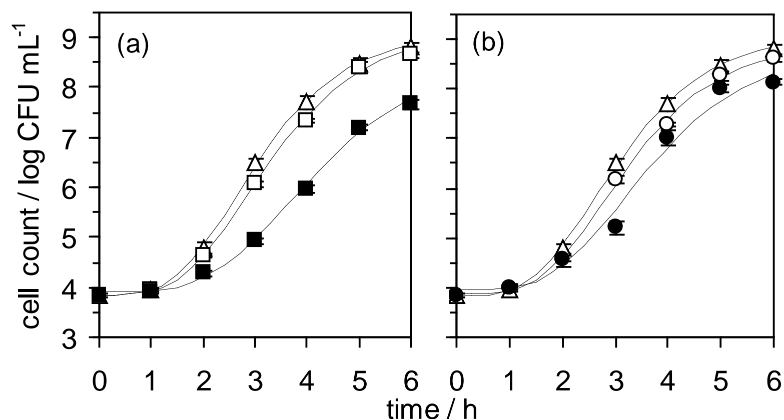

Figure 4.6 Growth of S. aureus at the initial cell concentration of 3.8 $\log_{10}$ CFU mL^{-1} in nutrient broth at 37°C in the presence of: (a) thymol and (b) carvacrol films. Observed growth in: (Δ) control film; (□) TL1 film; (■) TL2 film; (○) CL1 film and (•) CL2 film. Curve fitting of: (—) Gompertz model.

Table 4.12 Parameter estimates from Gompertz model for the growth of S. aureus at various initial cell concentrations.

Cell Conc. / $\log_{10}$ CFU mL^{-1}	AM Film	Estimated parameters*			RMSE
		μ_{max} / h^{-1}	t_{lag} / h	y_{max} / $\log_{10}$ CFU mL^{-1}	
1.7	Control	1.4^{a}	2.2^{a}	7.1^{a}	0.1041
	TL1	1.2^{ab}	2.3^{a}	6.9^{ab}	0.1075
	TL2	0.7^{c}	3.2^{c}	4.7^{d}	0.0927
	CL1	1.3^{a}	2.4^{a}	6.5^{b}	0.0940
	CL2	1.0^{b}	2.7^{b}	5.9^{c}	0.0714
3.8	Control	1.7^{w}	1.4^{x}	8.6^{x}	0.0893
	TL1	1.5^{x}	1.6^{x}	8.6^{x}	0.0072
	TL2	1.1^{z}	2.0^{z}	8.2^{y}	0.0475
	CL1	1.5^{x}	1.6^{x}	8.5^{x}	0.0695
	CL2	1.3^{y}	1.8^{y}	8.3^{xy}	0.0663

Notes:* Means in the same column followed by different letters are significantly different in Tukey's test (i.e. $p < 0.05$).

The plots of log concentration versus time showed sigmoidal growth of *S. aureus* in the nutrient broth in the presence of AM films. Similar to *E. coli*, in some cases, the AM films significantly lowered the μ_{max} and y_{max} and significantly extended the t_{lag} of *S. aureus* growth compared to the control film. However, the inhibitory effect was reduced at high initial cell concentration of *S. aureus* resulting in higher μ_{max} and y_{max} and lower t_{lag} than that at low initial concentration. In most cases, the AM activity of the films was in the order of TL2 > CL2 > TL1 ≈ CL1.

4.3.4 Factors Affecting AM Activity In Vitro

Agar Media Assays

Agar media studies are used to model experimental systems that mimic the gelled aqueous microstructure of some foods. In agar media the microorganisms are immobilized and grow as colonies (Broklehurst 2004). For a compound to be effective, it should diffuse through the agar media in order to reach the immobilized colonies (Parish and Davidson 1993). Due to the local accumulation of end-products, microbes on agar media are generally more sensitive to AM activity than the microbes in broth cultures (Broklehurst 2004). However, in the present study, the clear zones formed against bacteria were relatively small or were confined to the area underneath the film.

The aqueous solubility of a compound is critical since it governs the transfer of compounds to the microorganism (Sikkema *et al.* 1995). Thus, it would be expected that compounds of lower water solubility would show less activity even if solubility did not affect their activity in other situations (Griffin *et al.* 1999). The hydrophobic nature of thymol and carvacrol greatly reduce their diffusion in agar media and this may have accounted for the low activity of AM films observed on agar media. Furthermore, it is suggested that the AM agents in the film tend to diffuse from the film area which is in contact with the agar medium rather than to migrate from the cut margins of the films. Moulded films containing thymol or carvacrol at lower concentrations showed significant clear zones against *E. coli* or *S. aureus* in the agar disc diffusion assay (see Appendix C). The discernible difference shown by the films may be mainly due to the differences in the release kinetics (Mistry 2006) and may also be related to the film thickness and film processing conditions.

Broth Culture Assays

The kinetic parameters of bacterial growth in broth cultures, μ_{max} and t_{lag}, were compared as critical measures to understand the effect of different factors on AM efficacy of the films. Plots of $\sqrt{\mu_{max}}$ or $(t_{lag})^2$ versus AM concentration are presented in Figures 4.7 and 4.8. These plots are linear ($R^2 > 0.97$) with different gradients that are statistically significant. In addition, significant differences were found between the AM films containing either thymol or carvacrol and the different levels of bacteria as indicated by the different slopes.

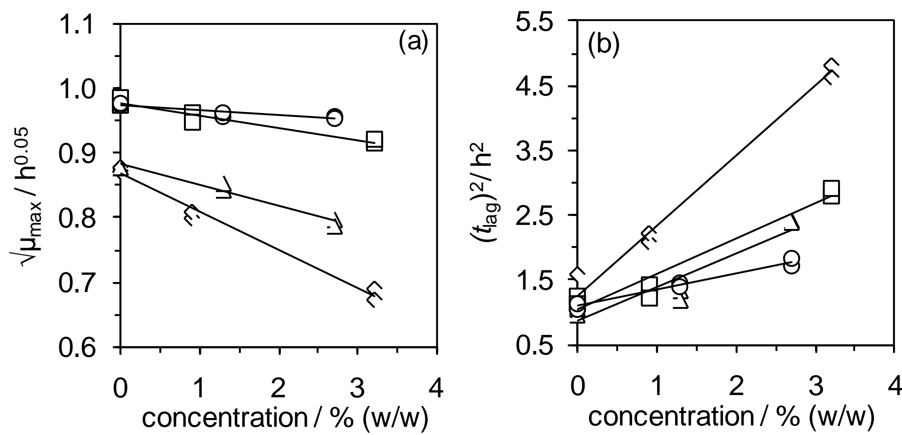

Figure 4.7 Plots of: (a) square root of μ_{max} versus AM concentrations and (b) square of t_{lag} versus AM concentrations for: (◊) thymol and (Δ) carvacrol films at 1.4 log_{10} CFU mL^{-1} and (□) thymol and (○) carvacrol films at 4.6 log_{10} CFU mL^{-1} of E. coli.

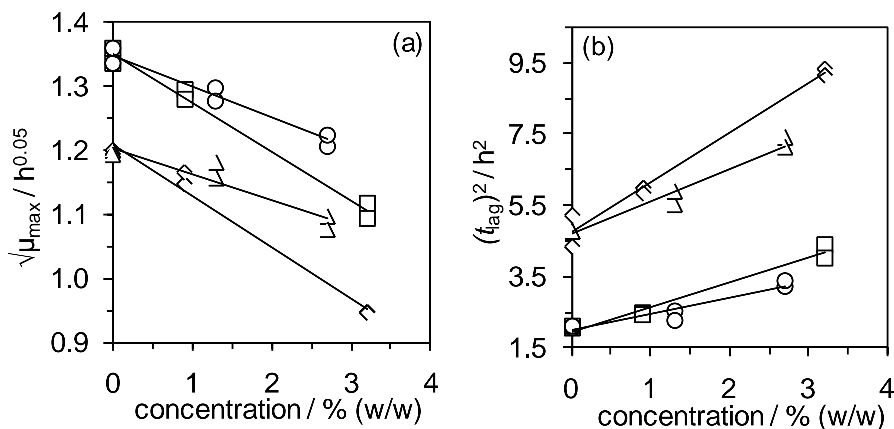

Figure 4.8 Plots of: (a) square root of μ_{max} versus AM concentrations and (b) square of t_{lag} versus AM concentrations for: (◊) thymol and (Δ) carvacrol films at 1.7 log_{10} CFU mL^{-1} and (□) thymol and (○) carvacrol films at 3.8 log_{10} CFU mL^{-1} of S. aureus.

In agreement with the findings on solid media (see Section 4.1.2), the significantly different slopes of the plots of $\sqrt{\mu_{max}}$ and $(t_{lag})^2$ AM films showed higher activity (compared to the control film) against *S. aureus* than against *E. coli* in broth culture. Despite the different inhibitory effects among the bacterial species and AM agents, with an increasing level of initial cell concentration, t_{lag} is reduced and μ_{max} is increased.

The effect of increasing bacterial concentration on the AM activity of films was apparent with an increase in μ_{max} and a reduction in t_{lag}. At high bacterial cell numbers, the bacterial growth rate may have exceeded the rate of diffusion of AM agents from the film to the broth medium. Thus, the amount of AM agent available in the broth culture may not have been sufficient to inhibit the growth or may not have reached lethal levels in the cell membranes. The higher concentrations of AM agents in the TL2 and CL2 films accounts, most probably, for the higher inhibitory activity shown by these films. The relative effectiveness of thymol films was found to be higher than that of carvacrol counterparts. For instance, although the TL1 film had only half the concentration of AM agent available per unit volume compared to that of CL1 film, it showed a higher AM activity against *E. coli*. This may be due to the higher inherent activity of thymol compared to that of carvacrol. Apart from the inherent AM efficacy, the differences in the release kinetics and/or solubility of these AM agents may have affected the inhibitory activity of these films (Griffin *et al.* 1999).

The AM agent concentrations in the films used in the present study are much higher than the concentrations of linalool in the films used by Suppakul (2004). However, the present films showed only marginal AM activity against bacteria in liquid medium. The slower release rates of compounds like thymol compared to that of linalool in the aqueous phase (Mistry 2006), where microbial proliferation takes place, is likely to impair their performance in aqueous media. The present study modeled the bacterial growth under optimum conditions, e.g. unlimited supply of nutrients, compared to limitations in the actual food substrates. However, at sub-optimal conditions such as lower temperatures (Lee *et al.* 2004), AM films may perform better than in the present study.

4.4 Characterisation of AM Films

In order to understand the effect of AM agents on the polymer properties, the extruded films were examined for their mechanical and thermal properties. The retention of AM agents during storage of the films and the release of AM agents into food simulants at various temperatures was also investigated.

4.4.1 Tensile Properties

The effect of AM agents on the tensile properties of extruded films was studied by measuring the peak load of films containing AM agents compared to the control film produced under the same extrusion conditions. The peak load of each film in both MD and TD is presented in Table 4.13.

Table 4.13 Tensile properties of extruded AM films

AM Film	**Peak Load* / N**	
	MD#	**TD≠**
Control	48.61 ± 3.0	45.74 ± 2.3
TL1	54.68 ± 3.1	45.34 ± 0.6
TL2	54.60 ± 1.8	43.51 ± 0.2
CL1	50.95 ± 1.7	45.04 ± 1.4
CL2	51.64 ± 1.4	45.69 ± 0.9

Note: *Mean (n = 5) with the standard deviation.
MD = machine direction; ≠ TD = transverse direction.

There were some differences between the control film and AM films for the peak load in the MD although these differences appear insignificant when the standard error is taken into consideration. No significant difference was observed between the control film and the AM films in the TD. These results are in agreement with a previous study (Mistry 2006) which showed the addition of 1.44% (w/w) thymol into an LDPE/EVA film did not significantly influence the tensile properties of the film. In general, the addition of a small amount of AM agent should not affect the physical integrity of the film due to the probability that these natural AM agents are located in the amorphous regions of the polymer structure (Han 2003). However, in agreement with the tensile

data previously reported for AM film containing thymol and linalool (Mistry 2006), for all films, the data obtained in the MD were higher than those obtained in the TD. The film surface defects, which arise during the film preparation, may have accounted for the lower TD peak loads.

4.4.2 Thermal Properties

The thermal data, in terms of melting temperature (T_m) and enthalpy of melting (ΔH), are summarised in Table 4.14. The thermal properties of pure LDPE used in the present study is also given in Table 4.14. As shown in Table 4.14, the addition of AM agents has no measurable effect on the thermal properties of the films. For the control film, the thermograms are comprised of a single peak representative of the LDPE/EVA blend. The T_m of all films was *ca*. 110-111°C. This is supported by the observations made on thermal properties of AM films containing linalool and methylchavicol where the addition of linalool or methylchavicol did not change significantly either ΔH or T_m (Suppakul *et al.* 2006).

Table 4.14 Thermal properties of extruded AM films

Resin/AM film	ΔH / J g^{-1}	T_m / °C
LDPE	68.52	113.5
LDPE/EVA	58.50	111.0
TL1	56.66	110.5
TL2	56.21	111.5
CL1	59.07	110.5
CL2	57.73	110.2

Comparing the melting behaviour of LDPE to that of LDPE/EVA film blends, both the ΔH and T_m of the latter are lower in the blend which is due to the EVA content. Typically, EVA copolymers are less crystalline than LDPE (Meszlényi and Körtvélyessy 1999) and as the vinyl acetate content increases, the crystallinity generally decreases (Hernandez *et al.* 2000).

4.4.3 Retention of AM Agents during Storage

During storage, depletion of volatile AM compounds by diffusion into the atmosphere may occur as a function of storage time and temperature. Therefore, AM agents were examined for their retention in the films during short- and long-term storage under different storage conditions.

Short –Term Storage

The concentration of the AM agent that is retained in the films, expressed as a proportion of the initial concentration, up to 28 days under different storage conditions is given in Figure 4.9 and Figure 4.10. When the films were stored in the open air (OA), by day 28, only 0.45% and 1.64% (w/w) thymol was retained in the TL1 and TL2 films respectively. Considering the initial thymol concentrations of 0.9% and 3.2% (w/w) in TL1 and TL2 films, respectively, this equates to *ca.* 50% retention. In addition to the OA storage, retention was also measured in stored films that were covered at room temperature (FC) and foil covered and refrigerated (FCR). The retention of thymol in TL1 film was 0.63% (w/w) for both FC and FCR storage while that of TL2 film was 2.2% and 2.0% (w/w) for FC and FCR storage respectively which corresponds to *ca.* 60-70% retention. The retention of thymol in each of the films under FC and FCR storage was not significantly different.

When stored under OA, the retention of carvacrol after day 28 was *ca*. 0.83% and 1.1% (w/w) in CL1 and CL2 films, respectively. This equates to *ca.* 60% and 40% retention of the initial concentrations of 1.3% and 2.7% (w/w) carvacrol in these films, respectively. Similar to thymol films, there was no significant difference in the FC and FCR storage of carvacrol films. Under both storage conditions, CL1 film showed about 1.1% (w/w) retention while that of CL2 film was about 2.2% (w/w) corresponding to about 80-87% of the initial concentrations.

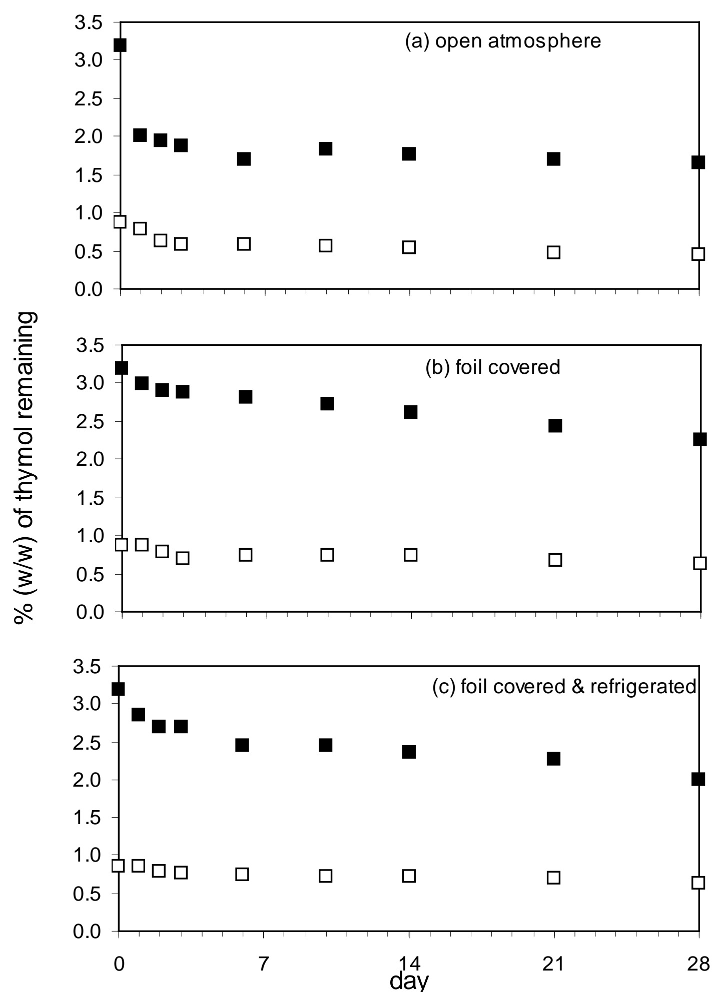

Figure 4.9 The percentage (w/w) retention of thymol during: (a) open; (b) foil covered and (c) foil covered and refrigerated storage. (□) TL1 film; (■) TL2 film.

In an attempt to enhance the retention of the AM agents during storage, conditions of FC and FCR were investigated. Storage of AM films under FC and FCR has a significant effect on the retention of both thymol and carvacrol during short-term storage (see Appendix F). These two storage systems retained 20% more thymol and 25-45% more carvacrol in the AM films compared to films stored in OA conditions. The results show that a rapid loss of AM agent from films occurred during the first few days of storage. All OA films attained equilibrium after *ca*. 3 d of storage whereas those stored under FC and FCR conditions attained equilibrium after *ca*. 10–14 d. Aluminium foil is considered to be an absolute barrier to aroma (Lamberti and Escher 2007) and this is was observed to be the case given the significantly higher retention of AM agent observed for films covered in foil compared to those in open storage.

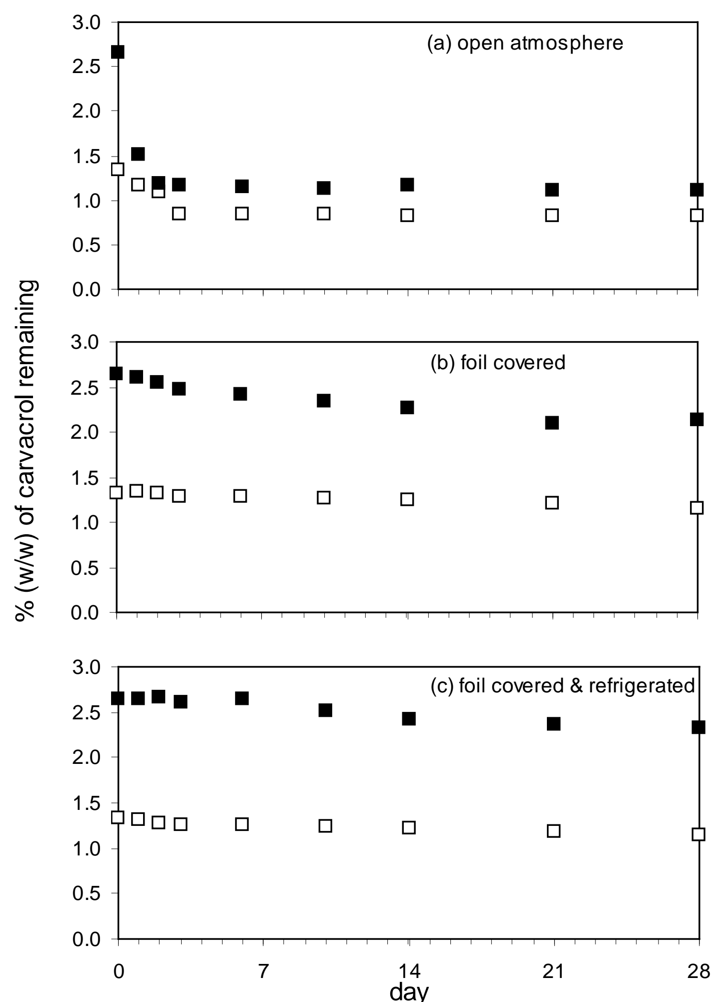

Figure 4.10 The percentage (w/w) retention of carvacrol during: (a) open; (b) foil covered and (c) foil covered and refrigerated storage. (□) CL1 film; (■) CL2 film.

Long –term Storage

The same films were evaluated for AM retention after 102 d to assess the ability of the films to retain AM agents during long-term storage. The films under OA showed higher peak values (data not shown) than the peak values at day 28 in their IR absorbance band at the wave number of 3450 cm^{-1} which corresponds to the hydroxyl group of thymol and carvacrol (see Appendix F). This may be due to the moisture absorbed by the polymer during storage. Therefore, only the retentions of FC and FCR are presented in Table 4.15.

Table 4.15 Retention of AM agents during long-term storage.

AM Film	**% Retention***	
	FC#	**FCR≠**
TL1	64.0	70.5
TL2	52.8	57.0
CL1	69.7	74.6
CL2	49.3	79.9

Note: *Percentage retained in the film after 102 d.
Foil covered.
≠ Foil covered and refrigerated.

As shown in Table 4.15, the retention of carvacrol in both CL1 and CL2 films under FCR was higher than that of the FC. Similarly, a higher retention of thymol was found in films stored under FCR than that of FC in both TL1 and TL2 films. The storage of films under FCR showed between 4% and 15% higher retention of carvacrol and an average of *ca.* 5% higher retention of thymol under FCR storage than that of FC. Reflecting the differences in the volatility of thymol and carvacrol (see Appendix A), the storage of AM films at low temperatures and covered in foil appears to have a more pronounced effect on the retention of carvacrol than that on thymol. Thus, having a barrier layer like aluminium foil and/or low temperature may have a significant advantage on the retention of highly volatile AM agents like carvacrol in AM films during long-term storage.

4.5 Release of AM Agent from Films

In this section, the extruded films were studied for AM agent release into the atmosphere and into food simulants in order to understand the kinetics of AM release from the polymer matrix.

4.5.1 Release of AM Agent to the Atmosphere

The release data from Section 4.4.3 was used to determine the kinetics and the effect of varying the amount and AM agent on the release of AM agents to the atmosphere. Plots

of the mass fraction of AM agent versus time are presented in Figure 4.11. From these plots it is evident that the initial rate of release of AM agent to the atmosphere is higher from the TL2 or CL2 films than that from the TL1 or CL1 films. In each case, however, the equilibrium is attained after 150 - 200 h.

Plots of $\ln(1 - m_t/m_\infty)$ versus time for the release to the atmosphere from films containing thymol and carvacrol up to 72 h are presented in Figure 4.12. The linearity ($R^2 > 0.98$) of these plots suggests that these systems follow first-order kinetics (see Equation 3.7) up to 72 h.

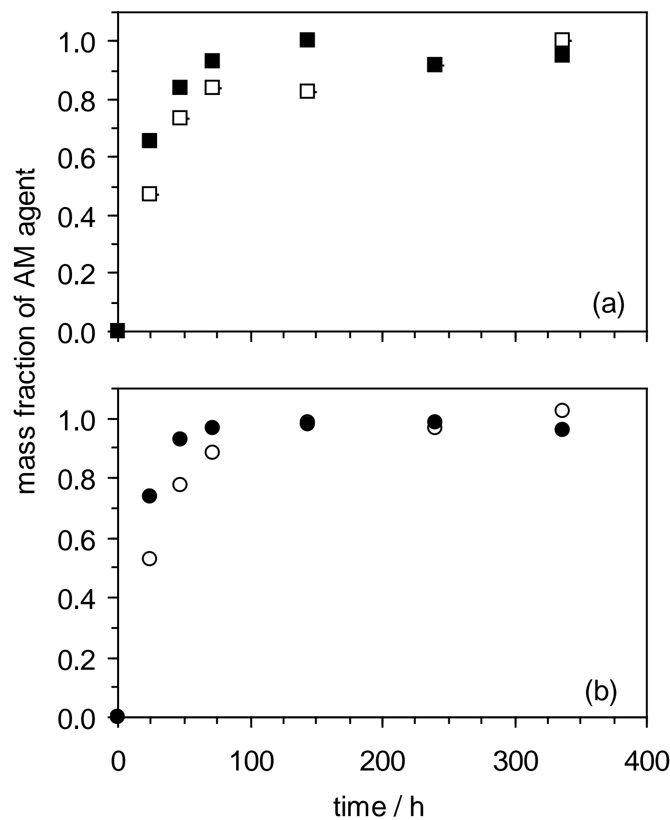

Figure 4.11 Plot of mass fraction mt/m∞ of (a) thymol and (b) carvacrol released to the atmosphere versus time from the films: (□) TL1; (■) TL2; (○) CL1 and (•) CL2.

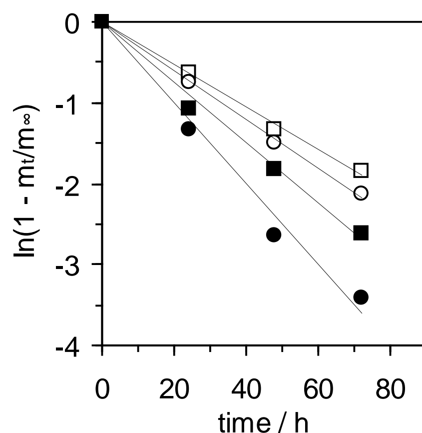

Figure 4.12 Plot of ln(1 - m_t/m_∞) versus time for the release of thymol and carvacrol to the atmosphere from the films: (□) TL1; (■) TL2; (○) CL1 and (•) CL2.

Values of the rate constants, k_2, for the kinetic analyses of release to the atmosphere are given in Table 4.16. The films containing thymol have lower k_2 values than carvacrol films, which suggests that the release of thymol to the atmosphere is slower than that of carvacrol. Moreover, the films with higher initial concentrations (TL2 or CL2) release AM agents at a faster rate than their respective films with lower initial concentrations (TL1 or CL1). This has been shown previously (Suppakul 2004) and it was suggested that it may be due to the greater driving force for mass transfer at higher AM concentrations. Nonetheless, the assumption of first-order kinetics seems to provide a sufficiently good fit to the data for the purposes of comparison in this work.

Table 4.16 Rate constant (k) of AM agent release from films to the atmosphere.

AM Film	**$k_2 \times 10^{-3}$ / h^{-1}**
TL1	26
TL2	37
CL1	30
CL2	50

The release rate of the volatile AM agents from the packaging system is highly dependent on the volatility, which relates to the chemical interactions between the volatile AM agent and the packaging materials (Han 2005a). Thus, the higher release of carvacrol may be attributed to its higher volatility (see Appendix A) and weaker interactions with the polymer matrix. The release to the atmosphere also reflects the propensity of the AM agent to be released into a package headspace. When the AM agent is vaporised into the headspace, it reaches the surface of the food and is absorbed by the food (Han 2005a). Therefore, carvacrol with a higher volatility and at a high concentration may reach the food surface more rapidly than in any other case. However, to maintain the surface concentration above the MIC of the target microorganism, it may be important to control the headspace concentration of AM agents.

4.5.2 Antimicrobial Agent Release in Food Simulants

The effect of food simulant and different temperatures on the release of thymol and carvacrol into food simulants were carried out using the films containing higher levels of AM agents (TL2 and CL2 film).

Effect of Food Simulant on the Release of AM Agent

The release of the AM agent carvacrol or thymol into various food simulants including isooctane, 95% (v/v) ethanol/water and 10% (v/v) ethanol/water was investigated. Plots of the mass fraction of thymol and carvacrol released into the simulants from AM films at 20°C versus time are presented in Figure 4.13. From these plots it is evident that the release of the agents into isooctane occurs faster than in any other simulant studied and that the slowest release is observed for 10% (v/v) ethanol/water. Similar plots were obtained for the release of AM agents at 10 and 15°C into isooctane, 95% (v/v) ethanol/water and 10% (v/v) ethanol/water (see Appendix F).

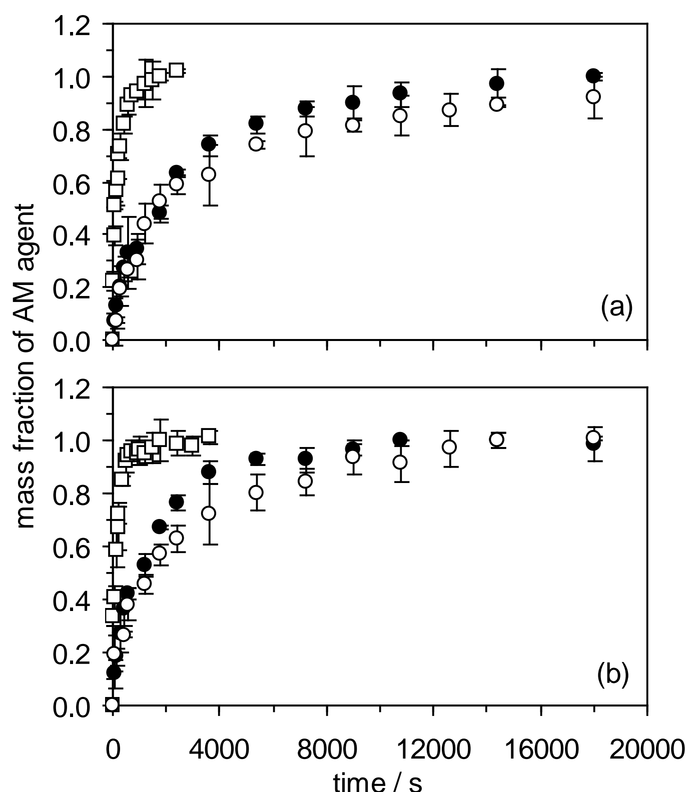

Figure 4.13 Plots of mass fraction m_t/m_∞ of (a) thymol and (b) carvacrol released at 20°C versus t into: (□) isooctane, (•) 95% (v/v) ethanol/water, and (○) 10% (v/v) ethanol/water.

At 20°C, the release of thymol and carvacrol into isooctane reaches equilibrium in *ca.* 8 and 10 min respectively. The release of thymol and carvacrol into 95% (v/v) ethanol/water reaches equilibrium in *ca.* 60 and 40 min, respectively, whereas the release into 10% (v/v) ethanol/water reaches equilibrium in *ca.* 120-150 min with a lag time (the time taken for any detectable release of AM agents) of *ca.* 5-10 min. Similarly, at 10°C and 15°C, equilibrium is attained faster in isooctane than in any other simulants studied and the slowest release is observed for 10% (v/v) ethanol/water (see Appendix F). The fast release of the AM agents into isooctane may be due to the swelling of LDPE in this solvent as reported by Helmroth and others (2003). The low solubility of the AM agents in polar aqueous solutions may also explain their faster release into a non-polar simulant such as isooctane (Cran *et al.* 2009).

Overall First-Order Kinetics Analysis

Plots of ln(1 - m_t/m_∞) versus time for the release of thymol and carvacrol into isooctane, 95% (v/v) ethanol/water and 10% (v/v) ethanol/water are presented in Figure 4.14. The first-order kinetics analysis (see Equation 3.8) show a reasonably good fit to the migration data, particularly up to about 90% of release of thymol and carvacrol. The plots also confirm that the release of thymol and carvacrol occurs fastest in isooctane and slowest in 10% (v/v) ethanol/water. Similar plots were obtained for the release of thymol and carvacrol into isooctane, 95% (v/v) ethanol/water and 10% (v/v) ethanol/water at 10 and 15°C (see Appendix F).

Table 4.17 lists the overall first-order rate constants and the initial release rates of thymol and carvacrol into various simulants at 10, 15 and 20°C. The results indicate that the values of v_0 and k_1 decrease consistently in the order: isooctane > 95% (v/v) ethanol/water > 10% (v/v) ethanol/water and in the order of 20°C > 15°C > 10°C. That suggest that the diffusion of these agents is expected to be low into aqueous or acidic foodstuffs (Cran *et al.* 2009) and to decrease with the decrease in temperature.

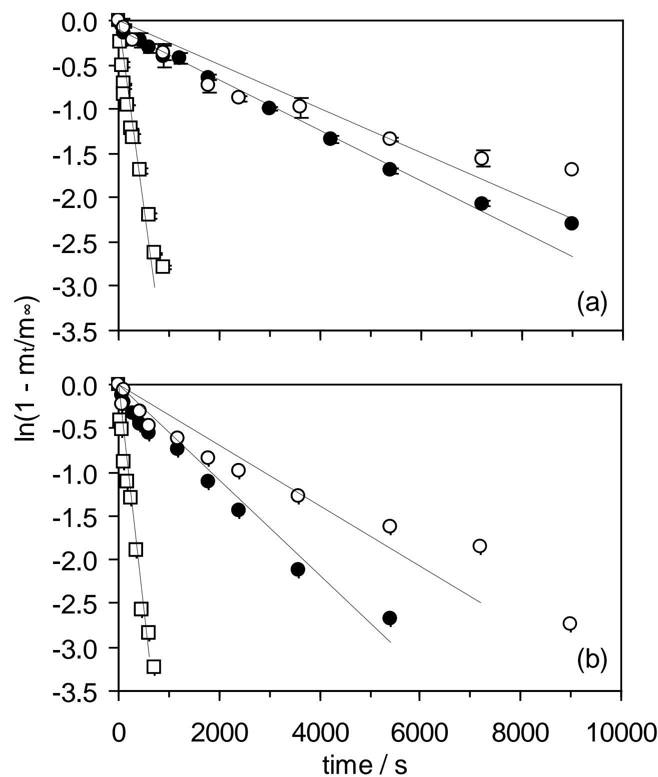

Figure 4.14 Plots of ln(1 - m_t/m_∞) versus t for the release of (a) thymol and (b) carvacrol into: (□) isooctane, (•) 95% ethanol, and (○) 10% ethanol at 20°C.

Short-Term and Long-Term Diffusion Analysis

Figure 4.15 to Figure 4.17 show the plots of m_t/m_∞ versus $t^{1/2}$ and $\ln(1 - m_t/m_\infty)$ versus t for the release of thymol into 95% (v/v) ethanol/water at 10, 15 and 20°C. The linearity of these plots confirms that the data are adequately described by the diffusion Equation 3.5 for short-term migration and Equation 3.7 for long-term migration of AM agents. Similar plots were obtained for the release of thymol into isooctane and 10% (v/v) ethanol/water at 10, 15 and 20°C and carvacrol into isooctane, 95% (v/v) ethanol/water and 10% (v/v) ethanol/water at 10, 15 and 20°C (see Appendix F).

Table 4.17 Effect of food simulant and temperature on the release of AM agents

AM Agent	Food Simulant	Temperature /°C	Diffusion Analysis		Kinetic Analysis	
			$D \times 10^{-14}$ / $m^2\ s^{-1}$	$k_1 \times 10^{-5}$ / s^{-1}	$v_0 \times 10^{-5}$ / $g\ s^{-1}$	$k_2 \times 10^{-5}$ / s^{-1}
Thymol	10% (v/v) Ethanol/water	10	1.1	2	0.5	5
		15	2.3	4	0.8	8
		20	6.2	8	1.7	14
	95% (v/v) Ethanol/water	10	2.8	8	0.8	10
		15	3.4	11	1.3	14
		20	7.4	23	3.1	26
	Isooctane	10	24.7	70	9.8	90
		15	47.6	110	17.7	156
		20	112.9	258	41.2	338
Carvacrol	10% (v/v) Ethanol/water	10	1.9	3	0.6	6
		15	3.8	11	0.8	14
		20	8.1	22	2.0	27
	95% (v/v) Ethanol/water	10	3.5	8	0.7	14
		15	6.9	16	1.8	27
		20	12.7	28	3.2	42
	Isooctane	10	31.4	97	10.5	119
		15	51.4	191	23.1	236
		20	133.8	409	52.2	490

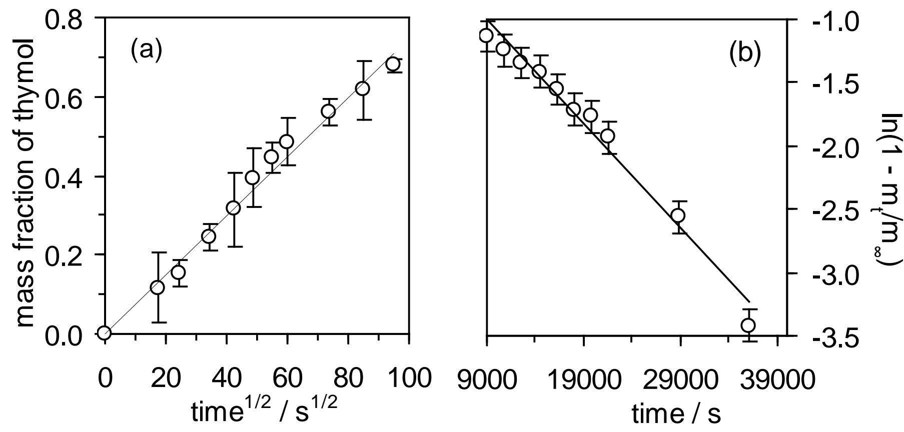

Figure 4.15 Plots of: (a) m_t/m_∞ versus $t^{1/2}$ and (b) $\ln(1 - m_t/m_\infty)$ versus t for the release of thymol into 95% ethanol at 10°C.

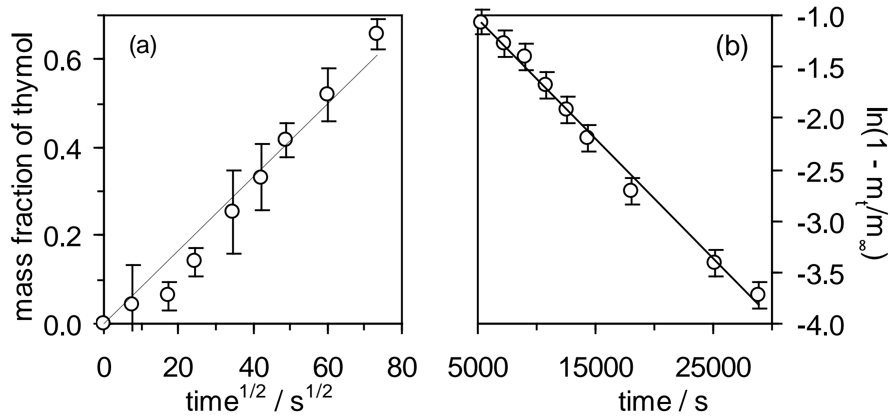

Figure 4.16 Plots of: (a) m_t/m_∞ versus $t^{1/2}$ and (b) $\ln(1 - m_t/m_\infty)$ versus t for the release of thymol into 95% ethanol at 15°C.

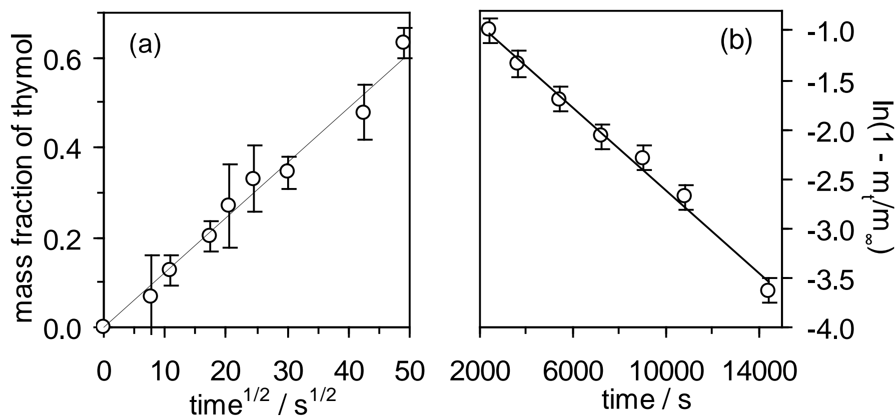

Figure 4.17 Plots of: (a) m_t/m_∞ versus $t^{1/2}$ and (b) $\ln(1 - m_t/m_\infty)$ versus t for the release of thymol into 95% ethanol at 20°C.

Values of D and k for the diffusion analyses of thymol and carvacrol in various simulants at 10, 15 and 20°C are presented in Table 4.17. The kinetic parameters decrease consistently in the order: isooctane > 95% (v/v) ethanol/water > 10% (v/v) ethanol/water. This reflects the decreasing order of solvent polarity of the simulants and the decreasing order of affinity of the simulant to the polymer substrate (Sajilata *et al.* 2007). The observed quick release of the AM agents into isooctane may be due to swelling of LDPE in this solvent (Feigenbaum *et al.* 2000; Helmroth *et al.* 2003). Ethanol is minimally absorbed by LDPE (Helmroth 2002) resulting in a lower diffusion rate of AM additives. The low solubility of the AM agents in aqueous media (see Appendix A) may have contributed to the slow release of the AM agent into 10% (v/v) ethanol/water. Thus, it can be assumed that the release of AM agents into aqueous foods would also be low which may reduce the possibility of developing off-flavors in these food products. Regardless of the food type, however, the relatively high vapour pressure of these agents may result in their extensive release into the food package headspace. Thus AM films containing carvacrol or thymol may be suitable for package/headspace/food systems (Cran *et al.* 2009).

Effect of AM Agent on the Release from Films

The effect of varying the AM agent on the release into food simulants at various temperatures was studied using thymol and carvacrol. Figure 4.18 shows plots of the mass fraction of thymol and carvacrol released into 95% (v/v) ethanol/water versus time at 20°C from which it is evident that the release of carvacrol occurs faster than that of thymol. Plots of $\ln(1 - m_t/m_\infty)$ versus time for release of thymol and carvacrol into 95% (v/v) ethanol/water at 20°C are shown in Figure 4.19 and confirm the faster release of carvacrol than that of thymol. Similar plots were obtained for the release of thymol and carvacrol at 10 and 15°C into 95% (v/v) ethanol/water and 10, 15 and 20°C into isooctane and 10% (v/v) ethanol/water.

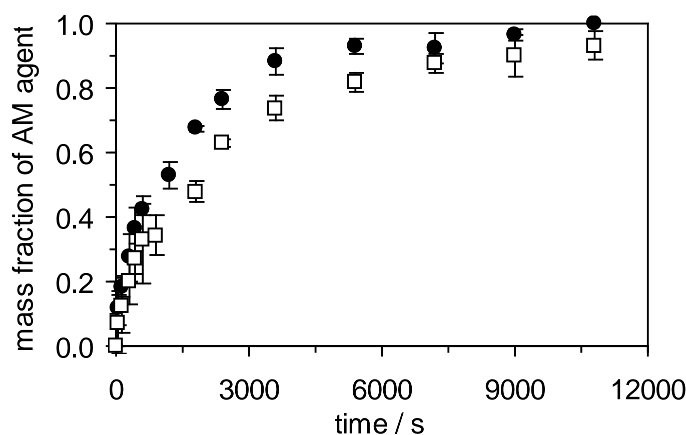

Figure 4.18 Plots of mass fraction m_t/m_∞ versus time for the release of (□) thymol and (•) carvacrol released into 95% ethanol at 20℃.

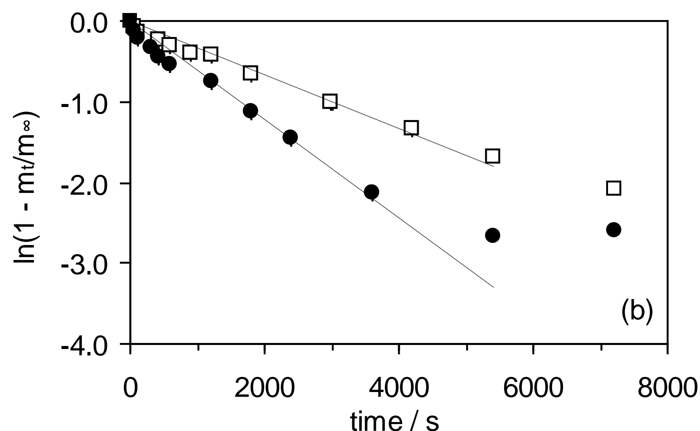

Figure 4.19 Plots of ln(1 - m_t/m_∞) versus time for the release of (□) thymol and (•) carvacrol into 95% ethanol at 20℃.

From the values of D, k_1, k_2 and v_0 for the diffusion and kinetic parameters given in Table 4.17, it is evident that the release of carvacrol into any solvent at all the studied temperatures is higher than that of thymol. This is in accordance with the release of the AM agents from the film into the atmosphere (see Section 4.5.1). The values of v_0 for thymol and carvacrol indicate that the initial release rates of thymol are significantly lower than that of carvacrol. For instance, v_0 for thymol and carvacrol into isooctane at 20°C are 41.2 × 10^{-5} g s^{-1} and 52.2 × 10^{-5} g s^{-1}, respectively. This suggests that thymol may be retained longer in the film because of a possible stronger intermolecular interaction between thymol and the polymer than that of carvacrol.

Effect of Temperature on the Release of AM Agent

The effect of three different temperatures, 10, 15 and 20°C, on the release of AM agents from the films was explored. In Figure 4.20, the effect of temperature on the release of the AM agents is presented. From this plot it is evident that the rate of release of AM agents is highest at 20°C whereas it is lowest at 10°C as expected. Similar plots were obtained for the release of thymol and carvacrol at 10, 15 and 20°C into 95% (v/v) ethanol/water and 10% (v/v) ethanol/water (see Appendix F).

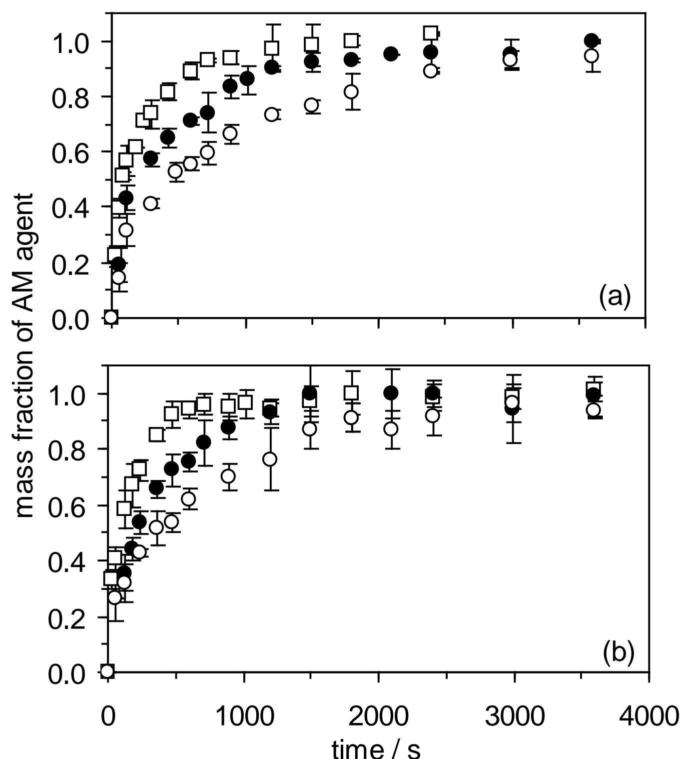

Figure 4.20 Plots of the mass fraction m_t/m_∞ versus time for the release of (a) thymol and (b) carvacrol into isooctane at: (○) 10; (•) 15 and (□) 20°C.

Figure 4.21 shows plots of ln(1 - m_t/m_∞) versus time for the release of thymol and carvacrol at various temperatures into isooctane. These plots confirm the faster rate of release at higher temperatures. Similar plots were obtained for the release of thymol and carvacrol at 10, 15 and 20°C into 95% (v/v) ethanol/water and 10% (v/v) ethanol/water (see Appendix F).

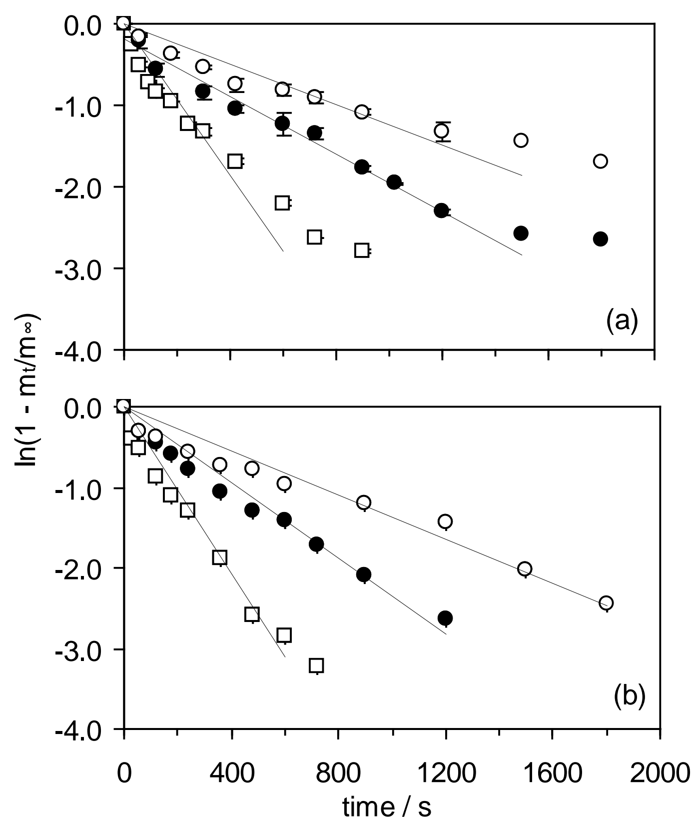

Figure 4.21 Plots of ln(1 - m_t/m_∞) versus time for the release of (a) thymol and (b) carvacrol into isooctane at: (○) 10; (•) 15 and (□) 20°C.

As depicted in Table 4.17, raising the temperature from 10 to 20 °C leads to a faster rate of migration of AM agents. It is generally known that an increase in temperature results in an increase in the rate of diffusion of molecules (Laidler 1965). The temperature dependence of the kinetic parameters, D and k, can be established from an Arrhenius plot derived from the logarithmic transformation of Equation 3.10 and Equation 3.11 versus the reciprocal of the absolute temperature. Figure 4.22 shows plots of ln(D) and ln(k) versus T^{-1} for the release of thymol and carvacrol into isooctane. Similar plots were obtained for the release of thymol and carvacrol into 95% (v/v) ethanol/water and 10% (v/v) ethanol/water (see Appendix F). The linearity of these plots, with R^2 values greater than 0.9, indicates that the diffusion of the AM agents obeys the Arrhenius equation.

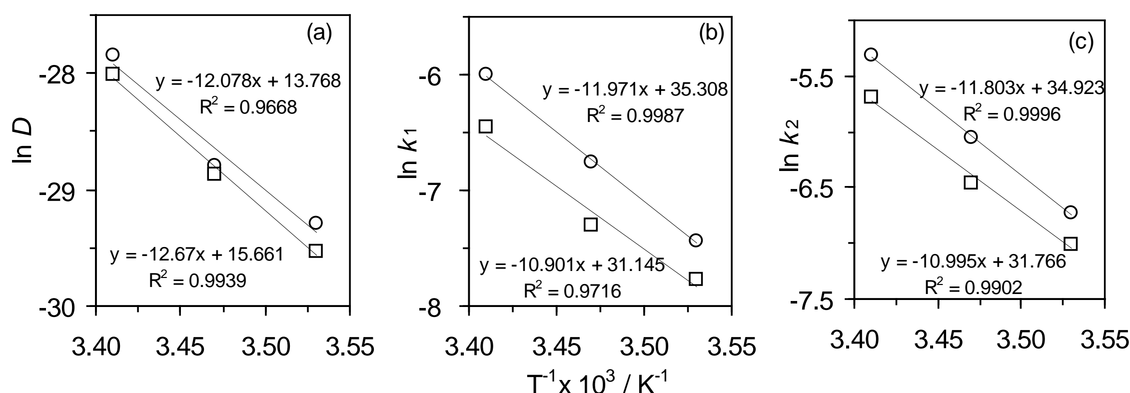

Figure 4.22 Plots of the logarithm of (a) D; (b) k_1 derived for the diffusion analysis and (c) k_2 versus the reciprocal of the absolute temperature for the release of: (□) thymol and (○) carvacrol into isooctane.

The activation energies for the release of AM agents from the films into different food simulants are presented in Figure 4.18. The dependency of diffusion on temperature is generally explained by temperature effects on the nature of adhesive forces at interfaces and on molecular mobility (Vojdani and Torres 1990; Zhu *et al.* 2006). At higher temperatures the mobility of polymer segments and diffusing molecules are enhanced and increase the diffusion coefficients (Zhu *et al.* 2006). Carvacrol show slightly higher activation energies than thymol systems. This difference is more significant in 10% (v/v) ethanol/water. The concentration of thymol in the film is slightly higher than that of carvacrol. The higher the concentration of AM agent in the polymer matrix, the activation energy for diffusion might be smaller (Cho *et al.* 2005).

The temperature dependency of diffusion could also be explained by the solvent properties (Igwe *et al.* 2006), Isooctane has the highest solubility in LDPE (Brydson 2000; Helmroth *et al.* 2003) and thus causes more free volume in the polymer, resulting in a higher release rate of the additive. At increased temperatures, the migration of AM agents is thus expected to increase with increasing isooctane penetration into the films (Suppakul 2004). Ethanol is hardly absorbed by LDPE (Helmroth *et al.* 2003).

Table 4.18 Activation energy (E_a) for release of AM agents from films into different food simulants.

Solvent	**AM agent**	E_a / J mol^{-1}		
		D^*	$k_1^{\#}$	$k_2^{\neq}$
Isooctane	Thymol	92	86	87
	Carvacrol	100	100	102
95% (v/v) ethanol/water	Thymol	86	71	70
	Carvacrol	89	86	76
10% (v/v) ethanol/water	Thymol	104	80	91
	Carvacrol	117	137	98

Note: *Activation energy calculated from D.
$^{\#}$Activation energy calculated from k_1 of diffusion process.
$^{\neq}$ Activation energy calculated from k_2 of chemical process

4.5.3 Extension to the Diffusion Model

The treatment of AM migration data in accordance with the idealized diffusion approach (Miltz 1987) can, in some cases, produce results that deviate from linearity (see Section 4.5.3). One explanation for the limited fit of the data relates to the assumptions that apply in the derivation of Equation 3.5. A better fit to the data may be achieved by moving the short-term/long-term boundary so that the non-conforming data are shifted to the long-term time domain, namely, a lower value of m_t/m_∞ for the transition from the short-term to the long-term migration regime (Cran *et al.* 2009).

To investigate this possibility further, a computer program was written that calculates the values of m_t/m_∞ for the approximate long-term solution of AM agent diffusion in an idealized or "infinite" system depicted by Equation 3.6 (Cran *et al.* 2009) and the exact long-term solution given by Equation 4.1 below (Crank 1975; Miltz 1987):

$$\frac{m_t}{m_\infty} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} \exp\left(\frac{-(2n+1)^2 \pi^2 D t}{l^2} \right) \qquad (4.1)$$

Figure 4.23 shows theoretical plots of m_t/m_∞, versus time for both the approximate and the exact solutions to the diffusion equation for an "idealized" system (Crank 1975; Miltz 1987) that have been calculated for the long-term period where the diffusion coefficient is 1×10^{-15} m^2 s^{-1} and film thickness of 50 μm (Cran *et al.* 2009).

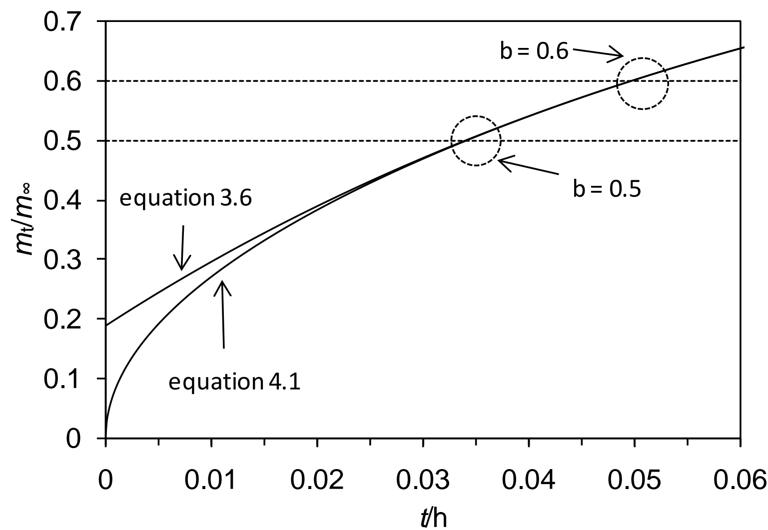

Figure 4.23 Theoretical plots of m_t/m_∞ versus time for the exact and approximate solutions to the diffusion equations that pertain to an "idealized" system (Crank 1975; Miltz 1987) where $D = 1 \times 10^{-15}$ m^2 s^{-1} and l = 50 μm. The summation calculations for the "idealized" solution were performed up to n = 40 terms.

It is apparent from Figure 4.23 that the two functions represented by Equations 3.6 and 4.1 remain almost convergent for values of m_t/m_∞ down to *ca*. 0.5. Indeed, this suggests that the definition of the short-term/long-term boundary (b) in the analysis can be shifted from b = 0.6 to b = 0.5 with little effect on the analytical result. The difference between the two functions at b = 0.6 was calculated to be 0.03% and at b = 0.5 the difference is 0.23%. This suggests that the error in assuming congruence of the two functions remains acceptably low if the short-term/long-term boundary is shifted downwards from b = 0.6 to b = 0.5 for the purposes of producing a more convenient data analysis (Cran *et al.* 2009).

Plots of m_t/m_∞ versus the square root of time (short-term diffusion analysis) where $m_t/m_\infty \geq 0.6$ or $m_t/m_\infty \geq 0.5$ for the release of thymol films into 95% ethanol at 10°C are shown in Figure 4.24. Inspection of Figure 4.24(a) reveals that the inclusion of the data up to the boundary $b = 0.6$ presents an apparent curvature in the plot which should, of course, be linear. A better fit to the short-term diffusion data is achieved by setting the short-term/long-term boundary at $b = 0.5$ as shown in Figure 4.24(b) (Cran *et al.* 2009).

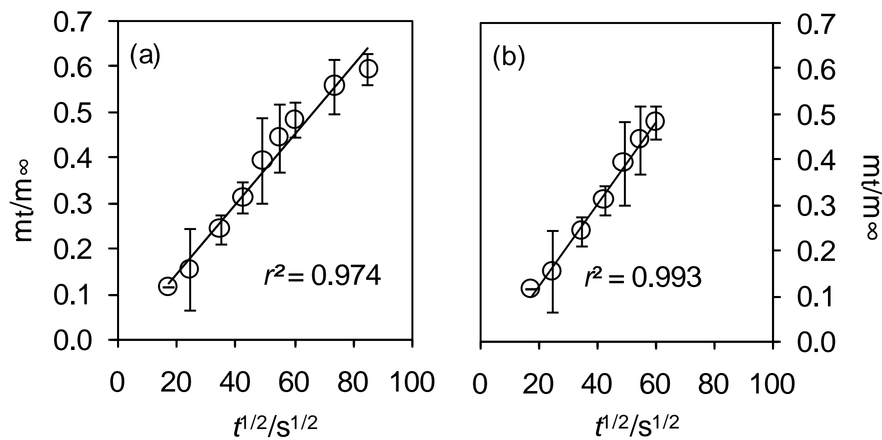

Figure 4.24 Plots of m_t/m_∞ versus the square root of time for the short-term release of thymol from films into 95% ethanol at 10°C where the boundary conditions are: (a) b = 0.6 and (b) b = 0.5.

Figure 4.25 shows plots of $\ln(1 - m_t/m_\infty)$ versus time (i.e. long-term diffusion analysis) where $m_t/m_\infty > 0.5$ or $m_t/m_\infty > 0.6$ for the release of thymol from films into 95% (v/v) ethanol/water at 10°C. It is apparent that also for the long-term diffusion data a better fit is achieved by moving the boundary from $b = 0.6$ to $b = 0.5$. Thus upon considering the data in Figure 4.24 and Figure 4.25 simultaneously it is clear that a better fit to the experimental data is achieved in both, the short term and the long term, by moving the boundary from $b = 0.6$ to $b = 0.5$ (Cran *et al.* 2009).

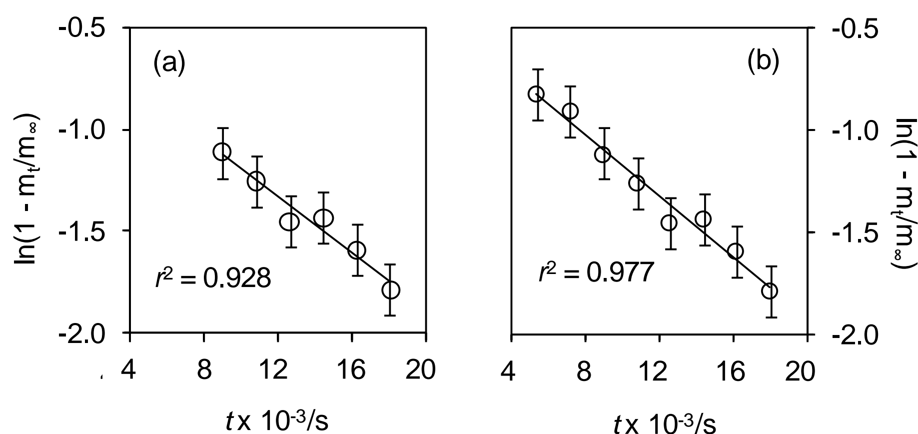

Figure 4.25 Plots of ln(1 – m_t/m_∞) versus time for the long-term release of thymol from films into 95% ethanol at 10 °C where the boundary conditions are: (a) b = 0.6 and (b) b = 0.5.

In order to demonstrate more clearly the possible flexibility in the definition of the short-term/long-term boundary when applying the idealized equations the data for a number of different systems were analyzed using b = 0.5 or b = 0.6 as the boundary condition. The goodness of fit of the model in the case of the short-term analyses was determined by calculating the respective linear regression coefficient, R^2. Table 4.19 presents these data for various selected systems. It demonstrates that in most cases a better fit of the idealized diffusion model is obtained in the case where the short-term/long-term boundary is moved from b = 0.6 to b = 0.5 (Cran *et al.* 2009).

Table 4.19 Comparison of linear regression coefficients obtained in the short-term analysis of various AM-containing systems for b = 0.5 and b = 0.6.

Sample	Food Simulant	Linear Regression Coefficient, r^2			
		Short-term analysis		Long-term analysis	
		b = 0.6	b = 0.5	b = 0.6	b = 0.5
Carvacrol L2 film	10% ethanol, 15°C	0.965	0.984	0.977	0.983
Carvacrol L2 film	95% ethanol, 20°C	0.978	0.991	0.932	0.915
Carvacrol L2 film	100% isooctane, 10°C	0.984	0.989	0.949	0.920
Thymol L2 film	95% ethanol, 10°C	0.974	0.993	0.928	0.977

4.6 Application of AM Films on Cheddar Cheese

In order to assess the usefulness of films containing different levels of thymol and carvacrol in the enhancement of food preservation, the films were used to package Cheddar cheese. This section summarizes the work on the effect of AM films on microbiology, physical and chemical properties, and in particular, sensory characteristics of the Cheddar cheese during storage.

4.6.1 Challenge Test

The extruded AM films were assessed for their AM effectiveness on Cheddar cheese by a challenge test involving the bacteria *E. coli* and *S. aureus*. The challenge test aimed at studying the fate of microorganisms on Cheddar cheese affected by the AM films under temperature abuse conditions (12°C). The effectiveness of AM films on bacterial inactivation was expressed and compared quantitatively by means of mathematical modelling.

Inactivation of E. coli

The influence of AM films on the inactivation of *E. coli* inoculated on Cheddar cheese is presented in Figure 4.26. The inactivation, given by the total viable numbers, of *E. coli* proceeded according to a biphasic pattern; a rapid inactivation phase followed by a slow inactivation phase termed “tailing” (Brul *et al.* 2007).

The observed data were fitted with Weibull and first-order biphasic models, which are commonly applied to describe the “tailing” behaviour in inactivation (Brul *et al.* 2007; Geeraerd *et al.* 2005). The biphasic model performed slightly better ($R^2 > 0.997$ and RMSE < 0.13) than the Weibull model ($R^2 > 0.98$ and RMSE < 0.18). Therefore, the first-order biphasic model was adapted to depict the inactivation curves of *E. coli*. The parameters of the biphasic model, P (the fraction of survivors in subpopulation 1) and the rate constants of both fractions (s_1 and s_2), were estimated and are summarised in Table 4.20. The statistical indices, R^2 and RMSE, of the fits are also included in this Table 4.20. The curves (represented by solid lines in Figure 4.26) drawn with these estimated parameters exhibited good agreements with the experimental data.

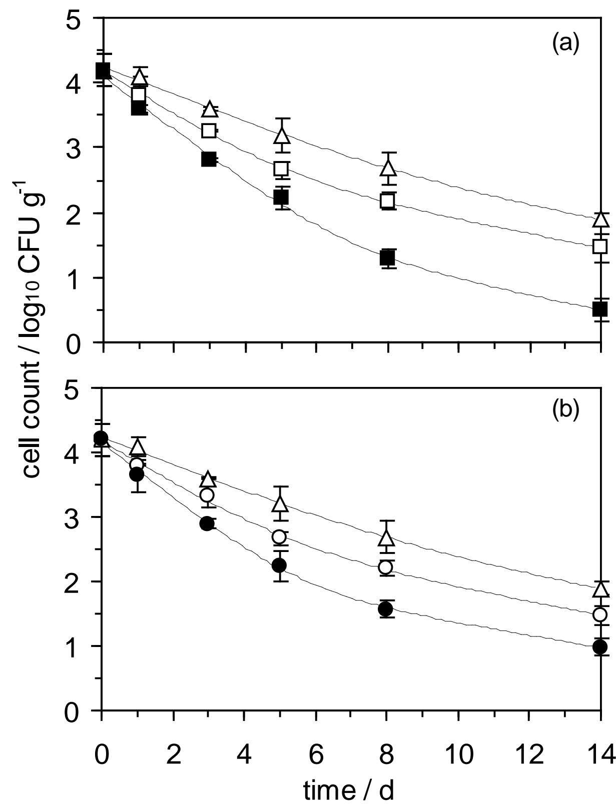

Figure 4.26 Inactivation of E. coli on Cheddar cheese wrapped with: (a) thymol and (b) carvacrol films. Observed growth in the presence of: (Δ) control film; (□) TL1 film; (■) TL2 film; (○) CL1 film and (•) CL2 film. (—) Curve fitting of first-order biphasic model.

Table 4.20 Parameters estimates of first-order biphasic model for the inactivation of E. coli on Cheddar cheese wrapped in AM films.

AM Film	Estimated parameters			RMSE	R^2
	P	s_1 / d^{-1}	s_2 / d^{-1}		
Control	0.931^{a}	0.52^{a}	0.20^{a}	0.0602	0.9981
TL1	0.943^{b}	0.79^{b}	0.24^{bc}	0.0495	0.9978
TL2	0.990^{d}	0.99^{e}	0.26^{c}	0.1305	0.9987
CL1	0.943^{b}	0.83^{c}	0.21^{ab}	0.0760	0.9991
CL2	0.986^{c}	0.96^{d}	0.22^{b}	0.0703	0.9975

Note: Means in the same column followed by different letters are significantly different in Tukey's test (i.e. $p < 0.05$).

The values of P and s_1 of the more sensitive population were higher in cheese samples packaged with films containing either thymol or carvacrol than in the control film. Furthermore, the P and s_1 values increased with an increasing concentration of AM agent in the films. Significant differences were observed also for the s_2 values where they increased with an increasing concentration of the AM agent in the films. The inactivation of *E. coli* by AM films was in the order of TL2 > CL2 > TL1 ≅ CL1. In agreement with these findings, Limjaroen *et al.* (2005) reported a dosage-dependent AM activity of polyvinylidene chloride (PVDC, Saran® F-310) films containing sorbic acid at different concentrations (0%, 1.5% and 3.0% (w/v)) against *L. monocytogenes* on Cheddar cheese. It is generally accepted that the higher the concentration of AM agent in the film, the more migration occurs and more AM agent is present in the medium (Ha *et al.* 2001; Limjaroen *et al.* 2005).

The biphasic model suggests the existence of two discrete subpopulations (Virto *et al.* 2004) of *E. coli* that differ in their sensitivity to AM films. The two sub populations decay independently according to first-order kinetics (Xiong *et al.* 1999), one being more resistant (in this case, sub population 2) to AM films than the other (Geeraerd *et al.* 2005). The sensitivity of P values and the rate constants to the AM film treatments suggests, however, that the reason for the observed biphasic survival curves may not be due to the existence of two different subpopulations (Lee *et al.* 2001; Virto *et al.* 2004), but may be caused by the AM films. Conversely, a microbial adaptation is quite improbable considering the fraction of the most sensitive population and its rapid inactivation (Virto *et al.* 2004). Thus, the biphasic nature of the inactivation curves may have resulted from a change in the concentration of the AM agents during the time of exposure of a homogeneous population of *E. coli* to the AM films. The release kinetics and subsequent interactions with the food matrix may result in a change in AM concentration available for inhibition. Migration of AM agents from the packaging film to the food follows usually a non-linear behaviour and a decrease in the diffusion coefficient occurs with time (Miltz 1987). The microbial inhibition by thymol or carvacrol is primarily related to their intrinsic hydrophobicity (Griffin *et al.* 1999; Nostro *et al.* 2007) and the same affinity (to the hydrophobic domain in cheeses) may

lower the effective AM concentrations resulting in a loss of AM action (Smith-Palmer *et al.* 2001).

Consistent with previous findings (Rupika *et al.* 2006; Suppakul 2004), *E. coli* showed a decline in viable numbers (even in the control film) over the storage period. With the high acid and high salt tolerance as well as survival during refrigeration and freezer storage temperatures, *E. coli* has the ability to survive in Cheddar cheese (Conner 1992; Guraya *et al.* 1998; Reitsma and Henning 1996). For instance, survival of *E. coli* in Cheddar cheese packaged in plastic bags or pouches that were stored at 6-7°C for more than 60 days has been demonstrated by several researchers (Reitsma and Henning 1996; Schlesser *et al.* 2006). In all cases, however, the viable count of *E. coli* decreased as a function of time (Reitsma and Henning 1996; Schlesser *et al.* 2006). In another study, survival accompanied by a cease in growth of *E. coli* on Cheddar cheese under sub-optimal conditions has been reported (Guraya *et al.* 1998).

Inhibition of S. aureus

The aim of the challenge test including a second bacterium, *S aureus*, was to understand the differences in the AM activity of the films against different bacteria. The viable counts of *S. aureus* during the challenge test are presented in Figure 4.27. Similarly to *E. coli*, the total viable counts of *S. aureus* declined over the storage period and appeared to demonstrate two successive kinetic steps of inactivation.

As in the case of *E. coli*, the first-order biphasic model (with $R^2 > 0.99$ and RMSE < 0.05) produced better fits than the Weibull model (with $R^2 > 0.97$ and RMSE < 0.08). Therefore, the parameter estimates of the biphasic model were used for comparisons. The parameter estimates and measures of goodness-of-fit are summarised in Table 4.21. According to RMSE and R^2 values, the biphasic model accurately describes the inactivation curves of *S. aureus* under all treatment conditions.

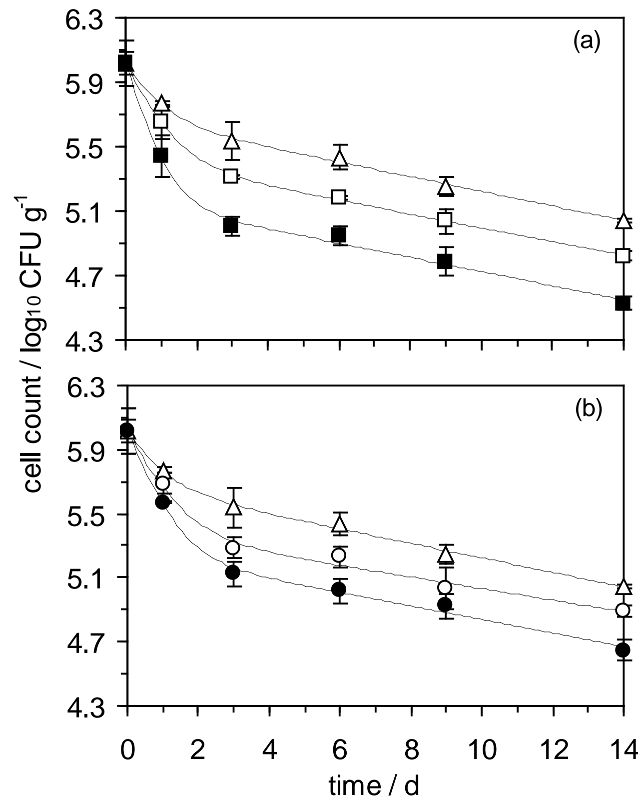

Figure 4.27 Inactivation of S. aureus on Cheddar cheese wrapped with: (a) thymol and (b) carvacrol films. Observed growth in the presence of: (Δ) control film; (□) TL1 film; (■) TL2 film; (○) CL1 film and (•) CL2 film. (—) Curve fitting of first-order biphasic model.

Table 4.21 Parameters estimates of first-order biphasic model for the inactivation of S. aureus on Cheddar cheese wrapped in AM films.

AM Film	Estimated parameters			RMSE	R^2
	P	s_1 / d^{-1}	s_2 / d^{-1}		
Control	0.5452^a	1.31^a	0.10^a	0.0285	0.9974
TL1	0.7460^b	1.34^a	0.10^a	0.0161	0.9994
TL2	0.8607^e	1.87^c	0.10^a	0.0483	0.9967
CL1	0.7682^c	1.32^a	0.08^a	0.0573	0.9926
CL2	0.8285^d	1.49^b	0.10^a	0.0451	0.9967

Note: Means in the same column followed by different letters are significantly different in Tukey's test (i.e. $p < 0.05$).

Similar to the inactivation of *E. coli*, the values of *P* and s_1 obtained in the presence of *S. aureus* are high in cheeses packaged with AM films in comparison to the control film and the values increase with an increasing concentration of AM agent in the films. This suggests that the biphasic survival of *S. aureus* is also a cause of change in AM concentration with time rather than the existence of sub-populations with different sensitivities. The s_2 values did not vary with the film treatment and therefore confirm the above hypothesis. This behaviour also suggests a loss of AM activity of films with time.

To compare the differences between *E. coli* and *S. aureus* on the AM effect, the relative inactivation rates ($s_{1(r)}$) were used (see Equation 4.2).

$$s_{1(r)} = \frac{(s_{1(AMfilm)} - s_{1(Control)})}{s_{1(Control)}} \qquad (4.2)$$

where, $s_{1(control)}$ is the inactivation rate of the more sensitive population by the control film and $s_{1(AMfilm)}$ is the inactivation rate of the more sensitive population caused by the AM films. The $s_{1(r)}$ values plotted against the AM concentration are presented in Figure 4.28. Except for relative rate constants of thymol values against *E. coli* (R^2 = 0.79), a linear relationship with relatively good correlation ($R^2 > 0.9$) was observed between the relative inactivation rates and AM concentrations.

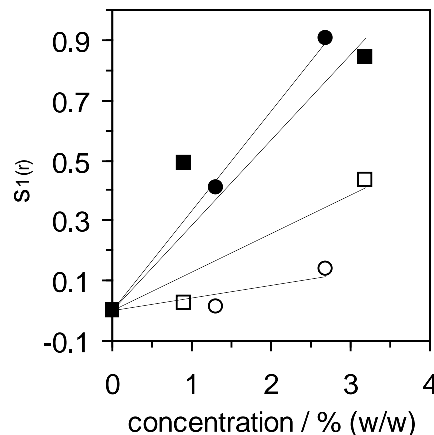

Figure 4.28 Plot of relative inactivation rate constants ($s_{1(r)}$) of S. aureus and E. coli versus AM concentration. (□) thymol films against S. aureus; (■) thymol films against E. coli; (○) carvacrol films against S. aureus and (•) carvacrol films against E. coli.

According to the relative rate constants of the more sensitive population, both thymol and carvacrol films performed better in the inactivation of *E. coli* than that of *S. aureus*. The superior resistance of *S. aureus* was also confirmed by the inactivation rate of the more resistant population (s_2) that was not significantly affected by the AM films compared to the control.

Gram-negative bacteria are generally more resistant to AM compounds than Gram-positive bacteria (Cosentino *et al.* 1999; Dorman and Deans 2000). In contrast to this hypothesis and the results from *in vitro* studies, in the present study *S. aureus* on Cheddar cheese was found to be more resistant to AM films than *E. coli*, a Gram-negative microorganism. In previous studies a higher sensitivity of *E. coli* than that of *S. aureus* to the effect of high pressure inactivation on Cheddar cheese was found (O'Reilly *et al.* 2000) and to the AM activity of a bacteriosin in cheese (Rodriguez *et al.* 2005). In cheese, inhibitory factors other than AM films and their interaction effects occur. Under such conditions, Gram-negative bacteria that are characterized by a thin peptidoglycan cell wall are expected to be more vulnerable to AM compounds than Gram-positive bacteria, which have a thicker peptidoglycan cell wall (Virto *et al.* 2006).

The evolution and sensitivity of bacteria observed on Cheddar cheese samples wrapped in AM films was different from those in the broth (see Section 4.3.3). In broth, an increase in the bacterial population occurred (even in the presence of AM films) compared to the decreased populations on cheese. It has been reported that the microbial resistance depends on the conditions applied (Buzrul and Alpas 2007). For instance, *L. innocua* which was resistant to AM films containing linalool on agar media showed a significant sensitivity to the same AM films on Cheddar cheese (Rupika *et al.* 2006; Suppakul 2004). The presence of other inhibitory factors (e.g. low pH and a_w levels, presence of salt and inherent AM compounds like bacteriocins) on cheese may have accounted for the differences in microbial survival and growth. Increased biocidal action of thymol and carvacrol has been reported at low pH levels (Paster *et al.* 1990; Ultee *et al.* 1998), low a_w (Guynot *et al.* 2003) and in combination with bacteriocins (Molinos *et al.* 2008; Pol and Smid 1999; Yamazaki *et al.* 2004). The different

temperatures of incubation and storage may also have contributed to the differences in the microbial evolution in the two systems (Smith-Palmer *et al.* 2001).

4.6.2 Efficacy of AM Film on Cheese under Storage

Cheddar cheeses packaged in AM films were stored under refrigeration (*ca.* 4°C) and subjected to microbial, physical and chemical quality analyses in order to understand the effect of AM films under actual storage conditions.

Effect on Microbiological Quality

The effect of AM films on microbiological quality was evaluated by examining the evolution of the microbial groups: TAB, LB, EC/C and YM. The change of TAB counts on Cheddar cheese over the storage period is presented in Figure 4.29. In all cheeses, after a rapid decrease in TAB numbers during first 5 days, the TAB counts increased slightly and then remained almost constant. The reduced oxygen atmosphere created by the packages may have contributed to the initial decrease in the TAB counts. The almost constant numbers may probably be due to the equilibrated gaseous atmosphere as result of some oxygen permeation through the packaging film. However, compared to the TAB counts on cheese packaged in the control film, significant log reductions in TAB counts were observed for cheese samples packaged in the AM films.

The duration of the suppressed growth of TAB by AM films varies with the type and concentration of AM agent as expected. The CL1 film was able to reduce the TAB numbers up to 16 days while the TL1 and CL2 films lasted for up to 22 and 28 days, respectively. Only the TL2 film showed a significant deviation of the TAB count from that of the control film during the 36 days storage period. However, the inhibitory effect of the AM films (compared to the control) decreases as the storage period progressed. A similar study on Cheddar cheese wrapped with linalool or methylchavicol films reported a suppressed TAB for a period of 9-15 days (Suppakul 2004). Films containing sorbic acid applied on Cheddar cheese reported a lower mesophillic aerobic bacterial (MAB) count (compared to the control) of up to 35 days. The inhibitory effect on MAB,

however, did not differ significantly between the films containing different concentrations (1.5% and 3.0% (w/w)) of sorbic acid (Limjaroen *et al.* 2005).

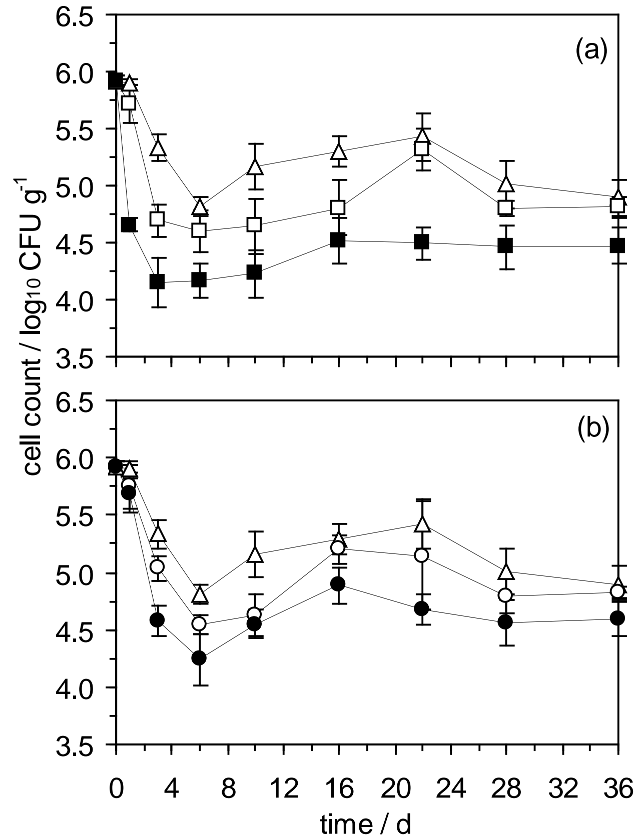

Figure 4.29 Evolution of total aerobic bacteria (TAB) on Cheddar cheese packaged in: (a) thymol and (b) carvacrol films. TAB counts in the presence of: (Δ) control film; (□) TL1 film; (■) TL2 film; (○) CL1 film and (•) CL2 film.

No noticeable growth of EC/C was observed during the storage period. The existence of EC/C in dairy products is indicative of poor sanitary conditions (Marshall 1992). Thus, the EC/C tests were conducted to detect AM activity of films with the possible bacterial recontamination of cheese samples during sample preparation and/or handling. In the absence of EC/C up to detectable levels, it is impossible to conclude about the degree of AM activity of films on EC/C growth. Similar to the present findings, Suppakul (2004) has not observed significant differences of EC/C counts between cheeses packaged in AM and control films while the EC/C counts of all cheeses were less than 0.5 CFU g^{-1}.

Growth of YM, which are normally present as contaminants in cheeses (Marshall 1992), was detected after 22 days of storage. The evolution of YM on Cheddar cheese wrapped in different films is given in Figure 4.30. The growth of YM was first observed after 22 days in cheese packaged with control films. Cheese samples packaged in CL1, CL2 and TL1 films on day 28 and cheese samples packaged in TL2 films on day 36 were examined for the presence of YM. The growth of YM in cheeses packaged in the AM films was significantly different from that in the control film and depended on the concentration of the AM agent in the film. An absence of visual growth of fungi up to about 10 days was reported for Cheddar cheese wrapped in LDPE film containing 1 g kg^{-1} Imazalil (Weng and Hotchkiss 1992). In another study, the mould growth was visible a week later in the cheese samples wrapped with the control film while no visual fungal growth was observed on Cheddar cheese for up to 2 months with films containing 0.34% (w/w) linalool or methylchavicol (Suppakul 2004).

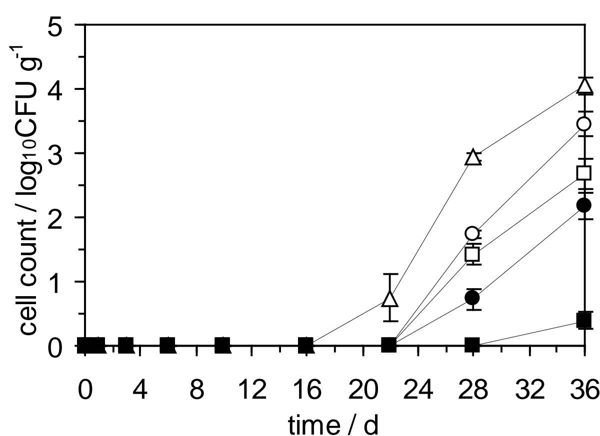

Figure 4.30 Evolution of yeasts and moulds (YM) on Cheddar cheese packaged in AM films. YM counts in the presence of: (Δ) control film; (□) TL1 film; (■) TL2 film; (○) CL1 film and (•) CL2 film.

The effect of AM films on a functional microbial group, LB, on Cheddar cheese was also assessed. The evolution of LB during the storage period is presented in Figure 4.31. The LB count on cheese packaged in films containing lower concentrations of thymol (TL1) or carvacrol (CL1) showed a significant reduction that continued up to about 10 days of storage. However, cheese packaged in TL2 or CL2 films was found to have significantly lower LB counts throughout the storage period.

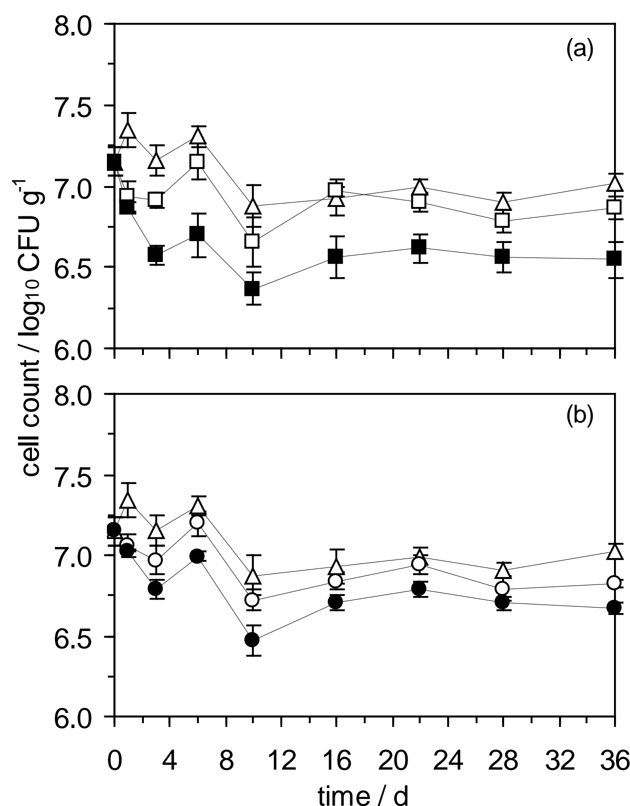

Figure 4.31 Evolution of lactic acid bacteria (LB) on Cheddar cheese packaged in: (a) thymol and (b) carvacrol films. LB counts in the presence of: (Δ) control film; (□) TL1 film; (■) TL2 film; (○) CL1 film and (•) CL2 film.

In agreement with the present findings, LB are reported to be relatively resistant to AM activity of these compounds (Bevilacqua *et al.* 2007; Dorman and Deans 2000). Conversely, inhibition of growth and lactic acid production of *Lactobacillus plantarum* and *Pediococcus cerevisiae* by oregano, which contains thymol and carvacrol in its EO fraction, have been reported elsewhere (Zaika and Kissinger 1981). Typically, species of *Lactobacillus* and *Pediococcus* results in improved flavour intensity, increased aroma and accelerated ripening (Fox *et al.* 2004). Thus, the resultant inhibition of LB by such AM films may have a negative effect on the quality of Cheddar cheese.

Effect on pH

The change in pH of the cheese samples during storage is presented in Figure 4.32. At the beginning of the storage period, no significant difference in pH between the packaging treatments is observed. Moreover, the recorded pH values of the cheeses are

consistent with normal Cheddar, with a pH range of 5.0 - 5.5 (Fox *et al.* 2004). This confirmed that pH did not become a limiting factor for the studied bacterial populations, TAB or LB, during the cheese storage suggesting that the above differences in the microbial growth among the samples can be ascribed to the differences in AM films only.

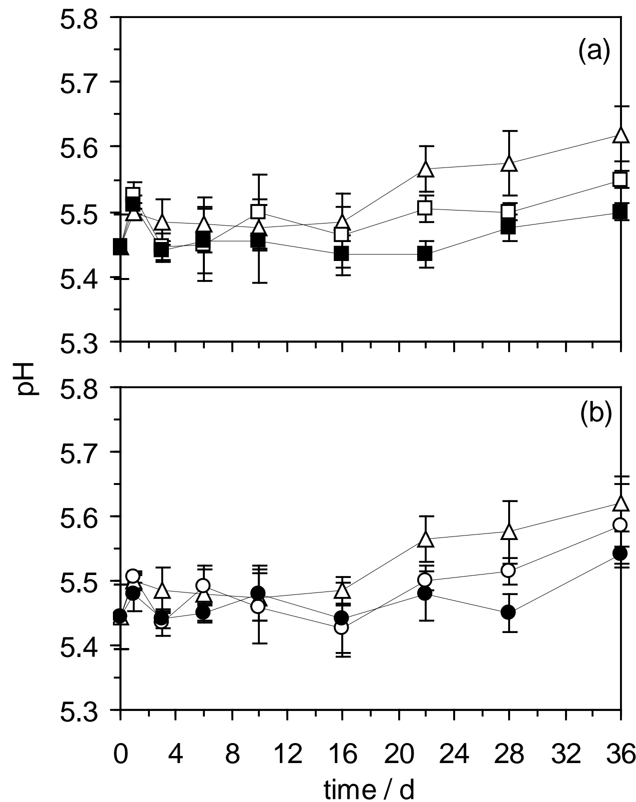

Figure 4.32 Change in pH of Cheddar cheese packaged in: (a) thymol and (b) carvacrol films during storage at 4°C. (Δ) control film; (□) TL1 film; (■) TL2 film; (○) CL1 film and (•) CL2 film.

At the end of the storage period, however, the pH of cheeses packaged in the AM films is significantly lower compared to that of the control film. The lowered pH may be attributable to a number of factors, including a metabolic shift of microbes naturally present in Cheddar cheese that may have been influenced by the AM films (Skandamis and Nychas 2001) and to the suppression of LB (Rynne *et al.* 2008). It has been reported that the ability of LB to produce acid is reduced upon its inactivation by OEO (Chouliara *et al.* 2007; Zaika *et al.* 1983). Therefore, the observed pH differences are more likely caused by the suppression of LB by the AM films.

4.6.3 Effect of AM Films on Sensory Quality of Cheddar Cheese

A sensory test was conducted to determine if the AM packaging causes a difference in odour and overall acceptability of Cheddar cheese during storage. It was aimed to identify whether a sensory difference, if any, has resulted from the AM packaging treatments and how panellists view the difference.

Detectable Odour Threshold of Thymol and Carvacrol

The odour thresholds of AM agents were determined in order to assess the possibility of detecting their odour at low concentrations by the panellists participating in the present study. The DOT and PT of thymol and carvacrol are summarised in Table 4.22.

Table 4.22 Odour thresholds of thymol and carvacrol.

Agent	Odour Threshold (ppm)		
	Observed		Reported
	DOT*	PT#	
Thymol	0.01	0.21	0.08 – 0.79 (Burdock 2005) 30.97 (Bitar *et al.* 2008)
Carvacrol	1.00	2.62	2.29 (Burdock 2005) 124.00 (Bitar *et al.* 2008)

Notes:* DOT, Detection odour threshold
PT, Probable threshold (the concentration at which a sample solution could be distinguished from the blank sample by 50% of the panellists).

The DOTs of thymol and carvacrol reported in this study are different from the odour threshold values published in the literature (see Table 4.22). Threshold values suffer from lack of reproducibility due to differences in methodology and experience of panellists (Ahmed *et al.* 1978). However, the PT values were in the range of the values for the odour thresholds of thymol and carvacrol mentioned by Burdock (2005). As indicated by the relative odour intensities, thymol is more readily perceived by the panellists than carvacrol. Odour detection thresholds are shaped by the molecular architecture of chemical stimuli. Significant differences in the magnitudes of the thresholds have been reported for isomers (Bitar *et al.* 2008). This demonstrates also the

ability of the panellists (participating in the present study) to differentiate a very low level of odour of AM agents.

Selection of Odour Descriptors

From the presented odour terms (see Appendix G), the terms "medicinal", "herb-like" and "minty", which scored 26%, 23% and 30% respectively, were selected to describe the odour of carvacrol and thymol. All other terms scored less than 10% for each in describing the odour of AM agents. The odour of Cheddar cheese was best described by the terms "creamy" and "buttery" having scores of 33% and 36%, respectively.

Triangle Test

Sensory analysis based on a series of triangle tests was conducted to differentiate between the cheeses packaged in different AM films. The results from these tests are summarised in Table 4.23. Despite the ability of the panellists to identify the odour of thymol and carvacrol at very low concentrations, there was no significant odour difference between cheeses packaged in L1 (TL1 or CL1) films and the control during the storage period. Moreover, no significant odour difference was perceived between cheeses packaged in L1 (TL1 or CL1) and L2 (TL2 or CL2) films, even in the first week.

Table 4.23 Difference between Cheddar cheeses packaged in AM films (triangle test).

Comparisons	**No. of correct judgements**				
	Week 1 ($n^{\#}$ = 16)	**Week 2** (n = 17)	**Week 3** (n = 12)	**Week 4** (n = 16)	**Week 5** (n = 15)
Control vs. CL1	8	4	3	5	8
Control vs. CL2	9^{*}	10^{*}	6	6	8
Control vs. TL1	8	8	4	7	7
Control vs. TL2	10^{*}	12^{**}	8^{*}	12^{**}	9^{*}
CL1 vs. CL2	4	8	7	2	3
TL1 vs. TL2	6	7	4	4	6

Note: # n = No. of panellists
* Comparisons are significant at $p < 0.05$ (95% confidence level).
** Comparisons are significant at $p < 0.01$ (99% confidence level).

Based on odour, however, Cheddar cheeses wrapped in CL2 films were significantly differentiated from that of the control film during the first two weeks of storage. Furthermore, significantly different odour differences were detected throughout the storage period between the cheeses wrapped in the TL2 film and cheeses wrapped in the control. The odour differences perceived by panellists between the cheeses packaged in the control film and cheeses packaged in L2-AM films suggests that the odour of the AM agent imparted to the cheeses is detectable only at higher concentrations. Although the concentrations of carvacrol and thymol in L2 films are not very different, based on odour, the panellists could differentiate between the cheeses packaged in TL2 films that are stronger for a much longer period than that of CL2 films. The much lower DOT of thymol may explain this difference.

Descriptive Sensory Analysis

A sensory evaluation based on quantitative descriptive analysis was carried out in order to understand how the panellists perceive the odour differences caused by the AM films. For simplicity, the scores for odour attributes: "creamy" and "buttery" were collectively taken as "Cheddar-like" odour while the scores for "minty", "herb-like" and "medicinal" were collectively taken as a "spicy" odour. The average scores for "Cheddar-like" and "spicy" odours during storage are presented in Table 4.24. These data suggest that the panellists noted significant differences in odour attributes of cheeses for different packaging films during storage.

Although, the panellists could not differentiate between the cheeses packaged in the control and L1 films during the triangle test based on odour, the differences between these packaging films became evident during the descriptive analysis. Except for the CL1 film in weeks 3 and 4, in all other cases the cheese samples packaged in AM films scored significantly lower values for "Cheddar-like" odour compared to the control. Although no consistency in the significant differences for "Cheddar-like" odour was observed, in most cases the lowest scores for "Cheddar-like" odour were associated with either the cheeses packaged in TL2 or CL2 films. The panellists' rankings for spicy odour intensity matched the AM concentration in the films at the 95% confidence level. Cheese packaged in TL2 film had a significantly higher score for spicy odour

throughout the storage period compared with all other samples. This was followed by the cheeses packaged in CL2 and TL1 films.

Table 4.24 Mean scores for odour attributes of cheeses packaged in AM films during storage.

AM film/Odour attributes	Average* score				
	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**
Cheddary odour #					
Control	4.88[d]	5.03[d]	4.25[c]	5.06[c]	4.93[e]
TL1	2.78[a]	3.47[b]	3.75[b]	4.06[b]	3.93[d]
TL2	3.16[b]	3.00[a]	3.33[a]	3.72[a]	3.73[c]
CL1	3.97[c]	3.91[c]	4.38[c]	5.00[c]	3.50[b]
CL2	3.03[b]	3.00[a]	3.83[b]	4.13[b]	3.27[a]
Spicy odours ≠					
Control	1.00[a]	0.60[a]	2.03[c]	1.33[b]	1.47[a]
TL1	2.48[cd]	1.88[c]	1.78[b]	2.25[c]	2.40[b]
TL2	2.54[d]	2.71[d]	2.58[d]	2.54[d]	2.84[d]
CL1	1.56[b]	1.44[b]	1.19[a]	0.92[a]	2.31[b]
CL2	2.46[c]	1.75[c]	2.06[c]	2.19[c]	2.62[c]

Notes:* Values in the same column followed by different letters are significantly different (i.e. $p < 0.05$). 1 = weak, 10 = strong.
Sum of the average scores of the odour terms "creamy" and "buttery'"
≠ Sum of the average scores of the odour terms "medicinal", "herb-like" and "minty".

Despite the highest preference in describing the "Cheddar-like" odour, in all cases, an average intensity of *ca.* < 5 was scored for "creamy" and "buttery" attributes. Hence, the Cheddar cheese samples used in the present study may resemble aged-Cheddar (Caspia *et al.* 2006) and the panellists may have chosen these two odour terms to describe the cheeses due to their familiarity with these terms. Cheese samples packaged in the control film containing no AM agent were also scored for spicy odour, although,

the scores were significantly low in weeks 1, 2 and 5. Due to the unavailability of a trained panel, panellists were trained for specific attributes over two to three training sessions to participate in the present study. Therefore, they may not have been able to distinguish the specific aroma attributes such as "fruity," and "pungent", which are noticeably higher in aged-Cheddar cheese (Caspia *et al.* 2006; Drake *et al.* 2005), from that of "spicy" odours of the AM agent.

The mean scores for the overall acceptance of Cheddar cheese samples packaged in the AM films during storage are summarised in Table 4.25. In the first week, the degree of acceptance of the cheese samples matched inversely the AM concentration in the films having lowest scores for L2 films and the highest score of acceptance for the control. At week 3, except for cheeses packaged in CL1, the acceptance scores for cheese samples packaged in the control film were less than those of cheeses stored in AM films.

Table 4.25 Mean acceptance of Cheddar cheese packaged in AM films during storage.

AM film	**Mean* overall acceptance**				
	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**
Control	6.94^{d}	5.13^{d}	4.42^{b}	5.88^{c}	4.47^{c}
TL1	5.69^{b}	3.31^{a}	4.83^{c}	6.31^{d}	4.80^{d}
TL2	5.25^{a}	4.81^{c}	4.58^{b}	5.06^{a}	4.13^{b}
CL1	6.13^{c}	4.56^{b}	4.08^{a}	5.63^{b}	4.53^{c}
CL2	5.31^{a}	4.44^{b}	4.83^{c}	5.81^{c}	3.87^{a}

Notes:* Means in the same column followed by different letters are significantly different (i.e. $p < 0.05$). 1 = dislike extremely, 5 = neither like nor dislike, 10 = like extremely.

The mixed results obtained for acceptance, specifically after week 3, and score intensities closer to "neither like nor dislike" (in most cases), indicate that the "spicy" odour is not necessarily perceived by the panellists as off-odour or taint in Cheddar cheese. This is confirmed by the results from the correlation analysis, given in Table 4.26, which indicates the relationship between sensory attributes. Although the relationship between "acceptability" and "spicy" odour is slightly negative, the correlation is not significant. Furthermore, there is no significant correlation between

acceptability and “Cheddar-like” odour. In contrast, thyme oil as low as 0.75% (w/w) in an AM coating prepared from soy or whey protein isolates, reduced the acceptability scores for pre-cooked shrimp (Ouattara *et al.* 2001). Application of chitosan films enriched with OEO, which predominantly contained carvacrol, had a negative impact on overall liking of bologna when the concentration increased up to 90 mg kg^{-1} (Chi *et al.* 2006). Thymol, when added to carrot broth, had a pungent odour and left a bad aftertaste which minimised the degree of acceptance or liking of the product while all samples containing carvacrol were unacceptable to the panellists (Valero and Giner 2006).

Table 4.26 Correlation between mean scores of odour attributes and the mean scores of acceptability of Cheddar cheese packaged in AM films.

AM film	Correlation between mean scores	
	Acceptability	**Cheddary odour**
Cheddary odour	0.325 0.133#	
Spicy odour	-0.255 0.219#	-0.781 0.000#*

Notes: # Probability (p) values.
* The correlation between mean scores is significant at p < 0.01 (99% confidence level).

In accordance with Table 4.26, there is a negative and significant correlation between “Cheddar-like” and “spicy” odour. The released odours of the AM agent may have masked the natural odours of Cheddar cheese. However, according to the acceptability scores, Cheddar cheeses with more complex odours may have an advantage over products with bland flavours in the application of AM films (Caspia *et al.* 2006; McEwan *et al.* 1989). It has been reported that thymol and carvacrol at levels up to 1.92% (w/v), evoked sensations described as “herbal”, “sweet”, “warm”, “penetrating”, and “tar-like” at supra-threshold concentrations jeopardizing the bland flavour in edible oils (Bitar *et al.* 2008).

4.6.4 Prediction of Storage Stability

On the basis of the data obtained for Cheddar cheese packaged in AM films under actual storage conditions, a model to predict the effect of AM films on the storage

stability was developed. Films were scored for the enhancement of storage stability based on two main criteria: (i) the microbiological quality and (iii) the sensory quality. In each case, the results from the control film were chosen as the reference.

Microbiological Quality

The reduction in TAB and YM growth by AM films were considered to be positive effects while that of LB considered to be a negative effect on microbiological quality or functionality of Cheddar cheese. Accordingly, a score of (+1) was given for each 0.1 log reduction (*LR*) in TAB and YM, after 6 and 36 days of storage, respectively, while that of LB after 36 days of storage was given a score of (-1). Similarly, a score of (+0.1) was given for each day (*D*) with significantly low counts of TAB while that of LB was given a (-0.1). For each day that delayed the onset of YM growth was given a (+0.5). Different scores were given for each criterion depending on their relative importance on the microbiological quality. For instance, the TAB count has little value in ascertaining the sanitary quality or contaminants of cultured dairy products like cheese. Detection of specific pathogens or EC/C counts and YM counts may be a reliable index of microbial quality. It is quite common that the spoilage of cheeses is due mainly to YM but not to TAB (Marshall 1992). Thus, higher scores were allocated for YM values. The sum of scores of *D* and *LR* was given as the microbiological quality index (I_{mq}) (see Table 4.27).

Table 4.27 Effect of AM films on the microbiological quality index (I_{mq}) of Cheddar cheese.

AM Film	Score for each criteria						I_{mq}
	TAB		YM		LAB		
	LR	***D***	***LR***	***D***	***LR***	***D***	
TL1	0.21	2.2	0.59	3	-0.16	-1.6	**4.24**
TL2	0.64	3.6	1.86	6	-0.32	-3.6	**8.18**
CL1	0.26	1.6	1.37	3	-0.12	-1.0	**5.11**
CL2	0.57	3.6	3.65	3	-0.44	-3.6	**6.78**

Despite the more negative effect on LBs, TL2 film showed the highest I_{mq} which was followed by CL2 film. Although the TL1 film showed higher inhibition of TAB and YM, due to its higher inhibition of functional bacteria, the overall microbiological quality of Cheddar cheese, indicated by the I_{mq} value, has been reduced more than that observed in the case of CL1 film.

Sensorial Quality

For the sensory quality analysis, the averages of scores from descriptive analysis during week 1 to 5 were used. The differences between the average scores for "Cheddar-like" and "spicy" odour and acceptance of AM films from that of the control film were calculated. None of the cheeses samples packaged in AM films were scored a value of zero, which indicates a completely unacceptable verdict for sensory quality. Thus, the positive differences of acceptance and "Cheddar-like" odour were taken as positive values and negative differences were taken as negative values. Conversely, the positive differences of "spicy" odour were taken as negative values and vice versa. The sum total of differences was taken to be the sensorial quality index (I_{sq}). The resultant differences of scores of sensorial attributes and I_{sq} for AM films are presented in Table 4.28.

Table 4.28 Effect of AM films on the sensorial quality index I_{sq} of Cheddar cheese.

AM Film	Score for each criteria			I_{sq}
	Cheddary odour	Spicy odour	Acceptance	
TL1	-1.23	-0.87	-0.38	**-2.48**
TL2	-1.44	-1.36	-0.60	**-3.40**
CL1	-0.68	-0.20	-0.38	**-1.26**
CL2	-1.38	-0.93	-0.51	**-2.82**

The lowest I_{sq} values were obtained for the cheese samples packaged in AM films with highest AM concentrations, i.e. for L2 films. Although, the concentration of thymol in

the TL1 film is less than the agent concentration of the CL1 film, the I_{sq} value of the TL1 film was *ca.* two times lower than that of the CL1 film. Thus, AM films containing carvacrol seems to have the advantage of higher DOT and PT of carvacrol in the application on Cheddar cheese.

Storage Stability Index

The summation of the values of I_{mq} and I_{sq} were taken as the storage stability index (I_{ss}). The values of I_{ss} are presented in Table 4.29.

Table 4.29 Effect of AM films on the storage stability index (I_{ss}) of Cheddar cheese.

AM Film	I_{mq}	I_{sq}	I_{ss}
TL1	4.24	-2.48	**1.76**
TL2	8.18	-3.40	**4.78**
CL1	5.11	-1.26	**3.85**
CL2	6.78	-2.82	**3.96**

The highest I_{ss} was achieved for Cheddar cheeses packaged in the TL2 film although it recorded the lowest I_{sq}. However, cheeses packaged in the TL1 film achieved the lowest I_{ss} due to the more negative effects of the film on I_{sq} and I_{mq}. No significant difference for I_{ss} was detected between the cheese samples packaged in CL1 and CL2 films. According to I_{ss}, the storage stability extension of Cheddar cheese by AM films are in the order of TL2 > CL2≈ CL1 > TL1. Although the storage stability indexes obtained in the present study are empirical values, such a model may give a reasonable prediction of effect of AM films on the storage stability of actual food products.

Chapter 5. Conclusions and Recommendations

Antimicrobial food packaging films containing the natural AM agents thymol and carvacrol were successfully developed by incorporating these agents into an LDPE polymeric substrate. Different film formulations were prepared to investigate the potential of the films to control the release of the AM agents. Films containing various concentrations of AM agents were then tested for AM activity both on laboratory media and on food substrates. The physical and mechanical properties and AM retention during storage of the films were evaluated in order to assess the effect that the addition of AM agent has on the ultimate properties of the film.

5.1 Conclusions

5.1.1 Effect of Film Processing Method and Additives

Several series of LDPE films containing thymol or carvacrol were successfully prepared using both compression moulding and extrusion film blowing. A laboratory-scale compression moulding press was used to prepare AM films to investigate the following effects: AM agent concentration; compounding an additive polymer (PEG and/or EVA) with LDPE; and mixtures of AM agents in the films. Antimicrobial films prepared by pilot-scale extrusion film blowing were used for further evaluation of AM activity and films characterisation. The retention of AM agents in the extruded films was significantly higher than the retention of thymol and carvacrol in the moulded films.

The addition of EVA in the LDPE matrix showed a significant effect on the retention of AM agents during thermal processing. Moreover, with the increasing concentration of EVA in the formulation, the retention of AM agents increased with a more pronounced effect on the retention of thymol than that of carvacrol. In the absence of EVA, PEG did not improve the retention of the AM agents in the polymer matrix. The results of AM activity for films containing various combinations of EVA and PEG, however, suggest

that PEG acts in binding the AM agent resulting in slowing the release of AM agent in the short-term.

5.1.2 AM Activity *In Vitro*

Optimum AM Concentration

Compression moulded films containing thymol and carvacrol at varying concentrations showed AM activity against bacteria *via* an agar disc diffusion assay. The relationship between the AM activity and the concentration in the films using three principal phases were obtained using a modified Gompertz function. The concentrations between the minimum inhibitory and maximum effective concentrations were identified as the optimum concentrations for AM activity. According to the inhibitory concentrations, Gram positive bacteria were found to be more sensitive to the AM activity of films containing thymol or carvacrol in comparison with Gram negative bacteria. Films containing thymol showed considerably higher antibacterial activity on agar media than did carvacrol. Accurate determination of the optimum AM agent concentration can enable a balance between the sensory acceptability, safety and AM efficacy.

AM Activity of Extruded Films

Inhibition of bacterial growth on solid media by AM films was confined to the area underneath the film. This suggests the need to have direct contact between the food surface and the AM films for effective control of microorganisms. The effect of AM films on the bacterial inhibition in liquid media was successfully modelled by both the Gompertz and the Baranyi models. Significantly different quantitative changes in growth profiles by AM films compared to that of the control film suggests three main effects of AM films on bacterial growth in liquid media: extension in lag period; reduction in growth rate; and/or reduction in the maximum population density. These effects, however, depend on the type of bacteria, initial bacterial cell concentration, type of AM agent and the concentration of AM agent. The thymol films inhibited bacterial growth more effectively than the carvacrol films. The difference between the AM films was most pronounced at higher AM concentrations and lower inoculum level of bacteria. Although the results on AM activity from *in vitro* studies are highly dependent

on the experimental conditions, their application allows a broad comparison of different film preparations. Ideally, all films should be subjected to a standard protocol involving a non-food medium to reduce the complexity of the assays.

5.1.3 Effect of AM Combinations

According to the conventional models, FIC and effect additivity, the films containing various combinations of thymol and carvacrol show mainly an antagonistic effect on inhibition of bacteria on agar media. Interaction effects of films containing AM combinations moved towards a positive effect with an increasing concentration of thymol in the combination and with increasing the incubation time. Interpretation of these results shows that it is difficult to anticipate the effects or to explain observed activity when considering binary mixtures of the natural antimicrobials used in this study. Furthermore, the conclusions on interaction effects may vary depending on the model used to interpret the results. Although the conventional models may be useful for initial screening, more robust and informative models may be required to understand the interaction effects of AM combinations in films.

5.1.4 Film Characterisation and Migration of AM agents

Tensile and Thermal Properties

Incorporation of thymol or carvacrol into the LDPE/EVA films did not significantly change either the MD or TD tensile properties of the film. In addition, there was no significant difference in the thermal properties of the AM films in comparison with the control film.

Migration of AM Agents from Films to Atmosphere

The retention of AM agent in the extruded films was examined under three different storage conditions: exposed to air at room temperature; covered in foil and stored at room temperature; and covered in foil and stored in a refrigerator. During short-term storage, a significantly higher retention of AM agents was obtained when the films were

covered in foil and covered in foil and refrigerated compared to that of open air storage. The AM retention, however, did not significantly increase with the reduced temperature of refrigeration during storage when the films were covered in foil. The effect of foil on the retention of carvacrol was higher than that of thymol. Under long-term storage, however, films covered in foil and refrigerated had a significantly higher retention of AM agents than the films covered in foil and stored at room temperature.

The release of the AM agents from film to atmosphere can be satisfactorily described by diffusion and first-order kinetics equations. From the kinetics analyses, it was revealed that the release of AM agents to atmosphere occurs very rapidly with the release of carvacrol from AM films being faster than that of thymol. Moreover, films containing AM agents at higher initial concentrations released the agent at a faster rate than films containing a lower initial concentration. Thus, carvacrol with higher volatility and at higher initial concentrations can be expected to volatilize rapidly into the package headspace and reach the food surface more rapidly than thymol.

Release of AM Agents into food simulants

The release of the AM agents from films into food simulants can be adequately and consistently described by short-term and long-term migration equations. Moreover, adequate fits to first-order kinetics were obtained. It is suggested that the release of AM agents from films to simulants is more complex and needs more detailed approaches towards the analysis. It can be inferred from the results that the release of the AM agents into isooctane was consistently high and was followed by release into 95% (v/v) ethanol/water whereas the release into 10% (v/v) ethanol/water was considerably slower. The release of carvacrol was found to be higher than that of thymol at any given condition. Moreover, an increase in the rate of release of AM agent with an increase in temperature was observed. The temperature dependency of the kinetic parameters was well described by the Arrhenius equation. A higher energy barrier for the release of AM agents was observed in aqueous solvents. Diffusion of AM agents from films containing thymol and carvacrol can also be modeled successfully using an extension of the model derived for the case of an idealized system involving an infinite "sheet" of polymer immersed in an infinite volume of food simulant. The extension technique involves

shifting the boundary condition separating the short-term and long-term diffusion data to 0.5 instead of the usual value of 0.6. Such a shift in the boundary condition can be theoretically made with little consequence to the numerical accuracy of any subsequent diffusion analysis and in some cases can improve the fit of the data.

5.1.5 Application of AM Films on Cheddar Cheese

Challenge Test

In the challenge test, the inactivation of *E. coli* and *S. aureus* inoculated on Cheddar cheese by AM films were successfully modeled by the first-order biphasic model. Compared to the control films, AM films showed a significant inhibitory action against both bacteria depending on the type of AM agent and its concentration. The inactivation kinetics nevertheless suggests that the biphasic nature of inactivation is a cause of effective AM concentration that changes over the period of microbial exposure. Furthermore, the challenge tests were found to be much more suitable than the *in vitro* experiments in the prediction of the AM activity of films for food applications.

Effect on Quality of Cheddar Cheese

The AM films had an effect on the microbial and physio-chemical attributes of Cheddar cheese under actual storage conditions. The AM films had both positive and negative effects on the microbiological quality by delaying the onset of YM growth, reducing the TAB counts and affecting the functional bacteria of Cheddar cheese. In the sensory studies, the presence of thymol was more readily perceived by the panelists than that of carvacrol and the panellists had the ability to identify the odour of AM agents at very low concentrations. Sensory analysis based on a triangle test was able to differentiate between the cheeses packaged in AM films containing higher concentrations of AM agents from those packaged in control films. The addition of higher concentrations of AM agents clearly imparted a noticeable odour to Cheddar cheese during storage. Significant differences in the odour attributes ‘Cheddary’ and ‘spicy’ were perceived on cheeses packaged in different AM films. Thymol had a more pronounced effect than carvacrol on the odour of Cheddar cheese. The acceptance of cheeses packaged in

control film was significantly higher than the cheeses packaged in AM films during the first week. However, after a few weeks of storage, the difference for acceptance became insignificant. Moreover, the intensity of acceptance scores revealed that the spicy odour detected on Cheddar cheeses packaged in AM films was not necessarily perceived as off-odour.

Effect on Shelf life of Cheddar Cheese

In the present study, among all of the quality parameters that may change during the storage of Cheddar cheese, the microbial and sensorial qualities in particular were considered the most representative in building the shelf-life model. The microbial quality of Cheddar cheese was affected by the type of AM agent and was proportional to the concentration of AM agent in the films. A better microbial quality was observed for cheeses packaged in thymol films. Regardless of the higher negative effect on functional bacteria, films with higher AM concentrations had a more pronounced effect on microbiological quality. The sensory quality progressively decreased with an increasing concentration of AM agent in the films while thymol films had a more negative effect. The shelf-life extension of Cheddar cheese by AM films was affected by the type and concentration of the AM agent. However, the overall quality did not necessarily increase with an increasing concentration of AM agent in the films. According to these results, the optimum AM concentration in films should be established with reference to the quality, safety and functionality of the product in its application.

5.2 Recommendations for Further Research

5.2.1 Blending Improvements

The production of the AM films required a double extrusion process involving compounding of LDPE pellets with EVA powder containing AM agents followed by extrusion film blowing. Although the retention of thymol and carvacrol after extrusion was reasonably high, retention of these AM agents in extruded films would be further enhanced by avoiding a double extrusion process. This may be achieved by using a

powdered form of both LDPE and EVA. The use of powdered forms of both polymers may also improve the blend morphology. Further morphological and rheological studies of AM/EVA/LDPE blends could be conducted to optimize the extrusion parameters.

5.2.2 Additives and System Characterization

Traditional liquid solvent/polymer extraction methods, such as Soxhlet extraction, involving dissolution and precipitation are generally time consuming and uneconomical. Moreover, the loss of volatile AM agents and solvent are very difficult to control and the extraction of AM agents from films may be affected by the film thickness. Therefore, application of more sensitive and effective methods of quantification such as on-line supercritical fluid extraction/chromatography could be explored or a combination of two or more methods could be applied.

Synergistic mixtures of antimicrobials are highly desirable for packaging applications. The use of synergistic natural AM combinations in food packaging films can potentially broaden the spectrum of AM activity and minimise the need for high concentrations of individual AM agents. Further research is needed to identify AM combinations exhibiting synergistic effects in the polymer substrate. The applicability of models based on multiple logistic regression and response-surface methodology, which are commonly used in assessing drug interactions could be explored. Furthermore, to understand the interaction effects of AM combinations in a polymer substrate, the determination of the release kinetics of combined AM agents is also recommended.

Further studies on the micro-structural characterisation may be conducted to understand the behaviour of AM agents at micro-structural level. For example, reasons for the higher retention of thymol than that of carvacrol could be explored.

5.2.3 Shelf-Life Studies

For a more accurate shelf life prediction, data on microbial, sensorial and physio-chemical may be combined with data moisture, oxygen and carbon dioxide barrier

properties of films That can also very crucial for the preservation of foods. Further studies are needed with regard to preservation of different types of cheeses as well as other microbiological sensitive food systems with the use of AM films containing thymol or carvacrol. Studies aimed at establishing the type of AM agent and optimum AM concentrations to maximize the shelf life, while at the same time maintaining good sensorial characteristics are required.

It is also important to further investigate the kinetics of microbial inactivation by AM films on real food at different growth conditions such as different temperatures and against different reference microorganisms. The antioxidant effects of films containing thymol and carvacrol could also be further assessed.

The controlled release of AM agent is required to maintain an adequate amount of AM agent on food surfaces over a longer period of time. Various material structures (e.g. barrier coating or layer) that will restrict the migration of the AM agent at the outer surface of the packaging and allow controlled release of AM agents to the food surface are recommended as an important field for further research.

The technique of FTIR spectroscopy offers a potentially valuable non-destructive method of additive quantification and film characterisation. This could be further explored by developing more accurate data acquisition and analysis in order to identify and quantify AM agents or mixtures of agents. In addition, the technique shows promise for the study of AM agent release kinetics and can potentially be used on-line for quality control purposes.

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Appendicies

Appendix A Material Properties

Table A.1 Typical properties of LDPE resin

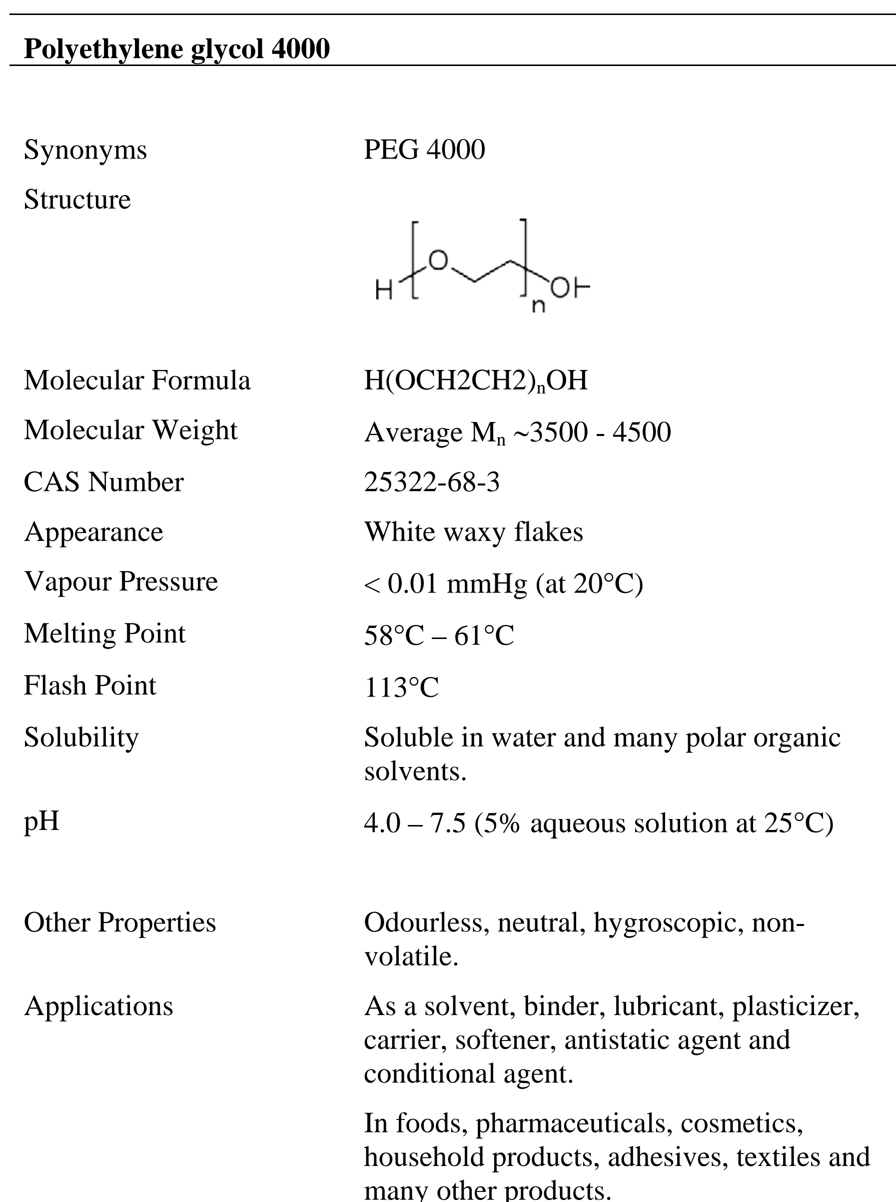
QenosTM ALKATHENE® XJF143/1700, Low-Density Polyethylene			
Description	Designed for a variety of lower gauge (20-50 µm) general purpose and blending applications. Does not contain any additives.		
Processing method	Blown film extrusion		
Applications	Food contact applications		
Manufacturer	Qenos Pty Ltd.		
Physical Properties			
Density	0.920 g cm^{-3}		ASTM D1505
Melt Index (190°C/2.16 Kg)	5.5 dg min^{-1}		ASTM D1238
Typical film properties (15 µm thickness; blow ratio 3.2 to 1)4			
Haze	3.5%		ASTM D1003
Gloss	74 units		ASTM D2457
Dart Impact	45 g		ASTM D1709
Tear Strength (N)	2.9 (MD)	0.8 (TD)	ASTM D1922
Tensile Yield (MPa)	9 (MD)	10 (TD)	ASTM D882
Tensile Strength (MPa)	22 (MD)	15 (TD)	ASTM D882
% strain at break	120% (MD)	670% (TD)	ASTM D882
Stiffness modulus (MPa)	150 (MD)	190 (TD)	ASTM D882
FDA Status	Complies with Food and Drug Administration Regulation 21 CFR 177.1520(c) 2.1 and AS2070-1999 section 4.1.1(a). Not applicable for holding food during cooking without the required compliance testing.		

Table A.2 Typical properties of EVA resin

Dupont™ ELVAX® 3120, Ethylene Vinyl Acetate

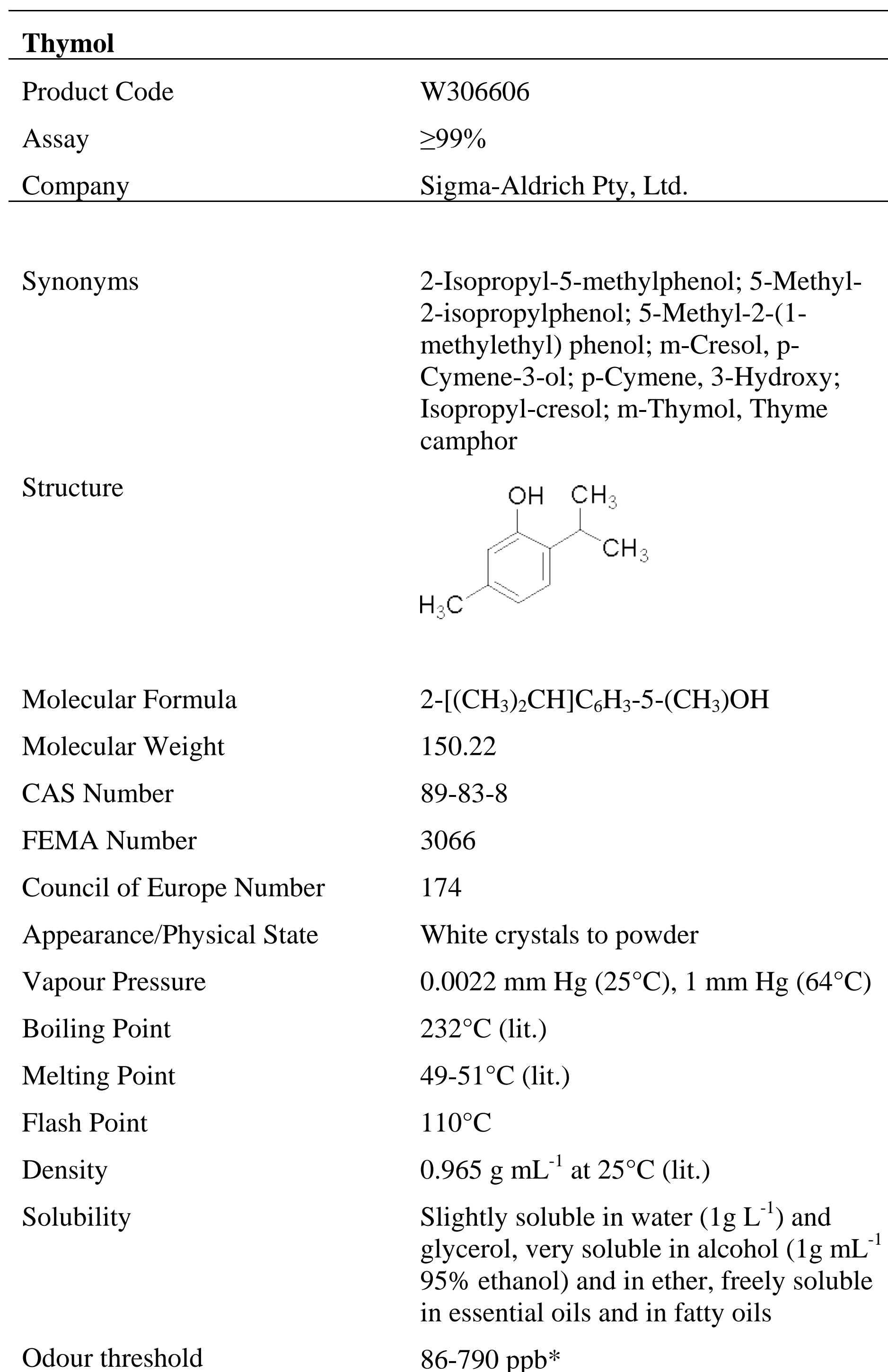
Product characteristics		
Processing method	Extrusion	
Composition	7.5% by weight Vinyl acetate	
Additive	Antiblock and slip	
Features	Copolymer	
Uses	Packaging	
Manufacturer	Dupont Packaging and Industrial Polymers	
Physical properties		
Density	0.93 g cm^{-3}	ASTM D792-ISO 1183
Melt Index (190°C/2.16Kg)	1.2 dg min^{-1}	ASTM D1238-ISO 1133
Vicat softening point	84°C	ASTM D1525-ISO 306
Melting point	99°C	ASTM D3417
Freezing point	82°C	ASTM D3417
Processing Information		
FDA Status	Complies with Food and Drug Administration Regulation 21 CFR 177.1350(a)(1). This regulation describes polymers to be used in contact with food, subject to finish food-contact- article meeting extractive limitations under the intended conditions of use as shown in paragraph (b)(1) of the regulation, for use in articles that contact food except for articles used for packaging or holding food during cooking.	
General processing information	Can be used in conventional extrusion equipment designed to process polyethylene resins. Applicable for blown film grades. Selection of desire temperature is subject to desire gauge, height of tower, cooling capacity, extruder hold-up time and machine variables.	

Table A.3 Typical properties of PEG resin



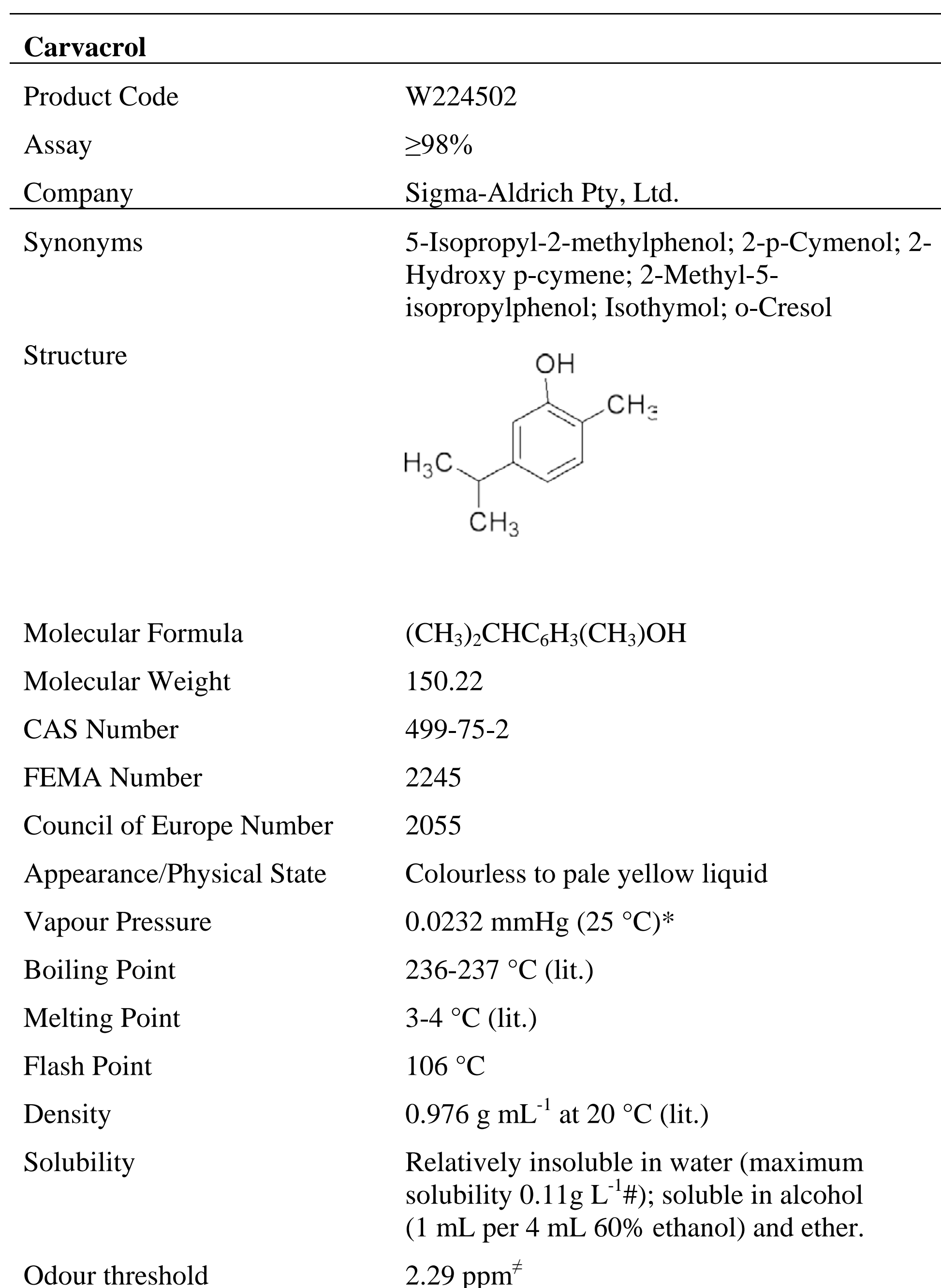
Polyethylene glycol 4000	
Synonyms	PEG 4000
Structure	H[O–CH2–CH2]nOH
Molecular Formula	$H(OCH2CH2)_nOH$
Molecular Weight	Average M_n ~3500 - 4500
CAS Number	25322-68-3
Appearance	White waxy flakes
Vapour Pressure	< 0.01 mmHg (at 20°C)
Melting Point	58°C – 61°C
Flash Point	113°C
Solubility	Soluble in water and many polar organic solvents.
pH	4.0 – 7.5 (5% aqueous solution at 25°C)
Other Properties	Odourless, neutral, hygroscopic, non-volatile.
Applications	As a solvent, binder, lubricant, plasticizer, carrier, softener, antistatic agent and conditional agent. In foods, pharmaceuticals, cosmetics, household products, adhesives, textiles and many other products.

Table A.4 Product characteristics and properties of AM agent thymol

Thymol	
Product Code	W306606
Assay	≥99%
Company	Sigma-Aldrich Pty, Ltd.
Synonyms	2-Isopropyl-5-methylphenol; 5-Methyl-2-isopropylphenol; 5-Methyl-2-(1-methylethyl) phenol; m-Cresol, p-Cymene-3-ol; p-Cymene, 3-Hydroxy; Isopropyl-cresol; m-Thymol, Thyme camphor
Structure	
Molecular Formula	2-[$(CH_3)_2CH$]C_6H_3-5-(CH_3)OH
Molecular Weight	150.22
CAS Number	89-83-8
FEMA Number	3066
Council of Europe Number	174
Appearance/Physical State	White crystals to powder
Vapour Pressure	0.0022 mm Hg (25°C), 1 mm Hg (64°C)
Boiling Point	232°C (lit.)
Melting Point	49-51°C (lit.)
Flash Point	110°C
Density	0.965 g mL^{-1} at 25°C (lit.)
Solubility	Slightly soluble in water (1g L^{-1}) and glycerol, very soluble in alcohol (1g mL^{-1} 95% ethanol) and in ether, freely soluble in essential oils and in fatty oils
Odour threshold	86-790 ppb*

*Adapted from Burdock (2005).

Table A.5 Product characteristics and properties of AM agent carvacrol



Carvacrol	
Product Code	W224502
Assay	≥98%
Company	Sigma-Aldrich Pty, Ltd.
Synonyms	5-Isopropyl-2-methylphenol; 2-p-Cymenol; 2-Hydroxy p-cymene; 2-Methyl-5-isopropylphenol; Isothymol; o-Cresol
Structure	OH, CH_3, H_3C, CH_3
Molecular Formula	$(CH_3)_2CHC_6H_3(CH_3)OH$
Molecular Weight	150.22
CAS Number	499-75-2
FEMA Number	2245
Council of Europe Number	2055
Appearance/Physical State	Colourless to pale yellow liquid
Vapour Pressure	0.0232 mmHg (25 °C)*
Boiling Point	236-237 °C (lit.)
Melting Point	3-4 °C (lit.)
Flash Point	106 °C
Density	0.976 g mL^{-1} at 20 °C (lit.)
Solubility	Relatively insoluble in water (maximum solubility 0.11g L^{-1}#); soluble in alcohol (1 mL per 4 mL 60% ethanol) and ether.
Odour threshold	2.29 ppm≠

* Adapted from Du et. al. (2008).
# Adapted from Afra et. al. (2006).
≠ Adapted from Burdock (2005).

Appendix B Reference Microorganisms

Table B.1 Characteristics and growth conditions of reference microorganisms (see sections 3.1.5)

	Gram-positive Bacteria		**Gram-negative Bacteria**		**Fungi**	**Yeast**
Genus	*Staphylococcus*	*Listeria*	*Pseudomonas*	*Escherichia*	*Aspergillus*	*Saccharomyces*
Species	*aureus*	*innocua*	*aeruginosa*	*coli*	*niger*	*cerevisiae*
Collection Acronym	UNSW*	ACM#	UNSW	UNSW	UNSW	UNSW
Accession Number	051300	4984	080400	080300	809000	703100
Equivalent to	ATCC≠ 13565 NCTC† 10652	ATCC 33090 NCTC 11288	ATCC 9027	ATCC 8739	ATCC 16404	
Isolated/Derived from	Ham involved in food poisoning			Faeces	Blueberry, North Carolina	Fermenting prunes
Growth Medium	Nutrient agar	Blood agar	Nutrient agar	Nutrient agar	Malt agar	Malt agar
Incubation Temperature	37 °C	37 °C	37 °C	37 °C	25 °C	25 °C
Incubation Time	24 h	24 h	24 h	24 h	1 week	48 h
Growth conditions	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Special Features and Usage	Prototype strain for production of enterotoxin A.		Assay of AM agents and AM preservatives	Assay of AM agents and AM preservatives	Assay of AM agents and AM preservatives	Non-flocculating

Notes: *UNSW = Culture collection, University of New South Wales, Sydney, Australia.
#ACM = Australian Collection of Microorganisms, Dept. of Microbiology, University of Queensland, Australia.
≠ATCC =American Type Culture Collection.
†NCTC = The National Collection of Type Cultures, United Kingdom.

Appendix C Supplemental AM Activity Figures

Supplemental figures for AM activity of moulded films containing different AM concentrations (see Sections 3.2.6 and 4.1.2).

Figure C.1 Inhibition of S. aurues by TF5 films containing 1.1% (w/w) thymol.

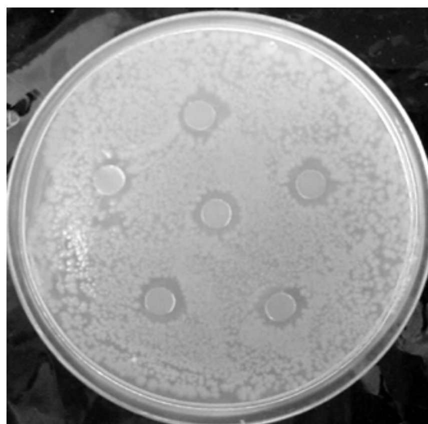


Figure C.2 Inhibition of E. coli by CF5 films containing 1.8% (w/w) carvacrol.

Supplemental figures for effect of PEG and EVA on AM activity (see Section 4.1.3).

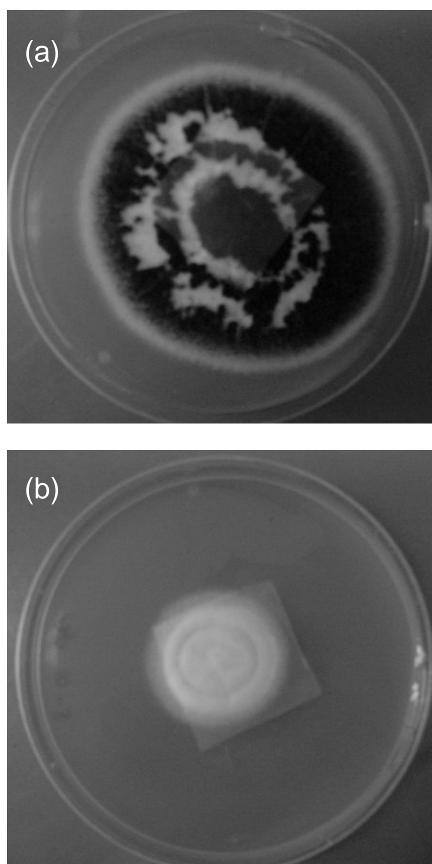


Figure C.3 Growth of A. niger after 1 week of incubation (a) control-film (b) AM film containing 1.9% (w/w) thymol, 2.5% (w/w) PEG and 10% (w/w) EVA.

Supplemental figures for AM activity of moulded films containing AM combinations (see Section 4.2.2).

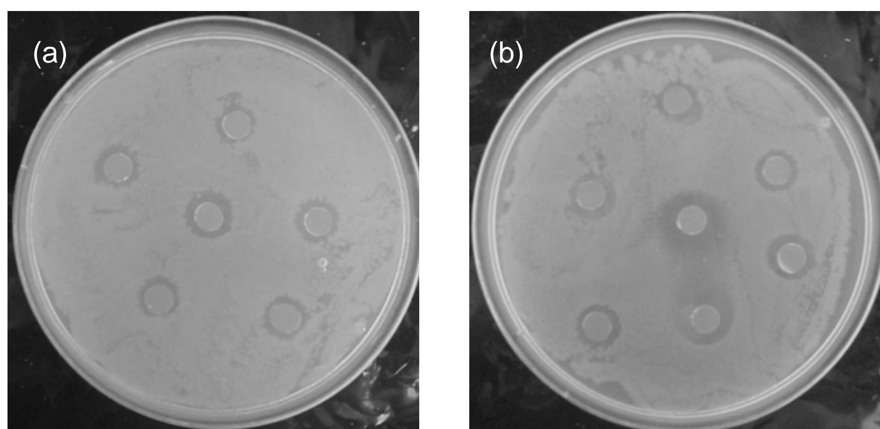


*Figure **C**.4 Inhibition of S. aurues by (a) AMI-film containing 1.1% (w/w) carvacrol and (b) AMC-film containing 1:1 thymol/carvacrol.*

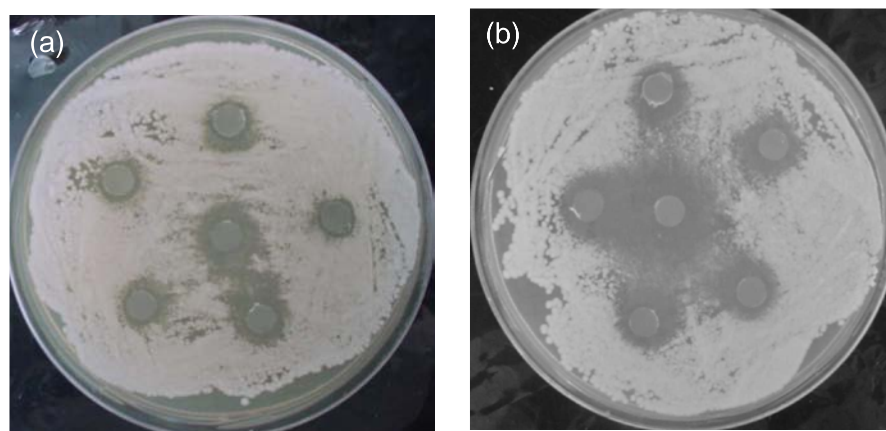


Figure C.5 Inhibition of S. cerevisiae by (a) AMC-film containing 1:2 thymol/carvacrol and (b) AMC-film containing 2:1 thymol/carvacrol.

Supplemental figures for AM activity of extruded films containing different levels of AM agents (see Section 4.3.2).

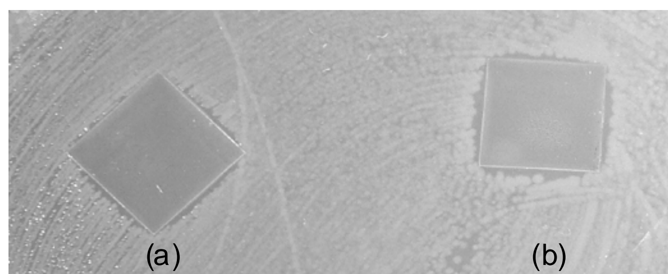


Figure C.6 Inhibition of E. coli by films containing 1.8% (w/w) thymol: (a) film cutting at 45/45 degree of extrusion direction; (b) film cutting at 0/90 degree of extrusion direction

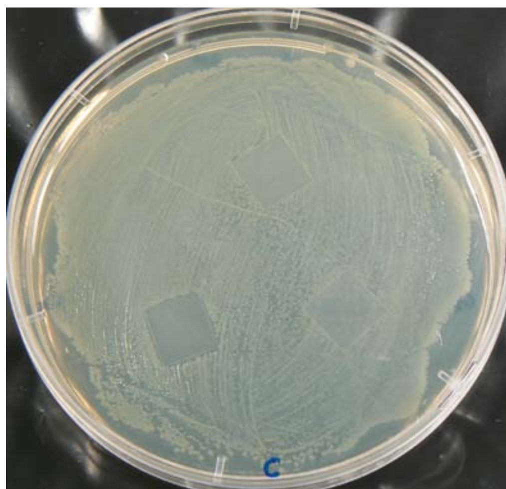


Figure C.7 Inhibition of E. coli by films containing 1.8% (w/w) carvacrol.

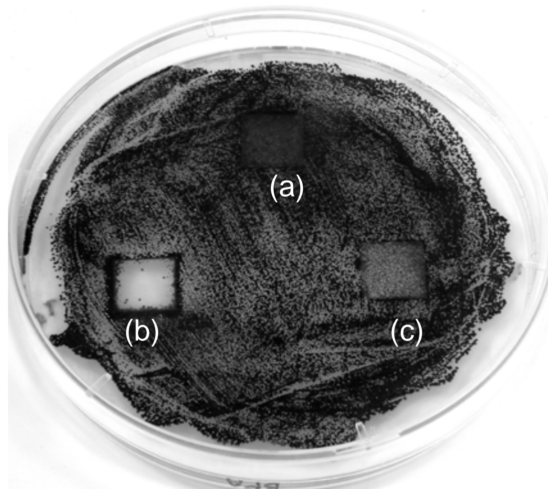


Figure C.8 Inhibition of S. aureus by extruded AM films: (a) control-film; (b) AM film containing 3.2% (w/w) thymol; (c) AM film containing 0.9% (w/w) thymol.

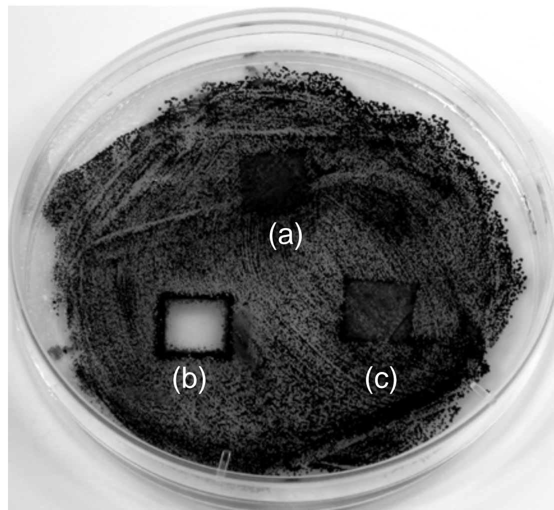


Figure C.9 Inhibition of S. aureus by extruded AM films: (a) control-film; (b) AM film containing 2.7% (w/w) carvacrol; (c) AM film containing 1.3% (w/w) carvacrol.

Appendix D Supplemental GC Analysis Figures

Supplemental figures for GC analysis (see Section 3.2.4).

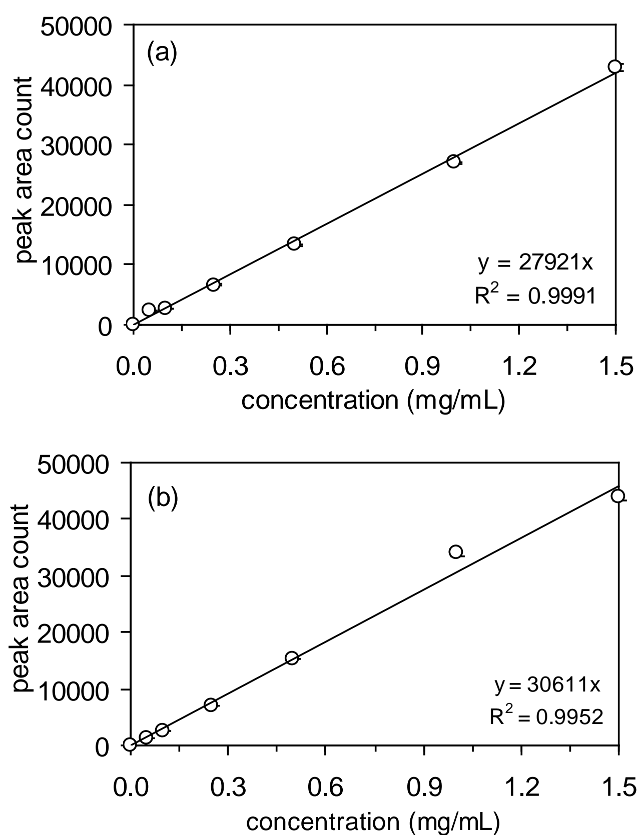


Figure D.1 Standard curves for AM agent quantification by GC analysis. The x axis represents the concentration of: (a) thymol and (b) carvacrol in the solvent isooctane.

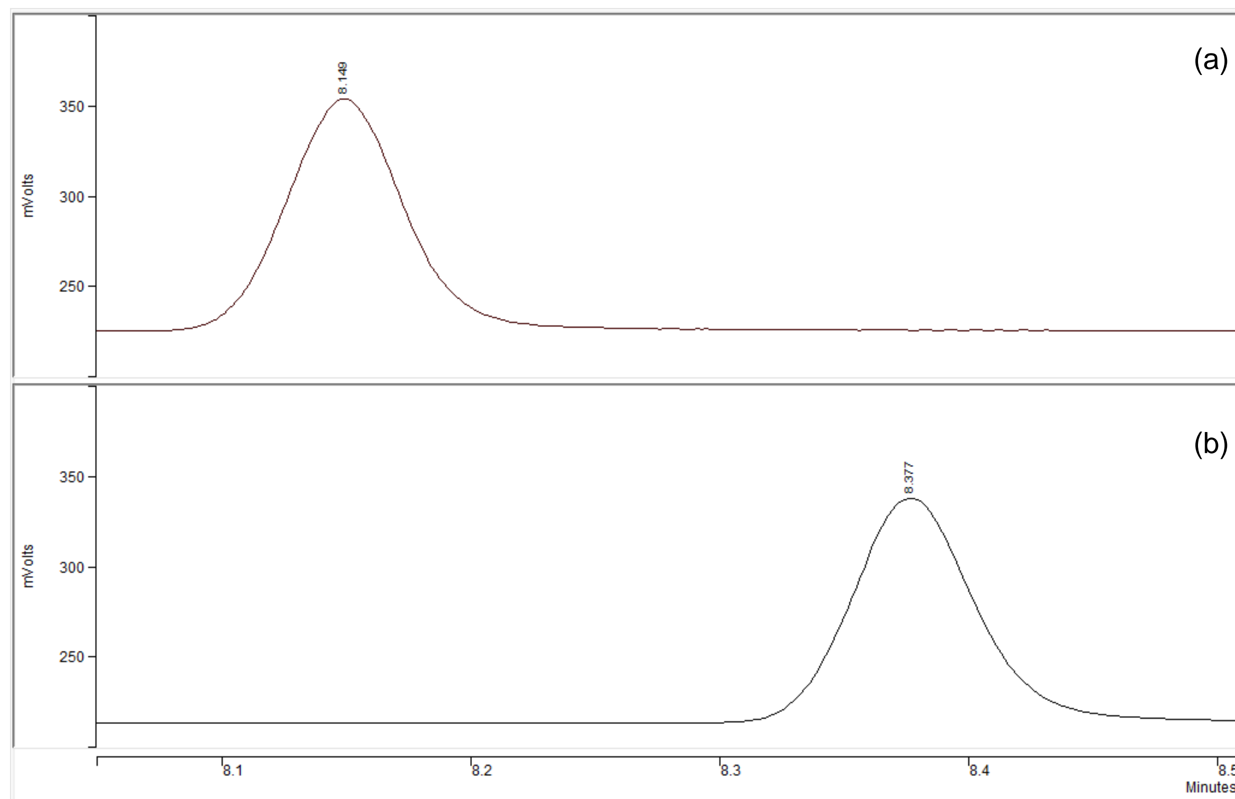


Figure D.2 GC chromatograms of AM agents dissolved in isooctane. (a) thymol 1.5 mg mL-1 peak at 8.149 min; (b) carvacrol 1.5 mg mL-1 peak at 8.377 min.

Appendix E Growth and Inactivation Models

Supplemental equations for growth and survival models used in the AM activity assay in liquid media (see Sections 4.3.3 and 4.6.1).

E.1 Modified Gompertz Model

The modified Gompertz model (Zwietering et al., 1991; Zwietering et al., 1990) is given by:

$$y = A\exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\right\}$$

where, y is the logarithm of the population size as a function of time (t), the maximum specific growth rate, μ_m, which is defined as the tangent in the inflection point; the lag time λ, which is defined as the t-axis intercept of this tangent; and the upper asymptote A, which is the maximal value reached.

E.2 Baranyi and Roberts Model

In the Baranyi and Roberts (Baranyi and Roberts, 1994) model, the variation in the number of cells (x) at time (t) is describe by:

$$\frac{d(x)}{d(t)} = \frac{q(t)}{q(t)+1} \cdot \mu_{\max} \cdot \left\{1 - \left[\frac{x(t)}{x_{\max}}\right]^m\right\} x(t)$$

where, $x_{\max}$ the maximum cell density, $\mu_{\max}$ the maximum specific growth rate, and parameter m characterise the curvature before the stationary phase. The lag phase is attributed to the need to synthesize an unknown substrate q that is critical for growth and $q(t)$ is the concentration of limiting substrate, which changes with time:

$$\frac{dq}{dt} = \mu_{\max} \cdot q(t)$$

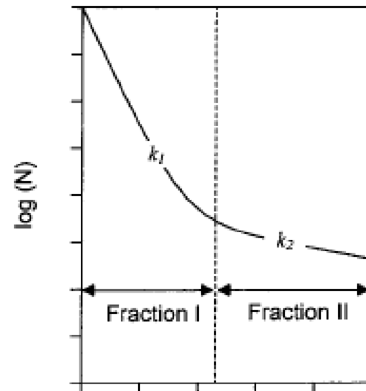
The initial value of $q(q_0)$ is a measure of the initial physiological state of the cells. A transformation of q_0 may define with the Baranyi-Roberts parameter (h_o), the lag time (λ) and $\mu_{\max}$ as:

$$h_o = \ln\left(1 + \frac{1}{q_o}\right) = \mu_{\max}\lambda$$

Thus the final model has four parameters, x_o, the initial cell number; h_o; $x_{\max}$; and $\mu_{\max}$.

E.3 Bi-phasic Model

This model consist of two fractions that follow independently first order kinetics (Xiong et al., 1999). The first inactivation fraction is designated as fraction I with the rate constant of k_1 (or s_1 in the present study). The second inactivation fraction is designated as fraction II with the rate constant k_2 (or s_2 in the present study) (see Figure E.1).



Then the fractions are expres

$$\frac{dN_1}{dt} = -k_1 N_1(t) \text{ and } \frac{dN_2}{dt} = -k_2 N_2(t)$$

where, N_1 and N_2 are the number of cells in the first and second fraction and t is the treatment time.

The surviving cells at time t are the sum of individual fractions:

$$N(t) = N_1(t) + N_2(t)$$

The analytical solution of the above equation is:

$$N(t) = N_0(f\mathrm{e}^{-k_1 t} + (1-f)\mathrm{e}^{-k_2 . t})$$

where, N_0 is the initial number of cells and f is the initial proportion of the of the first fraction (N_1/N_2).

Appendix F Supplemental AM Release Figures

Supplemental figures for AM release (see Sections 4.4.3 and 4.5).

Release to the Atmosphere

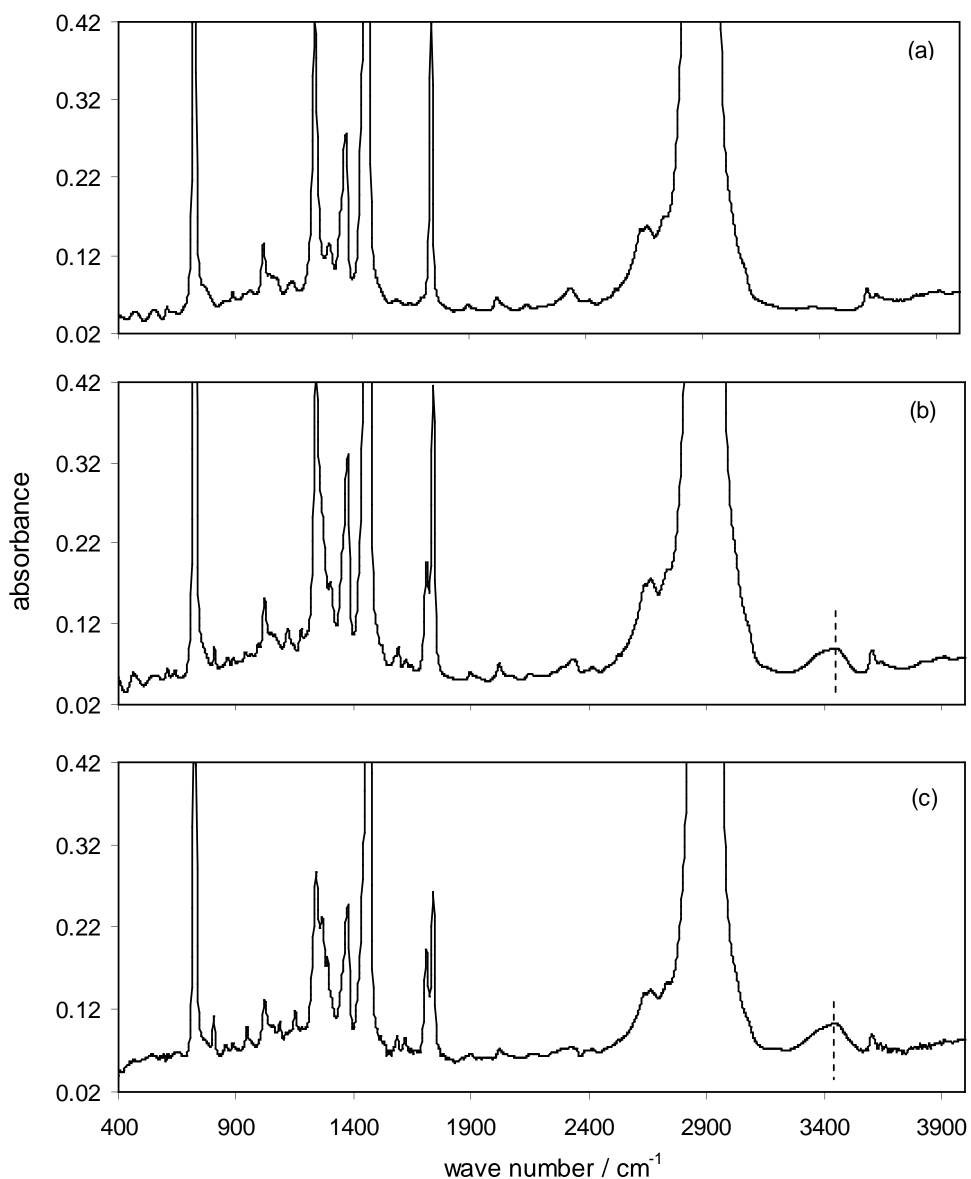


Figure F.1 FT-IR absorbance spectra of the: (a) control; (b) carvacrol and (b) thymol films in the regions between 400 – 4000 cm^{-1}. The wave number 3450 cm^{-1} corresponds to the hydroxyl group of AM agents.

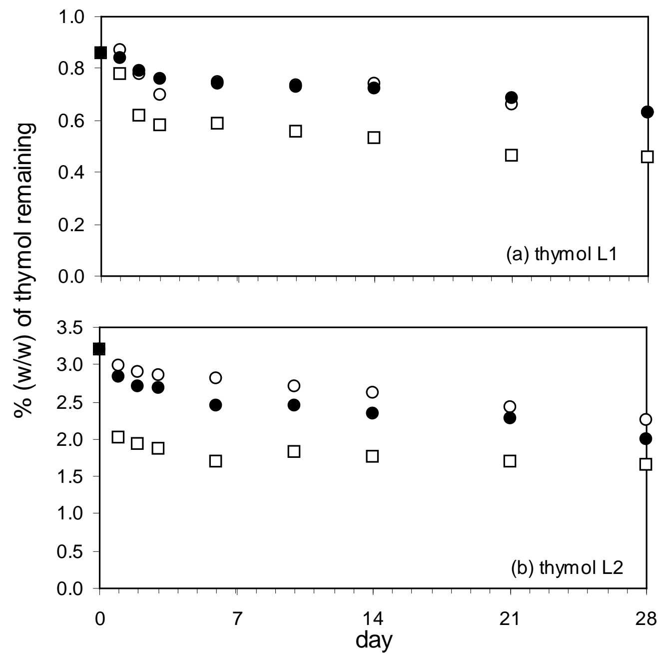


Figure F.2 The percentage (w/w) retention of thymol in the (a) thymol L1 and (b) thymol L2 films during: (□) open; (○) foil covered and (•) foil covered and refrigerated storage.

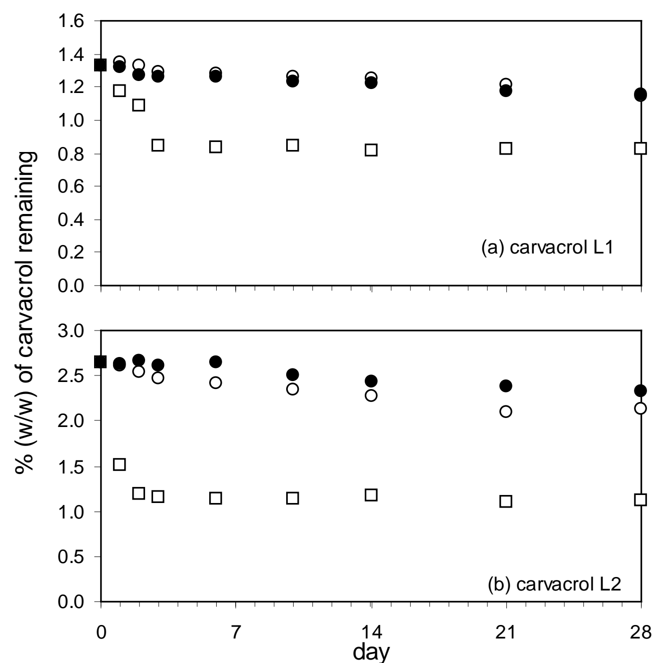


Figure F.3 The percentage (w/w) retention of carvacrol in the (a) carvacrol L1 and (b) carvacrol L2 films during: (□) open; (○) foil covered and (•) foil covered and refrigerated storage.

Effect of food simulant on the release of AM agent

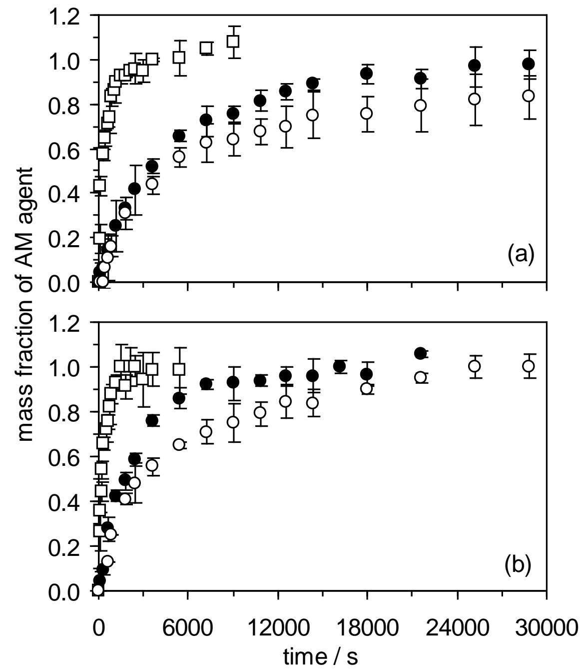


Figure F.4 Plots of mass fraction (m_t/m_∞) of (a) thymol and (b) carvacrol released at 15 ℃ versus time into: (□) isooctane, (•) 95% ethanol, and (○) 10% ethanol.

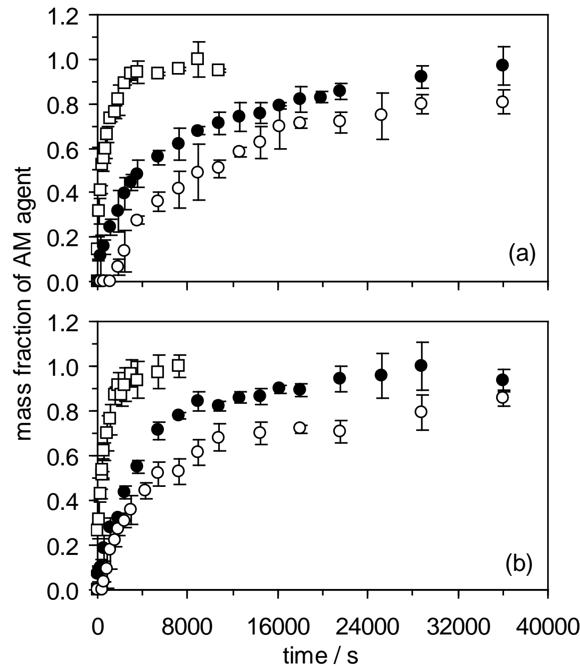


Figure F.5 Plots of mass fraction (m_t/m_∞) of (a) thymol and (b) carvacrol released at 10 ℃ versus time into: (□) isooctane, (•) 95% ethanol, and (○) 10% ethanol.

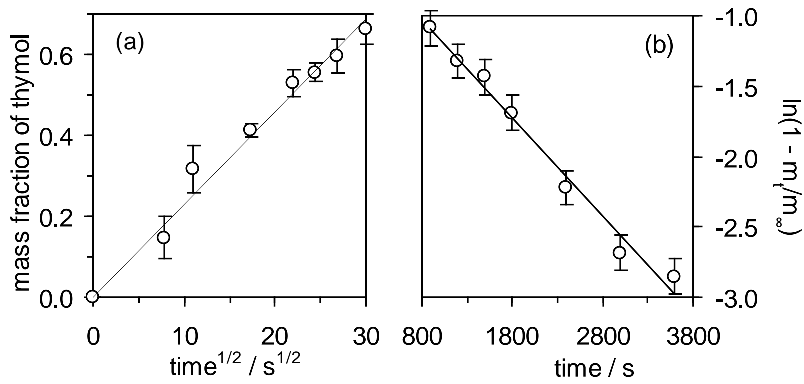


Figure F.6 Plots of: (a) (m_t/m_∞) versus $t^{1/2}$ and (b) ln(1 - m_t/m_∞) versus time for the release of thymol into isooctane at 10°C.

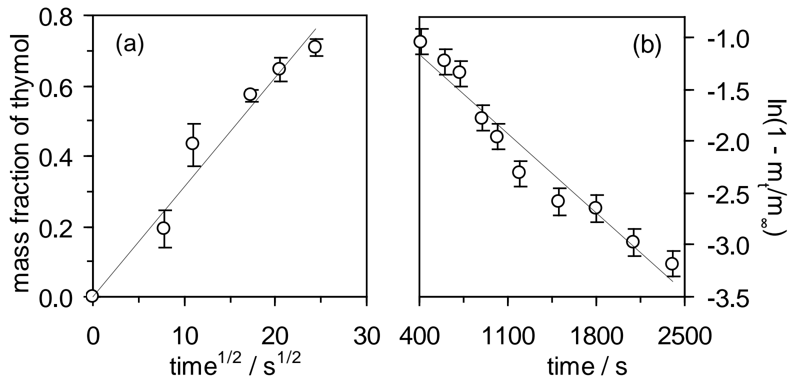


Figure F.7 Plots of: (a) (m_t/m_∞) versus $t^{1/2}$ and (b) ln(1 - m_t/m_∞) versus time for the release of thymol into isooctane at 15°C.

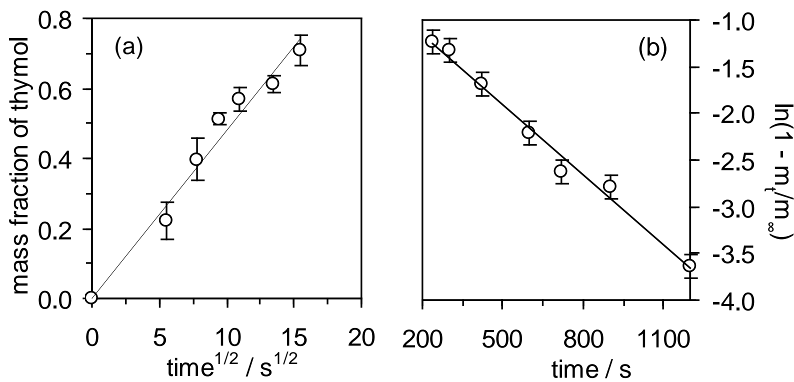


Figure F.8 Plots of: (a) (m_t/m_∞) versus $t^{1/2}$ and (b) ln(1 - m_t/m_∞) versus time for the release of thymol into isooctane at 20°C.

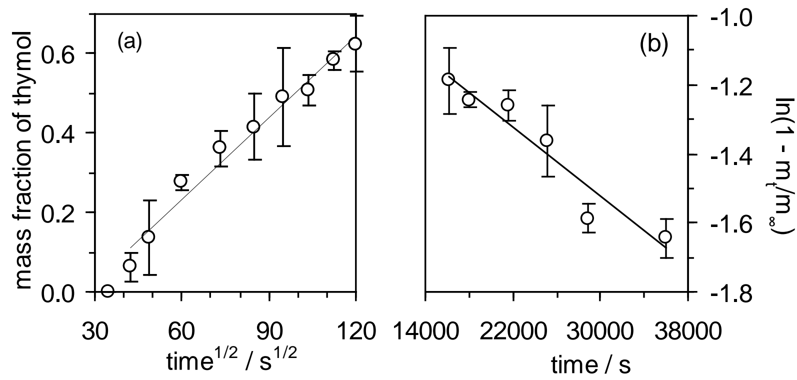


Figure F.9 Plots of: (a) (m_t/m_∞) versus $t^{1/2}$ and (b) ln(1 - m_t/m_∞) versus time for the release of thymol into 10% ethanol at 10°C.

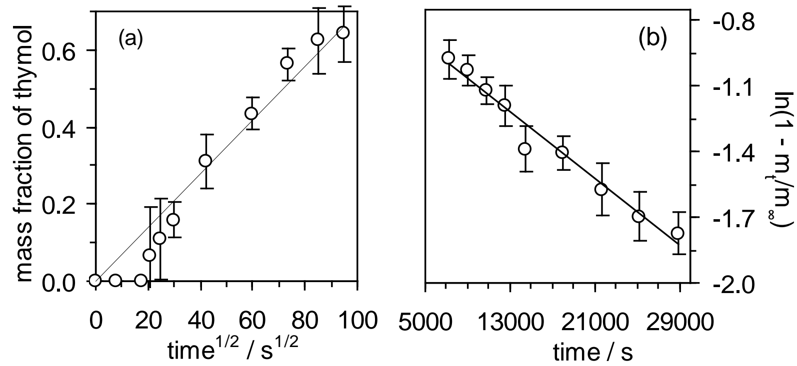


Figure F.10 Plots of: (a) (m_t/m_∞) versus $t^{1/2}$ and (b) ln(1 - m_t/m_∞) versus time for the release of thymol into 10% ethanol at 15°C.

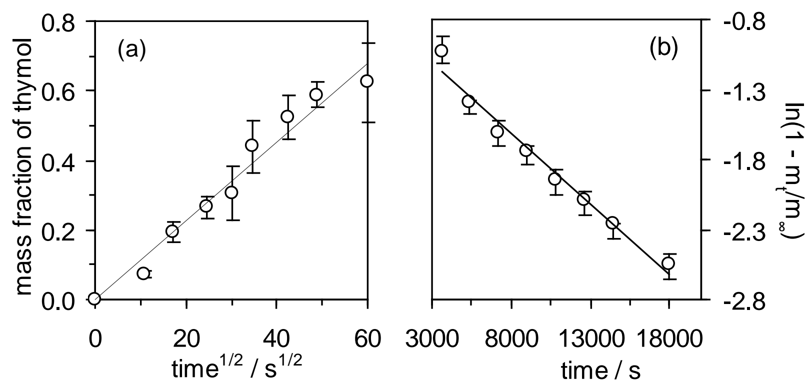


Figure F.11 Plots of: (a) (m_t/m_∞) versus $t^{1/2}$ and (b) ln(1 - m_t/m_∞) versus time for the release of thymol into 10% ethanol at 20°C.

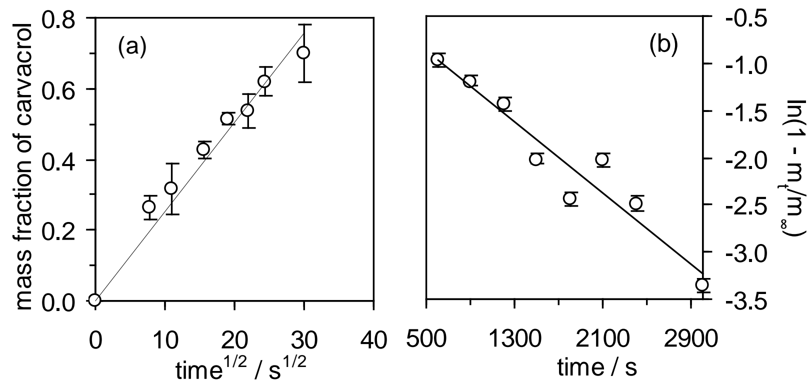


Figure F.12 Plots of: (a) m_t/m_∞ versus $t^{½}$ and (b) $ln(1 - m_t/m_\infty)$ versus t for the release of carvacrol into isooctane at 10°C.

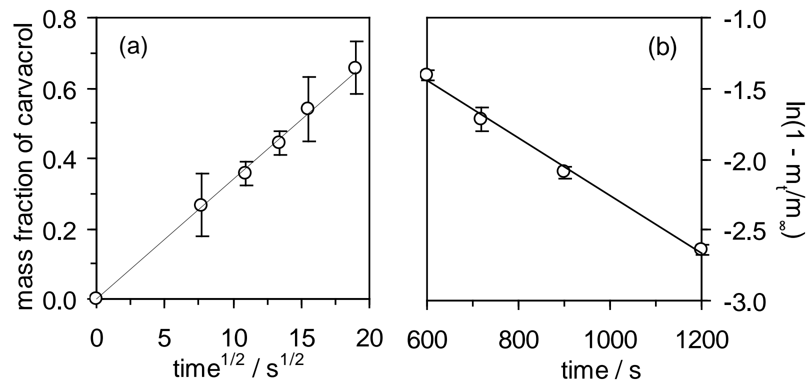


Figure F.13 Plots of: (a) m_t/m_∞ versus $t^{½}$ and (b) $ln(1 - m_t/m_\infty)$ versus t for the release of carvacrol into isooctane at 15°C.

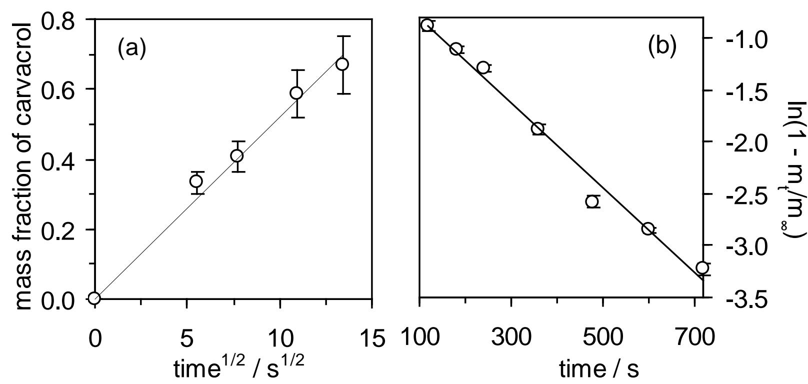


Figure F.14 Plots of: (a) m_t/m_∞ versus $t^{½}$ and (b) $ln(1 - m_t/m_\infty)$ versus t for the release of carvacrol into isooctane at 20°C.

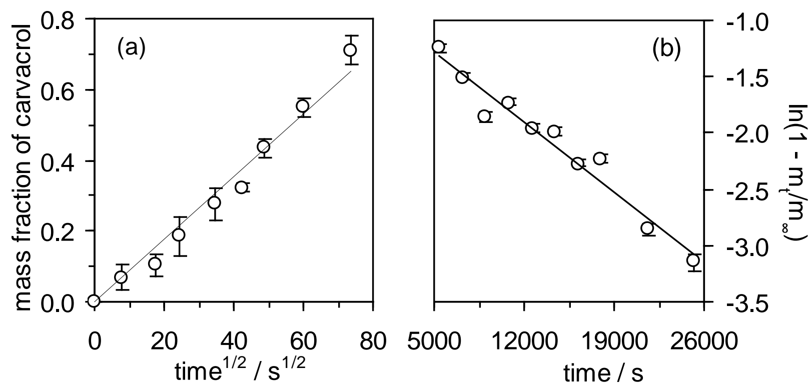


Figure F.15 Plots of: (a) (m_t/m_∞) versus $t^{½}$ and (b) ln(1 - m_t/m_∞) versus time for the release of carvacrol into 95% ethanol at 10 °C.

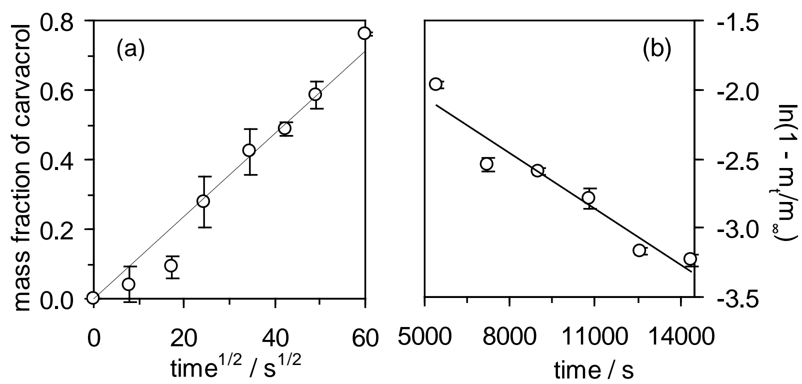


Figure F.16 Plots of: (a) (m_t/m_∞) versus $t^{½}$ and (b) ln(1 - m_t/m_∞) versus time for the release of carvacrol into 95% ethanol at 15 °C.

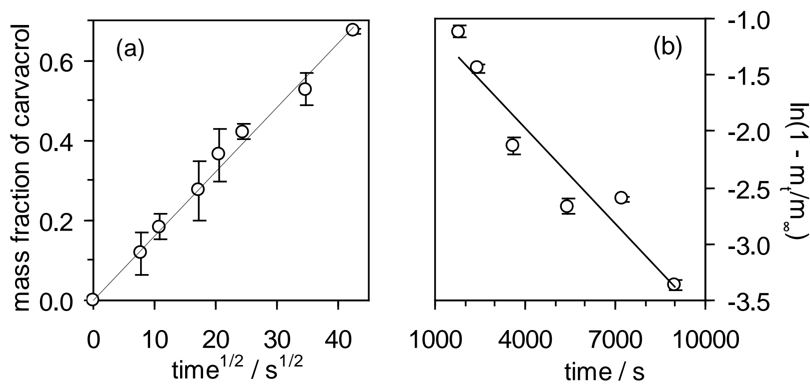


Figure F.17 Plots of: (a) (m_t/m_∞) versus $t^{½}$ and (b) ln(1 - m_t/m_∞) versus time for the release of carvacrol into 95% ethanol at 20 °C.

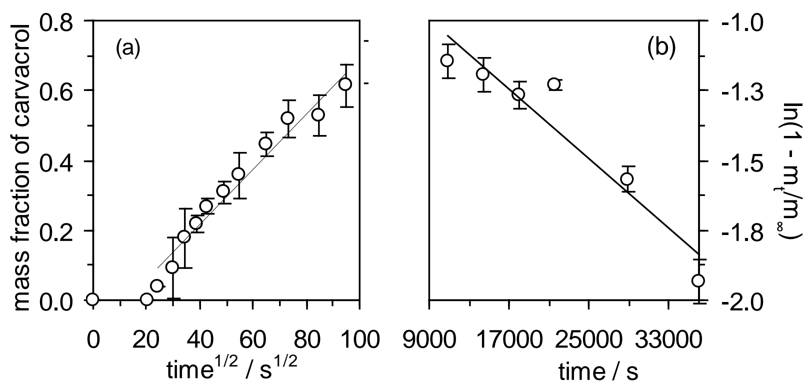


Figure F.18 Plots of: (a) (m_t/m_∞) versus $t^{1/2}$ and (b) $ln(1 - m_t/m_\infty)$ versus time for the release of carvacrol into 10% ethanol at 10°C.

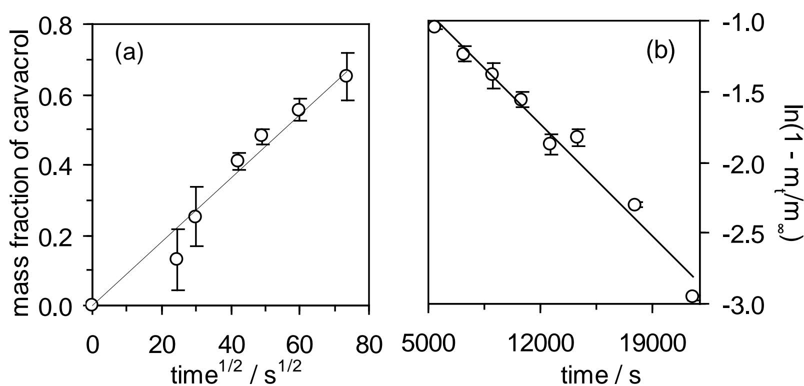


Figure F.19 Plots of: (a) (m_t/m_∞) versus $t^{1/2}$ and (b) $ln(1 - m_t/m_\infty)$ versus time for the release of carvacrol into 10% ethanol at 15°C.

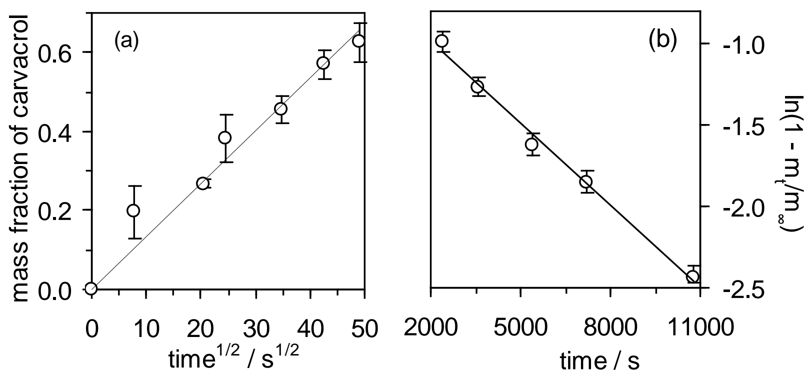


Figure F.20 Plots of: (a) (m_t/m_∞) versus $t^{1/2}$ and (b) $ln(1 - m_t/m_\infty)$ versus time for the release of carvacrol into 10% ethanol at 20°C.

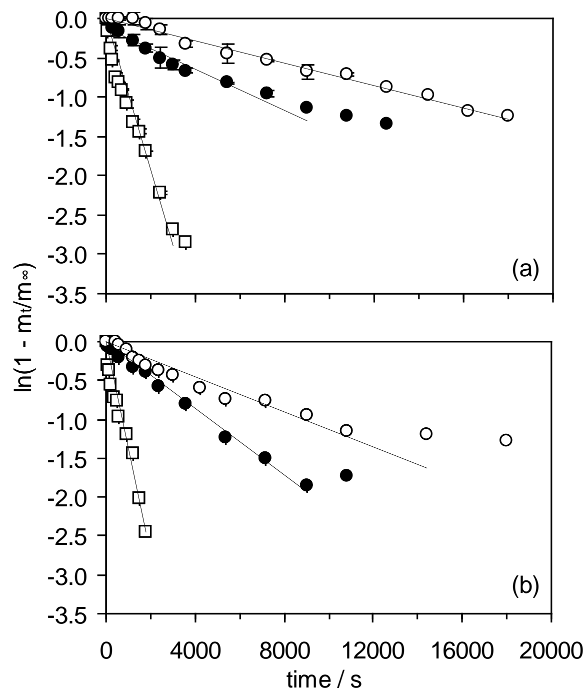


Figure F.21 Plots of ln(1 - m_t/m_∞) versus time for the release of (a) thymol and (b) carvacrol into: (□) isooctane, (•) 95% ethanol, and (○) 10% ethanol at 10°C.

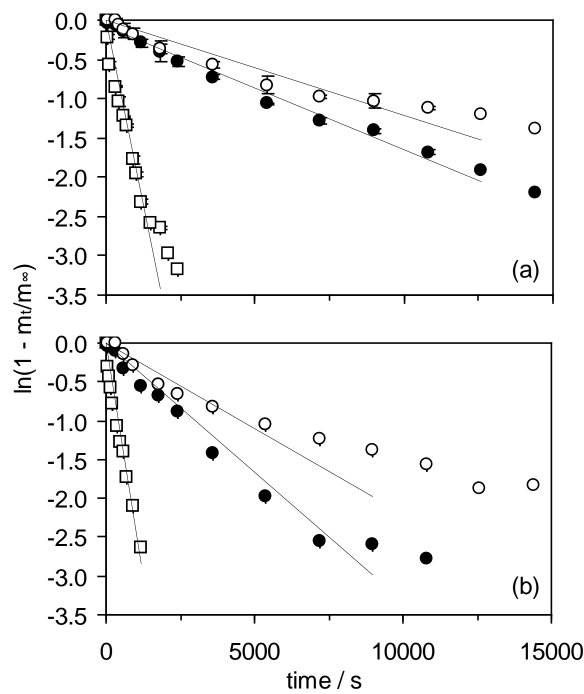


Figure F.22 Plots of ln(1 - m_t/m_∞) versus time for the release of (a) thymol and (b) carvacrol into: (□) isooctane, (•) 95% ethanol, and (○) 10% ethanol at 15°C.

Effect of temperature on the release of AM agent

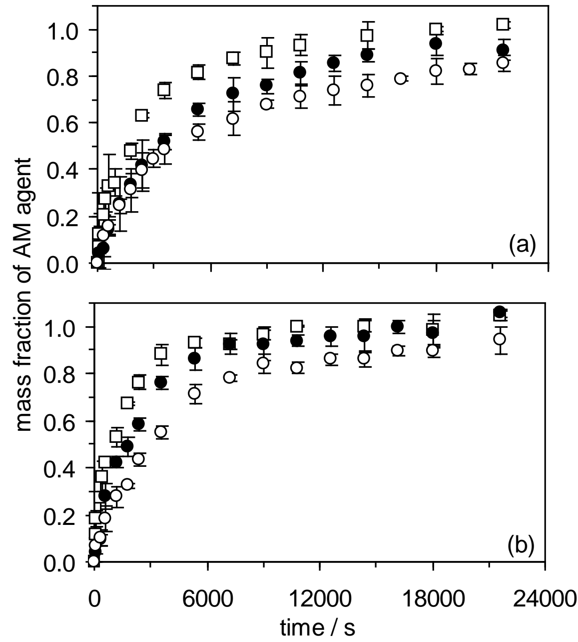


Figure F.23 Plots of mass fraction (m_t/m_∞) of (a) thymol and (b) carvacrol released into 95% ethanol at: (○) 10; (•) 15 and (□) 20 °C.

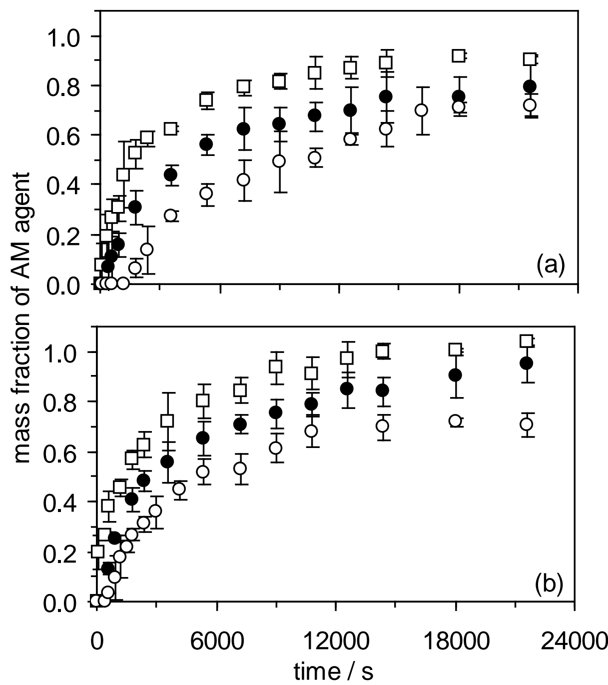


Figure F.24 Plots of mass fraction (m_t/m_∞) of (a) thymol and (b) carvacrol released into 10% ethanol at: (○) 10; (•) 15 and (□) 20 °C.

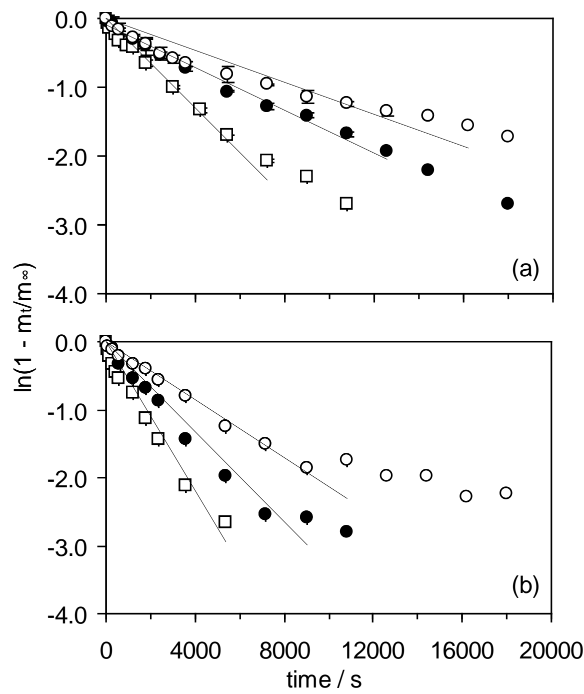


Figure F.25 Plots of ln(1 - m_t/m_∞) versus time for the release of (a) thymol and (b) carvacrol into 95% ethanol at: (○) 10; (•) 15 and (□) 20 °C.

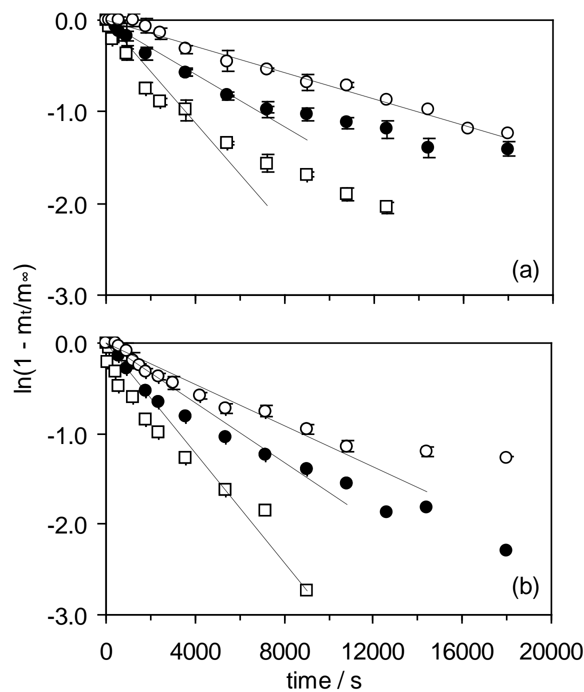


Figure F.26 Plots of ln(1 - m_t/m_∞) versus time for the release of (a) thymol and (b) carvacrol into 10% ethanol at: (○) 10; (•) 15 and (□) 20 °C.

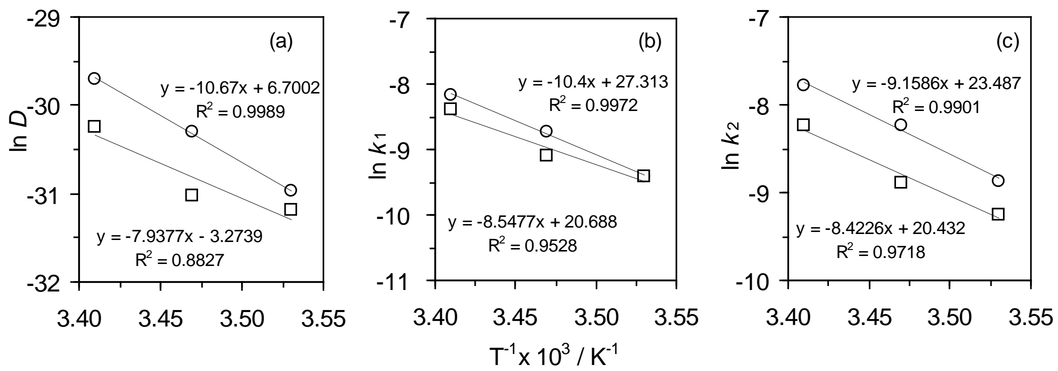


Figure F.27 Plots of logarithm of (a) D; (b) k of diffusion process and (c) k of chemical process versus the reciprocal of the absolute temperature for release of: (□) thymol and (○) carvacrol into 95% ethanol.

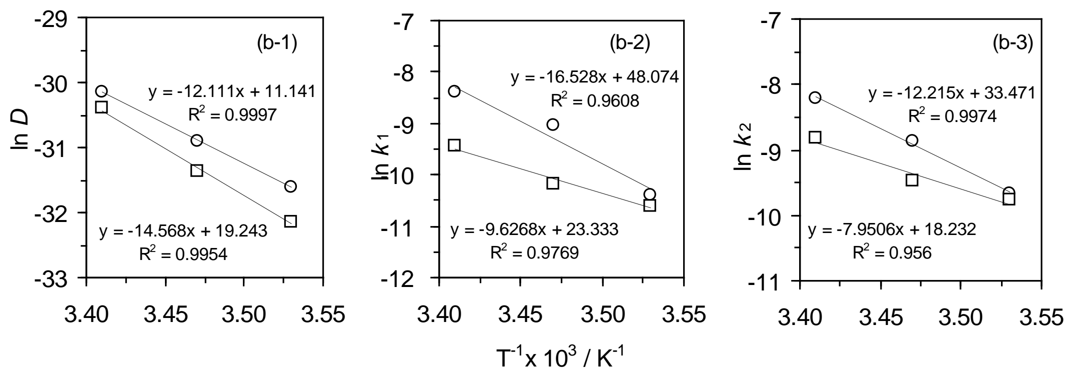


Figure F.28 Plots of logarithm of (a) D; (b) k of diffusion process and (c) k of chemical process versus the reciprocal of the absolute temperature for release of: (□) thymol and (○) carvacrol into 10% ethanol.

Appendix G Sensory Analysis

Sensory analysis information and data collection sheets (see Section 3.7.3).

INFORMATION TO PARTICIPANTS INVOLVED IN RESEARCH

You are invited to participate

You are invited to participate in a research project entitled Development and Evaluation of Antimicrobial Food Packaging Films Containing Natural Agents.

This project is being conducted by a student researcher Rupika Liyana-Arachchige as part of a PhD study at Victoria University under the supervision of Associate Prof. Kees Sonneveld from Packaging and Polymer Research Unit, faculty of Health, Engineering and Science.

Project explanation

Antimicrobial food packaging has the potential of preserving quality, extending shelf life and improving the safety of food products primarily by controlling the growth of undesirable microorganisms. The development of food packaging materials containing plant compounds emerged as a new technology to serve the consumer demands for safe, natural and convenient food products. The packaging materials used in the present study contains antimicrobial compounds present in herbs and spices. The plant extracts containing these compounds have been used from the ancient times as food preservatives or primarily as flavouring additives. However, their presence in food can change the sensorial qualities and acceptability. The objective of this study is to determine if the AM packaging causes a difference in odour and/or acceptability of cheddar cheese during storage.

What will I have to do?

Below you will find the definitions of the general terms or techniques used in the present study. These definitions will help you with understanding the assessment of samples and the basic sensory techniques used. The definitions of terms used in the descriptive sensory analysis will be provided separately at the time of evaluation.

Odourant: is a substance capable of eliciting an olfactory response.

Odour: is the sensation resulting from stimulation of the olfactory system.

Sheet G.1 Information to the participants involved in the sensory analysis (page 1).

Olfactory system: The olfactory system is basically the body's system of smell. Tiny molecules of odourants are inhaled by the nose. These molecules are trapped in the nose by ciliated (hairlike) nerve endings that pass the aroma on to receptor cells that then carry the molecules to the Olfactory Bulb. From the Olfactory Bulb, the aroma molecules are transported to the limbic system in the brain.

Odour adaptation: is the process by which one becomes accustomed to an odour or a temporary reduction in sensitivity to an odour following prolonged exposure to it.

Odour fatigue: occurs when total adaptation to a particular odour has occurred through prolonged exposure.

Descriptive sensory analysis: This method involves the detection (discrimination) and the description of both the qualitative and quantitative sensory aspects of a product. Panellists must be able to detect and describe the perceived sensory attributes of a sample.

Hedonic scale: A descriptive analysis technique most frequently employed in studies involving untrained consumer panels. It expresses the degree to which each of the characteristics is present in the test product by the assignment of some value to each attribute or to overall impression.

e.g. Acceptance scales used in 9 point-hedonic scale for acceptance

Like extremely
Like very much
Like moderately
Like slightly
Neither like nor dislike
Dislike slightly
Dislike moderately
Dislike very much
Dislike extremely

★ You have been provided with three coded samples. The samples may have the same or different odors. Please sniff the samples using shallow short sniffs.

★ **You will be asked to wait at least 30 seconds between smelling of samples in a set and 2 min between sniffing samples from different sets. This is very important to eliminate carryover effects, odour adaptation and odour fatigue. So you can evaluate the next sample impartially.**

★ This study continues up to 6 weeks. WE KINDLY REQUEST YOU TO BE PRESENT AT ALL THE SESSIONS STARTING FROM WEEK 1 TO WEEK 6. The exact date, time and venue will be notified by the researcher in advance.

Sheet G.1 Information to the participants involved in the sensory analysis (page 2).

What will I gain from participating?

The information provided by you will be used to assess consumers' acceptability of these products packaged in antimicrobial packaging films. This information will contribute to the advancement o knowledge.

How will the information I give be used?

The student researcher Rupika LiyanaArachchige will conduct the research, extract the data and assess them.
The principle supervisor Associate Prof. Kees Sonneveld and the associate investigator/s and/or co-investigator/s: Dr. Marlene Cran, Prof. Stephen W. Bigger and Prof. Joseph Miltz Packaging and Polymer Research Unit, School of Molecular Sciences (9919 8043) will also have accessibility to these data to organise and to extract the data.

What are the potential risks of participating in this project?

There will be no hazardous material involved in the study as the AM packaging materials are based on natural antimicrobials. If you are suffering from any **food related allergies** you are welcome to stay during this information session but **can not participate in the sensory study**.

All samples are microbiologically safe. However, **YOU ARE INSTRUCTED NOT TO TASTE THE SAMPLES.**

How will this project be conducted?

First of all, please remember not to taste or swallow the samples.
The sensory evaluation will be based on a test called "triangle test" and a system called "hedonic scale". We use triangle test to determine whether two samples are perceptibility different and hedonic scale to measure the degree of consumers' acceptance of the tested materials.

Triangle test: A test used to determine whether two samples are perceptibility different. In the triangle test, three samples are presented simultaneously; two samples are from the same formulation and one is from different formulation. Each panellist has to indicate which sample is the odd sample.
Hedonic test: You will be provided with 5 samples wrapped in various AM packaging materials. A questionnaire will be provided to you with different attributes that you need to consider in assessing this particular product. Please mark the degree of likeness for each attribute after smelling the sample. Please smell one sample at a time.

Sheet G.1 Information to the participants involved in the sensory analysis (page 3).

Who is conducting the study?

The student researcher Rupika Liyana Arachchige will conduct the research, extract the data and assess them.

The principle supervisor Associate Prof. Kees Sonneveld (9919 8043) and the associate investigator/s and/or co-investigator/s: Dr. Marlene J. Cran, Prof. Stephen W. Bigger and Prof. Joseph Miltz, Packaging and Polymer Research Unit, School of Molecular Sciences, Victoria University will have accessibility to these data.

Any queries about your participation in this project may be directed to the Principal Researcher listed above.

If you have any queries or complaints about the way you have been treated, you may contact the Secretary, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 phone (03) 9919 4781.

Sheet G.1 Information to the participants involved in the sensory analysis (page 4).

Test code: *Panelist No:*

Triangle Test

Date :

Type of sample: **DOT solutions**

Instructions:

1. You have been provided with five sets of three coded samples.
2. Within each set, two of the samples have the same odour and one is different.
3. Please sniff the samples in the containers from **left to right**, using shallow short sniffs.
4. Wait at least 30 seconds between samples.
5. Determine the **odd/different sample**.

	Codes			Indicate odd sample
Set 1				
Set 2				
Set 3				
Set 4				
Set 5				

Descriptive sensory analysis

From the terms presented, please **underline** **2 terms** of the odour that best represent the above samples.

Ashy	Phenol-like	Creamy	Buttery	Medicinal
Sulphur	Green	Minty	Sour-acidic	Pungent
Sweet	Woody	Herb-like	Fruity	Free fatty acid

Thank you for your time!

Sheet G.2 Score sheet for the triangle test of DOT solutions and odour term selection.

Descriptive sensory analysis

Odour descriptors

Odour term	Definition
Ashy	The light smokey/ashy aroma associated with burning tobacco such as cigarette smoke
Buttery	Of the nature of, or containing, butter
Creamy	The smell associated with creamy/milky products
Free fatty acid	Aromatics associated with short-chain free fatty acids
Fruity	The aromatic blend of different fruity identities
Green	Sharp, slightly pungent aromatics associated with green plant/vegetable matter
Herb-like	The aromatics associated with dry herbs
Minty	Aromatics associated with fresh mint; somewhat reminiscent of toothpaste. The sweet, green, earthy, pungent, sharp, mentholic aromatics associated with mint oils
Medicinal	Aroma characteristics of antiseptic-like products, such as band-aid, alcohol and iodine
Pungent	Physically penetrating sensation in the nasal cavity. Sharp smelling or tasting, irritant
Phenol-like	Aromatics associated with distinctive phenolic character
Sulphur	Aromatics associated with sulfurous compounds/rotten egg like
Sour-acidic	Odour associated with sour or acidic dairy products
Sweet	Blend of sweet aromas
Woody	Bar-like odour

Thank you for your time!

Sheet G.3 Information sheet of odour terms and their definitions.

Test code: *Panelist No:*

Triangle Test

Date :

Product : Cheddar cheese

Instructions:

You have been provided with three sets of cheese samples. Within each set, two samples are identical and one is different.

1. Please sniff the samples in the containers from **left to right**, using shallow short sniffs.
2. Wait at least 30 seconds between samples.
3. After sniffing each sample, select the odd/different sample
4. **Circle** the code number of the **odd/different** sample.
5. If you wish to comment on the reasons for your choice or if you wish to comment on cheese characteristics you may do so under remarks.

	Codes			Remarks
Set 1				
Set 2				
Set 3				
Set 4				
Set 5				
Set 6				

Thank you for your time!

Sheet G.4 Score sheet for the triangle test of Cheddar cheese.

Test code: *Panelist No:*

Descriptive Sensory Analysis

Date :

Product : Cheddar Cheese

Instructions:

You have been given 5 Cheddar cheese samples.

- Please **score** the cheese samples on a 0 to 9 scale for all parameters listed.

Odour Intensity	0 = Weak	9 = Strong
Acceptability	0 = Dislike extremely	9 = Like extremely

- Please score one sample at a time

Score sheet:

Attributes		**Sample Codes**				
Odour	Creamy					
	Buttery					
	Herb-like					
	Medicinal					
	Minty					
Acceptability						

Thank you for your time!

Sheet G.5 Score sheet for the descriptive sensory analysis of Cheddar cheese.