Measurement of Minimum Inhibitory Concentration (MIC) of Individual and Combinations of Essential Oil Volatiles in Food

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Abstract

The use of essential oil volatiles as natural food preservatives has received significant attention in recent years. Shelf life extension can be achieved through the appropriate design of active packaging systems that release volatiles into the product headspace at controlled rates. In some applications, the volatile concentrations required to delay or prevent spoilage can cause sensory changes to the product. The use of multiple volatiles and the potential for synergistic effects offer opportunities to minimise sensory effects in the pursuit of shelf life extension. The design of these systems requires a good knowledge of the target headspace volatile concentrations required to inhibit growth.

The aim of this research was to analyse the methods for measurement of minimum inhibitory concentration (MIC) for individual and mixtures of essential oil volatiles in food systems. Carvacrol and thymol, the predominant phenolic constituents of *Origanum vulgare* and *Thymus vulgaris* were selected as example active agents. Both are known to have strong antimicrobial activity.

The accuracy of the techniques normally applied to measure MICs against targeted microorganisms in the headspace is questionable. Volatile compounds released into the test headspace are absorbed by media and dish materials and lost into the environment. As such the MIC data collected are difficult to interpret and can even exceed saturated vapour pressures of the volatile compounds.

To demonstrate this, the Petri dish reversed method headspace dynamics was characterised during standard MIC tests. Factors influencing the sorption of volatiles by the culture media (Potato Dextrose Agar) and other parts of the system were investigated. The concentrations of antimicrobial compounds in the headspace were quantified using gas chromatography-mass spectrometry (GC-MS). The study showed that very low concentrations were found during MIC measurement and that the concentrations changed dynamically during the incubation period. The results demonstrated that absorption of vapour by the Potato Dextrose Agar (PDA) strongly influenced the headspace dynamics and was the main reason for the low volatile concentrations in the headspace.

The partitioning of carvacrol and thymol ($K_A/_W$ values 5.94×10^{-5} and 2.58×10^{-4} respectively) strongly favour the solid phase, providing a basis for the design of a new method to enable better MIC measurement. A new method based on pre-mixing the volatile compound into the liquid media was developed. Testing showed that headspace volatile concentrations quickly stabilised and remained constant throughout the incubation period, making MIC determination easier.

The potential of each compound and their binary combinations to inhibit growth were evaluated using the new MIC measurement method. This resulted in very repeatable results with much lower headspace concentrations than measured using traditional methods. To test for synergistic effects in the multiple volatile trials, an alternative data analysis approach was adopted. The inhibition time before growth observed in each sample was linearized and regressed against the thymol and carvacrol concentrations. This resulted in a simple model with a significant thymol/carvacrol interaction term, clearly demonstrating a synergistic, although minor effect. The study showed the measurement of stable and repeatable MIC values for individual and combinations of volatiles is possible using the new method. These findings strengthen the possibility of using natural essential oils as alternatives to chemicals to preserve food products.

The key disadvantage of the new method is the requirement to mix the liquid essential oils directly into the liquid media before solidification. This prevents its application to solid food systems. For solid food systems, a system capable of delivering stable flows of air with volatiles at high concentrations in the presence of high relative humidity was designed. With this system, well-controlled and stable air compositions were achieved over two days, making the system suitable for measurement of the inhibitory effects on spoilage organism growth. Although further optimisation of the design and control of this system is required, it has the potential for collection of accurate target headspace conditions for controlled volatile release active packaging design.

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List of Abbreviations

AI	Antifungal index
A_{GC}^{i}	Area of gas chromatogram peak from the injected volume of sample (Area).
CFU	Colony forming unit
C^{i}	Concentration of volatile organic compound (VOC) *i*, (mol.m^{-3})
C_{crv}	Carvacrol concentration (mol/m^{3})
DT	Detection time
EO	Essential oil
EVOH	Ethylene vinyl alcohol
FIC	Fractional inhibition concentration
Fh	Dry air flow rate for humidity (mL/min)
Fcrv	Dry air flow for carvacrol (mL/min)
FM	Mixture of dry air flow of carvacrol and humidity (mL/min)
GCMS	Gas chromatography–mass spectrometry
HDPE	High-density polyethylene
K_{GC}^{i}	Detector response or slope (mol. Area $^{-1}$) of a standard curve of VOC *i*.
LDPE	Low-density polyethylene
MAP	Modified atmosphere packaging
MFC1	Mass Flow Controller (mL/min) , humidity
MFC2	Mass Flow Controller (mL/min) , carvacrol liquid
MIC	Minimum inhibitory concentration
MLC	Minimum lethal concentration
OD	Optical density
OPP	Oriented Polypropylene

PD	Petri dish
PDA	Potato dextrose agar
PE	Polyethylene
PP	Polypropylene
ppm	Part per million
Psat	Saturated Vapour Pressure (Pa)
Pcrv	Partial Vapour Pressure for Carvacrol
P_h	Humidity Vapour Pressure (Pa)
$P_h/P_{h,sat\ (T2)}$	Humidity relative vapour pressure (Pa) at T_2°C
$P_{crc}/P_{crv,sat(T2)}$	Carvacrol relative vapour pressure (Pa) at T_2°C
RH	Relative humidity, (%)
RH_M	Relative humidity after mixture of carvacrol (%)
RH_2	Relative humidity at room temperature
Sp	Species
SPI	Soy protein isolate
T_1	Water bath temperature (°C)
T_2	Room temperature (25.0 ±1.0°C)
T_m	Temperature in mixing vessel (°C)
v/v	Volume per volume
Vol_{inj}	Injection volume of sample (m^3)
UV	Ultra-violet
w/w	Weight per weight

CHAPTER 1
PROJECT BACKGROUND

Many food products can be subject to spoilage by undesirable microbes such as fungi, yeast and bacteria (Appendini and Hotchkiss, 2002). Novel packaging technologies such as active packaging are being developed to extend the shelf life and improve the safety and sensory properties of fresh food (Ahvenainen, 2003). Additionally, active packaging may enhance food quality by reducing the need for additives and preservatives.

The principal commercial active packaging systems currently applied to food products have been summarized comprehensively by Suppakul *et al.* (2003) and Utto *et al.* (2005). Examples of active packaging systems are carbon dioxide scavengers and emitters (Smith *et al.*, 1995; Vermeiren *et al.*, 2000), oxygen scavengers (Ahvenainen and Hurme, 1996; Alvarez, 2000), ethylene scavengers (Brody *et al*., 2001), moisture absorption and control (Rooney, 1995; Ozdemir and Floros, 2004; Brody, 2005) and ethanol generators (Rooney, 1995; Brody *et al.*, 2001). Of these, active packaging systems which release active antimicrobial compounds such as ethanol, present an opportunity for prevention of spoilage organism growth and may be more widely used if system designs can be improved. The idea is to release antimicrobial compounds into the packaging headspace to prevent surface growth in foods, where a large portion of spoilage and contamination occurs (Vermeiren *et al.*, 2000; Brody *et al.*, 2001).

The antimicrobial activity of essential oils and their constituents has been extensively studied in recent years. The critical analysis of literature data by Suppakul *et al.* (2003), Burt (2004), Ceylan and Fung (2004) and Bakkali *et al.* (2008) showed that most of the oils tested have displayed some antimicrobial activity, and some have been shown to be more effective than others. Cavanagh (2007) highlighted that the inhibitory effect of some essential oils against fungi are greater in the vapour phase rather than through direct contact with liquid oil. The addition of naturally occurring plant volatiles, such as hexanal and 2-nonanone to the fresh products via product headspace, is known to have antifungal effectiveness and has been investigated by many researchers (Gardner *et al.*, 1990; Song *et al.*, 1996; Caccioni *et al.*, 1997; Song *et al.*, 1998; Wolford, 1998; Corbo *et al.*, 2000; Lanciotti *et al.*, 2003; Song *et al.*, 2007; Utto *et al.*, 2008; Almenar *et al.*, 2009). Similar studies have shown efficacy of selected essential oils against bacteria and fungi (Kim *et al.*, 1995b; Ben Arfa *et al.*, 2006; Chalier *et al.*, 2009; Gutierrez *et al.*, 2009b; Tzortzakis, 2009) and food-borne bacteria (Lopez *et al.*, 2005; Lopez *et al.*, 2007b; Nedorostova *et al.*, 2009).

The reported effectiveness of natural plant extracts suggests that further research is warranted to evaluate their antimicrobial activity and potential side effects in packaged foods (Vermeiren *et al.*, 2000). The advantages of using essential oils are because they are natural; generally recognised as safe (GRAS), and have a good wide spectrum of antimicrobial activity. It is likely that future research will make use of naturally-derived antimicrobial agents, bio-preservatives and biodegradable packaging materials to emphasize the qualities of antimicrobial packaging on food safety, shelf-life and environmental friendliness (Nicholson, 1998; Coma, 2008; Rodriguez *et al.*, 2008; Mastromatteo *et al.*, 2009).

One of the limitations of single-volatile-systems is the potential for modification of the sensory properties of fruit or foods. This is due to high concentrations being needed for some

volatiles to be effective as an antimicrobial agent and that may exceed the threshold for sensory impact, potentially altering the natural taste of the food. One approach to overcome this constraint is to combine multiple volatiles at reduced concentrations and hence avoid sensory changes. Pires *et al.* (2008) stated that natural antimicrobials are usually used in combination with others to provide hurdles to the growth of microorganisms without affecting sensorial and nutritional characteristics.

Some studies have shown that synergies between multiple volatiles could be achieved for some of the tested microorganisms. For example, a combination of the major constituents of basil oil (linalool and eugenol), enhanced the fungicidal effect for *Rhizopus stolonifer* and *Mucor sp*. even at low doses, compared to their effects when applied individually (Edris and Farrag, 2003). Pei *et al.* (2009) found that paired combinations between cinnamaldehyde/eugenol, thymol/eugenol, carvacrol/ eugenol and thymol/carvacrol revealed synergistic effects against pathogenic bacteria, e.g. *Escherichia coli*. Mixtures of cinnamon and clove essential oils at specific ratios have been reported to provide synergistic effects against *R. stolonifer (Sukatta et al., 2008)* and *Yersinia enterocolitica*, *Listeria monocytogenes* and *Bacillus cereus* (Goni *et al.,* 2009).

To be useful, volatile delivery systems that can be utilized to control the release of multiple volatiles into food products at specific concentrations are necessary. In this way, the volatile levels required to inhibit the microbial growth can be regulated over the duration of the food shelf life without reaching a threshold for sensory impact.

A key parameter required for the design and implementation of essential oil based antimicrobial systems is the target concentrations required in the headspace to inhibit or prevent spoilage. This is often referred to as minimum inhibitory concentration (MIC). In

synergistic systems, the total MIC of the combined volatiles is lower than the MIC of each individual component. If the concentration of volatiles required for inhibition is known, then the rate of release required to maintain this can be designed. Many methods for measurement of MIC exist and it is common that variations in reported MIC occur for the same system. The aim of this research was to analyse the systems for the measurement of MIC in food systems, for both individual and mixtures of essential oil volatiles. Through this analysis, reliable methods for MIC data that could be used in antimicrobial system design will be developed.

To achieve these goals the following objectives were proposed:

i. Select a model food and spoilage system together with at least two volatiles to use as case studies for the work.

ii. Characterise the dynamics of commonly used MIC measurement systems and demonstrate the underlying mechanism controlling them.

iii. Propose and test new methods to appropriately characterize MIC.

iv. Apply these methods to explore the synergistic effects of multi volatile systems.

v. Make suggestions on how to define the antimicrobial activity of essential oil volatiles in real food systems.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Since 1986, smart packaging systems have been progressively studied by researchers all over the world (Ahvenainen and Hurme, 1996). Labuza and Breene (1989) and Labuza (1996) were among the first to review the idea of active packaging. Rooney (1995), Alvarez (2000), Han (2005) and Kerry & Butler (2008) have compiled broader reviews of the field, including antibacterial packaging materials, intelligent packaging and scavenging systems for moisture and oxygen. The development of commercial active packaging systems has been led by Japan, mainly as oxygen scavengers and a range of emitters (Rooney, 1995). Active packaging systems technically have interactive functions to either control or monitor quality attributes targeted for the products (Rooney, 1995; Brody *et a*l., 2001).

This chapter reviews developments in active packaging systems with a specific focus on how to characterise the activity of potential natural volatile antimicrobial components that could be applied to these systems.

2.2 Overview of Active Packaging System Technologies for Food Products

Robertson (2006) highlights that active packaging has been classified in the literature by a number of differing definitions. Participants from the Antipack Project funded by the

European Commission defined active packaging "as packaging, which changes the condition of the packed food to extend shelf-life or to improve safety or sensory properties, while maintaining the quality of packaged food" (Ahvenainen, 2003). To sum up all definitions used by researchers (Ahvenainen and Hurme, 1996; Rooney, 2000; Vermeiren et al., 2000; Brody, 2002), Robertson (2006) has defined active packaging as "packaging in which subsidiary constituents have been deliberately included in or on either the packaging or the packaging headspace to enhance the performance of the package system".

An industry analyst (Harrington, 2011), reported that because of increasing food safety concerns, economic changes, demographic and health awareness, the global market for active and intelligent packaging is expected to double over the next decade. A report by an independent business information provider, Visiongain Research, stated that the global market for active, controlled and intelligent packaging is expected to grow 8.0% percent a year (Harrington, 2011). The report also indicated that the industry for active, controlled and intelligent packaging for foods and beverages will be worth US$17.23 billion in 2016, and was expected to increase to US$20.65 billion by 2021 (Harrington, 2011).

Table 2-1 lists some examples of active packaging systems.

Table 2-1: Examples of active packaging systems (Suppakul *et al.*, 2003; Ozdemir and Floros, 2004).

Type of active packaging system	Substances used and mode of action
Oxygen absorbing	Enzymatic systems (glucose oxidase-glucose, alcohol oxidase-ethanol vapour) Chemical systems (powdered iron oxide, catechol, ferrous carbonate, iron-sulphur, sulphite salt-copper sulphate, photosensitive dye oxidation, ascorbic acid oxidation, catalytic conversion of oxygen by platinum catalyst)
Carbon dioxide absorbing/ emitting	Iron powder-calcium hydroxide, ferrous carbonate-metal halide
Moisture absorbing	Silica gel, propylene glycol, polyvinyl alcohol, diatomaceous earth
Ethylene absorbing	Activated charcoal, silica gel-potassium permanganate, Kieselguhr, bentonite, Fuller's earth, silicon dioxide powder, powdered Oya stone, zeolite, ozone
Ethanol emitting	Encapsulated ethanol
Antimicrobial releasing	Sorbates, benzoates, propionates, ethanol, ozone, peroxide, sulphur dioxide, antibiotics, silver-zeolite, quaternary ammonium salts
Antioxidant releasing	[a]BHA, [b]BHT, [c]TBHQ, ascorbic acid, tocopherol
Flavour absorbing	Baking soda, active charcoal
Flavour releasing	Many food flavours
Colour containing	Various food colours
Anti-fogging and anti-sticking	Biaxially oriented vinylon, compression rolled oriented [d]HDPE
Light absorbing/regulating	UV blocking agents, hydroxy benzophenone
Monitoring	Time-temperature indicators
Temperature controlling	Non-woven micro-perforated plastic
Gas permeable/breathable	Surface treated, perforated or microporous films
Microwave susceptors	Metallised thermoplastics
Insect repellent	Low toxicity fumigants (pyrethrins, permethrin)

[a]BHA (Butylated hydroxyanisole) , [b]BHT (butylated hydroxytoluene), [c]TBHQ (tert-Butylhydroquinon), [d]HDPE (High density polyethylene

2.2.1 Active Antimicrobial Packaging System

Several of the systems described in Table 2-1 above are aimed at preventing or inhibiting microbial spoilage (e.g. ethanol or antimicrobial release). The interest in antimicrobial active packaging as a subset of packaging has dramatically increased in the past 10 years, as shown in Table 2.2. Kim *et al.,* (2008) stated that "the combination of various active packaging technologies with antimicrobial packaging has not been fully used. Nevertheless, the development of such combination systems in response to consumers' growing demands can be foreseen in the near future. In addition, the increasing attention to renewable sources of energy and more sustainable packaging will also force higher obligations on industry and government to support research and development and commercialization of antimicrobial packaging systems based on natural products".

Han (2000) wrote that active antimicrobial packaging is a promising and rapidly rising technology in which antimicrobial agents are incorporated into or coated onto food packaging materials to prolong the shelf-life of the packed food, usually by extending the lag phase and reducing the growth rate of microorganisms. The package contains an active component that interacts beneficially with the food or the internal gaseous phase in the package headspace, resulting in an extension of shelf life. Active packaging technologies actively inhibit mould and/or bacteria growth by releasing an additive with antimicrobial properties. This agent can be either immobilized within the package structure or incorporated into the substrate, pad, or sachet (Ceylan and Fung, 2004; Fisher and Phillips, 2008; Gutierrez *et al.*, 2008).

Table 2.2: The number of Articles in SCI journal published in international journals during 1991 to present (Dec 2014) through Keyword Science citation index.

Year	Antimicrobial Packaging	Active Packaging	Antimicrobial Packaging & Active Packaging
1991 – 2000	43	329	10
2001	10	41	1
2002	19	65	6
2003	23	68	8
2004	24	63	10
2005	32	72	17
2006	38	83	18
2007	50	109	19
2008	51	107	23
2009	74	135	33
2010	90	143	42
2011	119	309	32
2012	134	334	52
2013	165	385	69

Source: ISI Web of Knowledge (Database SCI-expanded) modified from Kim *et al.,* (2008) (as December 2014)

Controlled release technologies, are widely used in pharmaceutical applications, mainly in drug delivery systems (Langer and Peppas, 1981), and their design and performance under various conditions are well established (Brayden, 2003). Design processes for antimicrobial agent release systems are less formalised but have been explored for many years. For example, a patent for incorporating sorbic acid into foods and food wrappers to inhibit mould growth was obtained by Gooding/Best Foods in 1945 (Kerry and Butler, 2008). Many researchers have incorporated sorbic acid and potassium salts into food packaging materials to reduce microbial spoilage. For example, these compounds were mixed into:

- wax layers on cheese (Melnick and Luckmann, 1954; Smith and Rollin, 1954),
- wet wax coatings on packaging paper (Ghosh *et al*., 1977) and
- edible protein coatings on intermediate moisture foods (Torres and Karel, 1985).

Several categories of antimicrobials compounds have been tested for antimicrobial packaging applications. These are organic acids, fungicides, bacteriocins, proteins, enzymes, inorganic gases, spices, silver-substituted zeolite, and others (Devlieghere *et al.*, 2003; Suppakul *et al.*, 2003). Comprehensive reviews on antimicrobial food packaging have been published by Brody (2001; 2002; 2005), Brody *et al.,* (2008), Appendini and Hotchkiss (2002), Suppakul *et al.,* (2003), Han (2005), Joerger (2007) and Coma (2008).

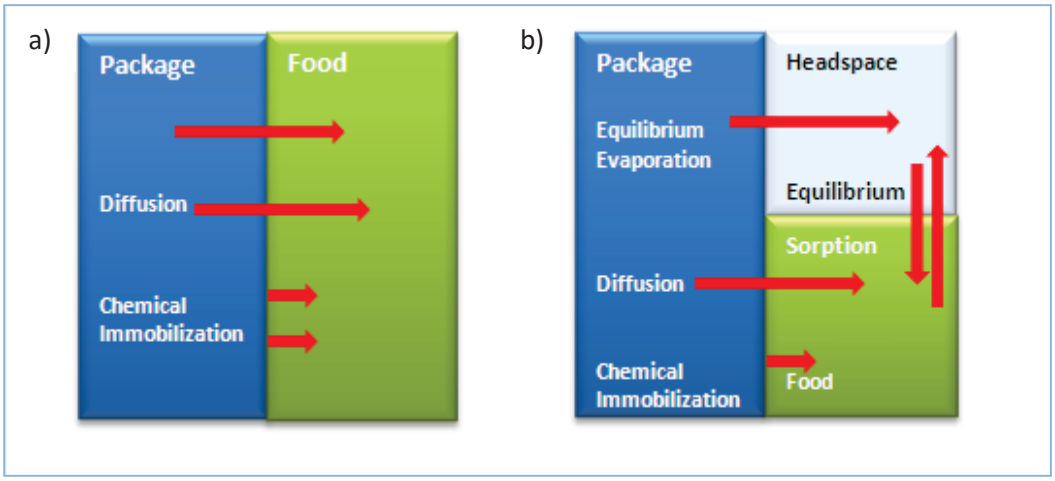
Han (2000), Appendini *et al.* (2002) and Suppakul *et al.* (2003) categorized antimicrobial packaging systems into several structures which are:

- Incorporation of volatile and non-volatile antimicrobials directly into polymers.
- Coating or adsorbing antimicrobial agents onto polymer surfaces.
- Immobilization of an antimicrobial agent to polymers by ionic or covalent linkages
- Use of antimicrobial trays or pads

- Use of polymers that are inherently antimicrobial.
- Addition of sachets containing volatile antimicrobial agents

Most food packaging systems approximate either a package/food system or a package/headspace/food system as depicted in Figure 2.1. A package/food system is a solid food product in contact with the packaging material, or a low-viscosity or liquid food without headspace. Individually wrapped ready-to-eat meat products, ''sous-vide'' cooked products and deli products are good examples. Most other systems (such as fresh horticultural products) include a headspace, providing three phases which interact. The relative behaviour of active substances in food packaging system is also shown in Figure 2-1 which demonstrates direct contact and vapour phase antimicrobial systems and their release mechanisms. System a) releases antimicrobial agents through diffusion directly to the food product, while system b) releases volatile antimicrobial by evaporation into the head space where it can then be absorbed into the food system.

Figure 2-1: **Food packaging systems and relative behaviour of active substance (Redrawn from Han (2000)**



These headspace systems are of particular interest in this study and are reviewed in more detail below.

2.2.2 Antimicrobial Packaging Using Gas-Based Systems

There are two categories of gas-based antimicrobial systems (Kerry and Butler, 2008) which are**:-**

I. Sachets

These systems modify the gas composition inside the package by releasing or absorbing gaseous components and have been used in meat, pasta, bakery and vegetable products. The sachets may contain moisture absorbers, oxygen scavengers, ethanol vapour generators, organic acids and surfactants.

II. Gas Flushing

In gas flushing, the headspace of the package is replaced by flushing with a specific gas composition prior to sealing, thereby modifying conditions within the system that inhibit microbial spoilage. The advantages of these systems are to eliminate the risk of accidental rupture of sachets and unintentional consumption of their content.

An example of gas flushing is the application of sulphur dioxide (SO_2) to control mould in the storage of berries and grapes in produce boxes which are palletized and stretch-wrapped (Kerry and Butler, 2008). The use of systems for the controlled release of sulphur dioxide from packaging is widely used in post-harvest fruits and vegetables to extend shelf life. For example, Guzev *et al.* (2008) found that > 0.4 ppm of sulphur dioxide was required to prevent *Aspergillus carbonarius* spoilage in table grapes. Even though sulphur dioxide has been found to be effective, concern has been raised that high concentrations of sulphites may affect the organoleptic properties of food (Adams, 1997). Another drawback of SO_2 fumigation is sulphite residue. The Food and Drug Administration (FDA) has set a maximum tolerance to sulphite residues at 10 mg/L while the European Union has forbidden the use of SO_2 (EU

Directive 95/2/CE). For this reason, more recent research has focused on using natural antimicrobials to reduce the use of synthetic fungicides.

For instance, the use of thymol, eugenol and menthol (Valero *et al.*, 2006; Martinez-Romero *et al.*, 2007), or carvacrol (Martinez-Romero *et al*., 2007) in combination with modified atmosphere packaging (MAP) has been used to improve table grape quality during storage. The addition of essential oils in MAP to other fruit has also been investigated to maintain quality and extend shelf-life (Serrano *et al*., 2008).

Matan *et al.* (2006) used filter paper absorbed with various combinations of clove and cinnamon oils to release volatiles into a modified atmosphere pouch so as to inhibit growth on inoculated plates. Pongjaruwat (2007) demonstrated the utilisation of silica gel as a porous material having high surface areas to adsorb clove oils which subsequently continuously released eugenol and other volatiles into a packaging system.

Tables 2-3 and 2-4 show some of the developments of antimicrobial film packaging with incorporated thyme and oregano oils and its major constituents, carvacrol and thymol which act as the active compound.

Table 2-3: Some of the developments of films using thyme and oregano oils and its major constituents carvacrol and thymol, reported in literature

Active Systems			
Films	Active ingredients	Summary	References
Chitosan-based film	Thyme, clove and cinnamon essential oils at 0.5, 1 and 1.5% v/v.	Thyme essential oil showed the highest antimicrobial efficacy against the gram-positive and gram-negative bacteria tested.	Guynot *et al*. (2003)
Oregano fortified PP and PE/EVOH film (4% w/w)	Oregano essential oils	Oregano-fortified PP films released higher levels of carvacrol and thymol compared to PE/EVOH. Shelf-life tests demonstrated that the antifungal activities of the films persisted for more than two months after their manufacture.	Lopez *et al.* (2007a)
High-density polyethylene (HDPE)	Carvacrol	The release of carvacrol from the polymer matrix can be controlled to make it suitable to be used in active packaging formulations.	Peltzer *et al.* (2009)
Zein-based film	Thymol	Thymol release rate decreased with the increase of the film thickness of both mono and multilayer films, without spelt bran addition.	Mastromatteo *et al. (*2009)
Soy Protein Isolated (SPI)-coated papers	Carvacrol	Increasing storage temperature (5, 20, 30°C) and RH (60, 80 and 100%) led to an increase of carvacrol diffusivity. The temperature and RH dependence of carvacrol release was related to the glass transition phenomenon and its effect on chain protein mobility and carvacrol diffusivity.	Chalier *et al.* (2009)
Nanocomposite Low-density Polyethylene (LDPE) + Organo-modified montmorillonite (clay)	Carvacrol	Antimicrobial properties of carvacrol-containing films showed significant activity against several bacterial strains.	Persico *et al.* (2009)

Continued from the previous page.

Active Systems			
Carvacrol-grafted chitosan nanoparticles	Carvacrol	Chitosan nanoparticles were grafted with eugenol and carvacrol. The in vitro diphenyl picrylhydrazyl assay and MICs test confirmed that the essential oil component grafted chitosan nanoparticles possessed both antioxidant and antibacterial activities.	Chen *et al.* (2009)
EVOH coating in Polypropylene (PP) film	Carvacrol	The partition equilibrium for carvacrol in the complex film largely favours (10,000-fold) the EVOH layer in dry conditions, although in humid conditions the solubility in both polymers is very close (4-fold). Kinetically, the presence of humidity increases the value of diffusion for carvacrol in EVOH from $3x10^{-19}$ m^2/s in dry conditions to $3x10^{-15}m^2/s$ in a wet environment.	Cerisuelo *et al.* (2012)
Polypropylene (PP) based film.	Carvacrol, Thymol	Carvacrol and thymol were incorporated in polypropylene (PP) films at three different concentrations: 4, 6 and 8 wt. % of both additives as well as an equimolar mixture of them. The presence of active components increased stabilization against thermo-oxidative degradations, with higher oxidation induction parameters. Thymol showed higher inhibition against bacterial strain present in food compared with carvacrol.	Ramos *et al*. (2012)
Polypropylene (PP) based film.	Carvacrol, Thymol	The migration of carvacrol and thymol as antioxidant (AO), from polypropylene (PP) packaging films containing the studied compounds at 80 g/kg separately and an equimolar mixture of them into food simulants was investigated. The release kinetics of AOs from PP films showed a Fichian behaviour with diffusion coefficients ranging from 1 to 2×10^{-14} m^2/s. The obtained results suggest that carvacrol and thymol show a potential use as AOs for active packaging for extending the shelf-life of food products.	Ramos *et al.* (2014)

LDPE (Low-Density Polypropylene), PP (Polypropylene), PE (Polyethylene),
EVOH (Ethylene vinyl alcohol), RH (Relative Humidity)

Table 2-4: Summary of active packaging systems that can release active compounds into the package headspace and their reported application in the food product.

Active Systems				
Active Agent	Carrier	Configuration	Food System	References
Eugenol-thymol-carvacrol	Sterilized gauze	Loose	Table grapes	Guillen *et al.* (2007)
2-nonanone	Alumine (F-1 80/100 mesh)	Sachet (Metallocene Polyethylene)	Strawberries	Almenar *et al.* (2007), Almenar *et al.* (2009)
Carvacrol	Gauzes	Loose	Table grapes	Martinez-Romero *et al*. (2007)
Methyl jasmonate, ethanol, tea tree oil and garlic oil	Filter paper strips	Loose	Fresh cut tomatoes	Ayala-Zavala *et al*. (2008c)
Cinnamon essential oil	Paraffin	Applied Coating to Paper	Sliced bread	Rodriguez *et al*. (2008)
Cinnamon essential oil	PP film	Incorporating in PP film	Bakery product (54 samples)	Gutierrez *et al*. (2009c)
Allyl-isothiocyanate, trans-cinnamaldehyde, garlic oil, and rosemary oil.	Soy Protein Isolate (SPI)	SPI Coated to OPP-PE film	Fresh sprout	Gamage *et al*. (2009)
Allyl isothiocyanate, cinnamaldehyde, and carvacrol	Wet filter paper	Loose	Spinach and lettuce	Obaidat and Frank (2009)
Allyl isothiocyanate	Polymeric resin (Accurel® XP200)	Sachet (HDPE)	Mozzarella cheese	Pires *et al*. (2009)
Oregano essential oil	perforated polyethylene, cellulose, and polyethylene	Adsorbent pads	Chicken drumstick	Oral *et al*. (2009)
Oregano essential Oil	Incorporated in ethylene-vinyl alcohol (EVOH)copolymer	PP/EVOH	Packaged Salad	Muriel-Galet *et al.* (2013)
Carvacrol	Incorporated in ethylene-vinyl alcohol (EVOH)copolymer	PP/EVOH/PP	Salmon	Cerisuelo *et al* (2013)
Carvacrol	Chitosan/ cyclodextrin film	Incorporating in Chitosan/cyclodextrin film	Chicken Breast fillets	Higuares *et al.* (2014)

OPP (Oriented Polypropylene), HDPE (High-Density Polyethylene), PP (Polypropylene).

According to Kerry *et al.* (2006) the development of a whole range of active packaging systems has attracted many researchers to investigate using essential oils as an antimicrobial agent incorporated into the packaging. Some examples include:

- The development of antimicrobial films by using essentials oil by Lopez *et al.* (2007). This was a new cinnamon-base active paper packaging system for use against *Rhizopus stolonifer*,
- the development of new antimicrobial active packaging for bakery products using cinnamon oil (Gutierrez *et al*., 2009c) and
- Incorporation of allyl isothiocyanate and trans-cinnamaldehyde as an antimicrobial compound in soy protein isolate coated oriented polypropylene/polyethylene (OPP/PE) films in sprout packaging (Gamage *et al.*, 2009).

2.2.3 Design of Volatile Release Systems

The design of optimised volatile release systems to extend shelf life is complex and must consider a number of different competing transport phenomena. Utto *et al*. (2008) studied and developed model-based design principles for volatile release active packaging systems using hexanal vapour into intact orange-red tomatoes (Royale), as an illustrative system. Similarly, Han (2000) summarised various models for mass transfer, sorption and diffusion of antimicrobial agents through films and food systems.

The key mechanism is to provide a source of the volatile either through the inclusion of sachets, trays or through it being absorbed into the packaging film itself. The rate it is released can be controlled through manipulation of the area, permeability and sorption properties of the entrapping component of the packaging. Once released into the packaging headspace, it can be

absorbed by the food material, by other packaging components (e.g. an outer film) and/or lost to the surroundings. The concentration of the active agent in the headspace is the result of a balance between these different rate processes.

The goal of the packaging system designer is to match the release rates with the losses to achieve a stable headspace concentration that is able to inhibit the growth of spoilage organisms on the product surface. A critical design parameter for volatile antimicrobial packaging systems is the target headspace concentration to be effective at extending product shelf-life. For this reason, the evaluation of the methods to determine this for different volatiles is the main objective of this work.

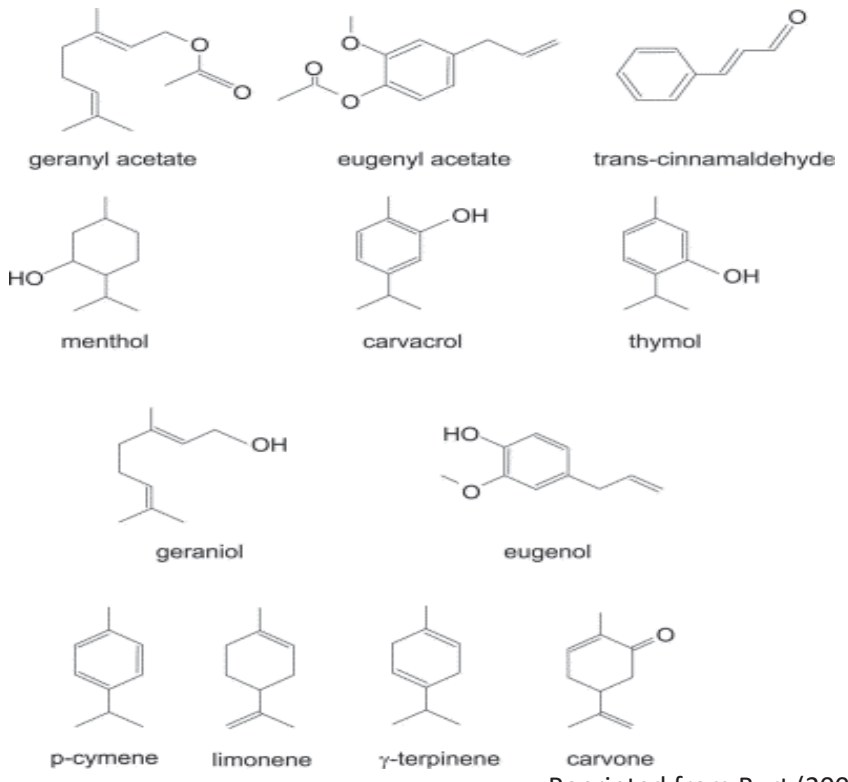
2.3 Essential Oils and their Antimicrobial Properties

Essential oils (EO) have been recognized for their antimicrobial and antifungal properties and are widely used in medicine and the food industry. Bassole *and* Juliani *(*2012) stated that "The essential oil term was used for the first time in the 16th century by Paracelsus von Hohenheim, who referred to the useful component of a drug as 'Quinta essential' ", (Guenther and Langenau, 1950). The first reference to the uses of EOs for therapeutic reasons was found in the Ebers papyrus. This document listed in detail more than 800 EOs remedies and treatments and showed that myrrh was a favourite ingredient, often mixed with honey and other herbs, because of its ability to inhibit bacterial growth. The first bactericidal experiment of EOs is said to have been carried out by de la Croix in 1881 (Boyle, 1955). However, since those times the use of EOs in medicine have gradually decreased as their use as flavour and fragrances increased (Guenther, 1948).

The antimicrobial activity of essential oils has been extensively studied and demonstrated against a number of microorganisms, usually using direct contact antimicrobial assays, such as diffusion or dilution methods (Gutierrez *et al.*, 2008; Nedorostova *et al.*, 2009), and has been

reviewed comprehensively by many authors (Burt, 2004; Simic *et al.*, 2005; Yonzon *et al.*, 2005; Veldhuizen *et al.*, 2006; Vagionas *et al.*, 2007).

Numerous studies have investigated the major components of essential oils such as eugenol, thymol, carvacrol, terpineol, geraniol, linalool, carvone, citronellol, limonene, cinnamaldehyde and eucalyptol (Arras and Grella, 1992; Kim *et al.*, 1995a; Bagamboula *et al.*, 2004; Valverde *et al.*, 2005; Valero *et al.*, 2006; Nostro *et al.*, 2007; Liolios *et al.*, 2009). The chemical structure of many of these compounds are shown in Figure 2.2. The antimicrobial activity of whole essential oils has been reviewed comprehensively by Kalemba and Kunicka (2003), Burt (2004), and Bakkali *et al.* (2008).



Reprinted from Burt (2004)

Figure 2-2: Structure formulae of selected components of essential oils.

Since the essential oils consist of complex mixtures of numerous molecules, Bakkali and co-workers (2008) point out that the biological effects may be either the result of a synergism of all molecules or only those of the major molecules present at the highest levels. Several investigators have concluded that complete essential oils have higher antibacterial activity than a mixture of the major components, which suggests that the minor components are critical to the activity and may contribute to a synergistic effect (Gill *et al.*, 2002; Burt, 2004; Nazer *et al.*, 2005; Carraminana *et al.*, 2008). For example, Ultee *et al.,* (2000b) showed that a mixture of major components with weaker activity components can reveal a synergistic effect. However, Consentino (1999) and Ipek (2005) demonstrated that the phenolic components, such as thymol and carvacrol, the major constituents of some essential oils, are mainly responsible for the antibacterial properties.

A summary of the major components of essential oils that exhibit antibacterial properties is presented in Table 2-5.

Table 2-5: Major component of essential oils that exhibit antibacterial properties.

Common name of Essential Oils	Latin Name of plant source	Major Components	Approximate Composition[b] (%) of total volatiles	References
Cilantro	*Coriandrum sativum* (immature leaves)	Linalool E-2-decanal	Trace - 26 Trace - 20	Delaquis *et al.* (2002), Matasyoh *et al.* (2009)
Black Mustard	Brassica nigra	Allyl-isothiocyanate Allyl sulfide	>98 <1	Suhr & Nielsen (2003), Mejia-Garibay *et al.* (2015)
Cilantro	*Coriandrum sativum* (seeds)	Linalool	11 – 80	Delaquis *et al.* (2002), Msaada *et al*. (2007)
Orange (Peel)	*Citrus sinensis*	Limonene	>95	Jose Velazquez-Nunez *et a*l. (2013)
Cinnamon	*Cinnamomum zeylandicum*	Trans-cinnamaldehyde	50 – 60	Baratta *et al.* (1998), Matan *et al.* (2006)
Oregano	*Origanum vulgare*	Carvacrol Thymol g-Terpinene p-Cymene	Trace-80 Trace-64 2 –52 Trace-52	Bozin *et al.* (2006), Ai-Turki *et al.* (2008)
Rosemary	*Rosmarinus officinalis*	a-pinene Bornyl acetate Camphor 1,8-cineole	2 –25 0 –17 2 –14 3 –89	Baratta *et al.* (1998), Del Campo *et al.* (2000), Carraminana *et al.* (2008)
Sage	*Salvia officinalis L.*	Camphor a-Pinene h-pinene 1,8-cineole a-tujone	6 –15 4 – 5 2 –10 6 –14 20–42	Marino *et al.* (2001), Edris (2007)
Clove (bud)	*Syzygium aromaticum*	Eugenol Eugenyl acetate	75–85 8 –15	Chaieb *et al*. (2007), Matan *et al*. (2006)
Thyme	*Thymus vulgaris*	Thymol Carvacrol g-Terpinene p-Cymene	10–64 2 – 11 2 –31 10–56	Cosentino *et al.* (1999), Marino *et al.* (1999), Arras and Usai (2001), Guynot *et al.* (2003), Kalemba and Kunicka-Styczynska (2003), Bagamboula *et al.* (2004), Lopez *et al.* (2007b)
Basil	*Ocimm basilicum*	Linalool Estragole Eucalyptol	42 – 57 1 - 27 8	Edris and Farrag (2003), Bagamboula *et al.* (2004), Lopez *et al.* (2005)

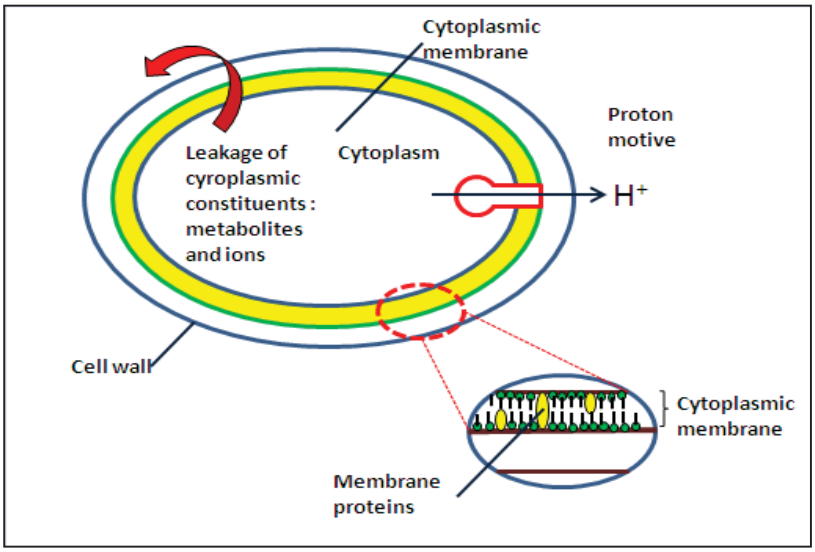
[a]Essential oils which have been shown to exert antibacterial properties in vitro or in food models and for which the composition could be found in the literature. [b]Percentage of totals volatiles rounded to the nearest whole number.

2.3.1 Mechanism of Antimicrobial Agents

Natural antimicrobial compounds have an ability to interfere with the metabolism of microorganisms in the same way that synthetic preservatives do. This reactivity also affects the food system or the package and high concentrations are sometimes required to compensate for their inactivation (Ayala-Zavala et al., 2008a).

The critical analysis of literature data by Kalemba and Kunicka-Styczynska (2003), Suppakul *et al.*(2003), Burt (2004), Ceylan and Fung (2004) and Bakkali *et al.* (2008) showed that most oils tested had antimicrobial activities. Numerous reports have emphasized that components of some plant essential oils, for example, thymol, carvacrol, linalool, cinnamaldehyde and eugenol, are chiefly responsible for the antimicrobial properties, providing a spectrum of antimicrobial activity (Bagamboula *et al.*, 2004; Burt, 2004; Chalier *et al.*, 2007; Rodriguez *et al.*, 2007; Rodriguez *et al.*, 2008; Gutierrez *et al.*, 2009a). These components may act as either bactericidal or bacteriostatic agents, depending upon the concentration used (Kalemba and Kunicka-Styczynska, 2003). The mode of action of essential oils against microorganisms is under debate, but some studies suggest that these compounds penetrate the cell and disturb the cellular metabolism (Kalemba and Kunicka-Styczynska, 2003; Burt, 2004).

Figure 2-3 illustrates the location and mechanism of action of essential oils towards microorganisms.



Redrawn from Burt (2004)

Figure 2-3: **Locations and mechanisms in the bacterial cell thought to be sites of action of essential oil components: degradation of the cell wall (Helander *et al*., 1998); damage to cytoplasmic membrane (Sikkema *et al.*, 1994; Ultee *et al.*, 2000a; Ultee *et al.*, 2002); damage to membrane proteins (Ultee *et al*., 1999); leakage of cell contents (Helander *et al*., 1998); coagulation of cytoplasm and depletion of the proton motive force (Ultee *et al.*, 1999; Ultee and Smid, 2001)**

Some studies suggest that phenols such as carvacrol and eugenol, disturb the cellular membrane and react with the active sites of enzymes and by inactivating or destroying the genetic material (Kim *et al.* 1995b). Kalemba and Kunicka- Styczynska (2003) reported that the mode of action differs between compounds. Edris and Farrag (2003) identified that there was a relationship between the chemical structures of the most abundant compounds in the essential oil and the antimicrobial activity.

The mechanisms of action against microorganisms was discussed by researchers, for example, degradation of the cell wall (Helander et al., 1998); damage to cytoplasmic membrane (Sikkema et al., 1994; Ultee et al., 2000b; Ultee et al., 2002); damage to membrane proteins (Ultee et al., 1999); leakage of cell contents (Helander et al., 1998); coagulation of cytoplasm and depletion of the proton motive force (Ultee et al., 1999; Ultee and Smid, 2001). Similarly Shao *et al.* (2013)

found the main reason for tea tree oil antifungal activity was due to increases in membrane leakage.

Pauli (2005) reviewed the action of essential oils on Candida for medical application. Mechanisms cited largely involved uptake of the oils into the cell membrane, accumulating between the lipid bilayers and altering membrane fluidity and function. The resulting disruption to the lipid packing in the membrane causes membrane leakage.

Lambert *et al.* (2001) showed using innovative techniques that thymol and carvacrol increased membrane permeability through a staining technique, dissipated pH gradients through a fluorescent probe and caused leakage of inorganic compounds in *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

For most of the mechanisms identified in the literature, the concentration of the active agent in the cell membrane is of key importance. Pauli (2005) explained that the antimicrobial activity can be linked to the lipophilicity of the compounds which can be characterised by the octanol/water partition coefficient (K_{ow}). This is the equilibrium concentration ratio of the compound of interest between octanol and water. Octanol is used as a model compound with similar properties to membrane material. The higher the partition coefficient, the more lipophilic the compound will be, meaning higher dissolved concentrations would be present in the membrane (Pauli 2005, Sikkema *et al.* 1995). It makes sense then, that compounds with higher partition coefficients would be expected to have higher antimicrobial activity.

While octanol/water partition coefficients are used in pharmacology to explain the relative antimicrobial actions of different compounds, other aspects of the compound are also important. Maffei *et al*. (2001) for example, showed in a model system of root cells from cucumber perfused with peppermint essential oil at different concentrations, that the essential

oils with higher K_{ow} (such as limonene) did not damage membrane integrity as much as oils with lower K_{ow} (such as menthol). These oils with lower octanol/water partition coefficient however did have higher solubility and therefore more accumulation of essential oil during the perfusion process would have occurred. As such, the dynamics of how the essential oil is added also impacts cellular damage.

Ben Arfa *et al.* (2006) compared the antimicrobial activity of carvacrol, eugenol, menthol and two synthesised carvacrol derivatives against several bacteria, yeast and a fungus. They compared activity measured when the antimicrobial compounds were delivered in the liquid and gas phases. In both cases, the order of antimicrobial effectiveness of the different compounds was the same and broadly followed the order of hydrophobicity. They showed however that free hydroxyl groups as part of the molecule functions to act as a proton acceptor and reduces the pH gradient across the cell membrane. From this it is clear that the molecular structure as well as its hydrophobicity/lipophilicity are important to a compounds antimicrobial activity.

While the microbiological and pharmacological literature refer to the partitioning behaviour of antimicrobial compounds, there is no reference to it in the food literature. Despite this, it appears to be a valuable tool in understanding antimicrobial activity and how to design antimicrobial systems.

2.3.2 The Use of Volatiles as Antimicrobial Agents

Several attempts have been made to utilize the volatile nature of essential oils for inhibition of microbial spoilage (Ward *et al*., 1998; Edris and Farrag, 2003; Ayala-Zavala *et al*., 2008b). For example, natural plant volatiles such as trans-2-hexenal (Corbo *et al*., 2000), hexanal (Utto *et al*., 2008) and 2-nonanone (Almenar *et al*., 2009) have well known antifungal activity but limited

post-harvest use due to their high volatility. It has been shown that the antifungal effectiveness of the volatiles varies depending on their concentration and the treatment duration (Hamilton-Kemp *et al*., 1996; Corbo *et al*., 2000; Almenar *et al*., 2007).

Some examples of the use of volatiles for antimicrobial packaging systems have been reported in the literature. For example the quality and safety of table grapes was improved by the combination of modified atmosphere packaging (MAP) and eugenol, menthol or thymol (Valverde *et al.*, 2005; Valero *et al.*, 2006) and eugenol, thymol or carvacrol (Guillen *et al.*, 2007; Martinez-Romero *et al.*, 2007).

Essential oils such as clove oil (Matan *et al*., 2006) and thyme oil (Cosentino *et al.*, 1999; Inouye *et al.*, 2000) are important sources that can generate antimicrobial vapours under typical food storage conditions, however the effect of blending two natural antibacterial agents in the vapour phase in the active packaging system has still not yet been explored extensively. One study by Goni *et al.,* (2009) demonstrated a synergistic effect of the combination of clove and cinnamon oils (1:1, v/v) in the vapour phase via *in vitro* control media towards *Y. enterocolitica and S. cholaraesuis*.

Inouye *et al.* (2000) investigated the relative antimicrobial activity of essential oils when contacted via the vapour phase. They found three levels of activity; citron, lavender and tea tree oils exhibited fungistatic behavior, perilla and lemongrass oils showed fungicidal action and cinnamon bark and thyme oils showed intermediate action. The amount of essential oils deposited onto the mycelia and agar was broadly correlated with the volatility of the oils, suggesting some activity could be due to rapid uptake by the mycelia directly from the vapour phase. In the same study, it was shown that inhibition of fungal growth was better for vapour contact compared with direct solution contact. Careful analysis of the results however, show that the concentrations used for direct contact (uniformly distributed throughout the media)

are or similar magnitude to the absorbed concentration in the vapour contact method when measured as a volume average. If it is considered that the equilibrium solid phase concentrations for the vapour contact experiments are at least 4x higher than the concentrations used in the direct contact method and that the surface of the agar would have been much closer to this equilbrium value, the observed results are not surprising. These results show that the dynamics of volatile release and absorption must be considered when interpreting experimental results.

Mejia-Garibay *et al.* (2015) made similar conclusions from a comparison of direct vs vapour phase contact experiments for black mustard essential oil on Aspergillus and Penicillium fungi. They say vapour phase release exhibits greater fungacidal activity because of lower concentrations needed to inhibit growth in vapour phase experiments. They do point out that diffusion into the agar medium was slow, and therefore with vapour phase experiments, the surface of the media is much higher than the average concentrations in the media. The main component of black mustard essential oil is allyl-isothiocynate which has a high vapour pressure (487 Pa stp) and a high water to air partition coefficient K_{wa}=101 (U.S. Environmental Protection Agency [EPA], 2012).

Reported vapour phase minimum inhibitory concentrations for tea tree oil were lower for vapour phase experiments compared with direct solution contact methods (Shao *et al.* 2013). If the data is analysed in more detail however, it can be seen (as discussed above), that the comparison is not equal. The active compounds in tea tree oil are 1-8-cineole and terpin-4-ol. The reported air phase concentrations required to inhibit growth correspond to 14x saturation for 1-8-cineole and 1224x saturation for terpin-4-ol (EPA, 2012). This is clearly not possible, and therefore some time is required before all the added essential oil can be released from the filter paper used as the vapour source. If the water-air partition coefficient is considered (222, and 2004 for 1-8-cineole and terpin-4-ol respectively: EPA, 2012), then it can be seen that both

compounds are highly soluble in the agar media. As such it is expected in vapour phase experiments, that the surface of the media (where growth is occuring), will receive much higher concentrations of essential oil, than that used in the direct contact experiments.

Much more similar MIC values were reported for direct solution and vapour contact of orange peel essential oil on *Aspergillus favus* (Vekazquez-Nunez *et al.* 2012). In this study MIC values were reported as 8 $g.L^{-1}$ air for vapour contact and 16 $g.L^{-1}$ media for direct contact although it was commented that the direct contact method was faster. On the surface of these results (and the authors conclusion), it also appears that the vapour phase provides a more effective anitimicrobial activity. On further analysis however it becomes apparent that the reported vapour phase MIC corresponds to 1.43 atm (greater than atmospheric pressure) and >1000 x the saturated vapour pressure of the main orange peel oil component, limonene (EPA, 2012). This is clearly not sensible. The release and absorption of the limonene from the filter paper source, will take time and could explain the observed difference in time required to observe antimicrobial effectiveness.

From this analysis it can be concluded that the essential oil concentration of importance for anitimicrobial activity, is the concentration in the spoilage organism membrane. At the food surface there is a three way equilibrium between the membrane of the organism, the surrounding media/food and the headspace. As a consequence, the headspace concentration, together with the partitioning behaviour between the phases is a useful measure of the level of antimicrobial compound required for effectiveness. Furthermore, the dynamics of release in antimicrobial activity measurements must be considered carefully when interpreting data, especially if it is to be used as a target for active packaging controlled release targets.

2.3.3 Minimum Inhibitory Concentration

Minimum inhibitory concentrations are important to verify the resistance of microorganisms to an antimicrobial agent as well as to examine the activity of new antimicrobial agents. A lower MIC is an indicator of a better antimicrobial agent. Generally, minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial required to inhibit the visible growth of a microorganism after overnight incubation. If measured appropriately, MIC could potentially be used as the target for controlled release active packaging designs.

In food applications, it is important to gain accurate knowledge of minimal inhibitory concentrations (MICs) in order to minimize the amount used and to reduce the risk of sensory impact while maintaining antimicrobial effectiveness. Most researchers cite MICs as a measure of the antibacterial performance (Burt, 2004). However, different researchers use different MICs definitions making reported MICs difficult to compare. A list of the most frequently used terms in antibacterial activity testing of essential oils is presented in Table 2.6.

From Table 2-6, it can be concluded that the MIC definition can be set according to the objective of the experiment. The level of the active compound concentration required to inhibit the growth of the microorganism can vary, depending on the target period of the incubation. In order to evaluate the efficacy between method and active compound, the definition of MIC should be standardized (Guynot *et al*., 2005; Ben Arfa *et al*., 2006).

Table 2-6: Terms used in antibacterial testing

Term	Definition, with reference to concentration of Essential Oil	References
Minimum inhibitory concentration (MIC)	Lowest concentration resulting in maintenance or reduction of inoculums viability	Carson *et al.* (1995b), Pei *et al.* (2009)
	Lowest concentration required for complete inhibition of test organism up to 48 h incubation	Canillac and Mourey (2001)
	Lowest concentration inhibiting visible growth of test organism	Delaquis *et al.* (2002)
	Lowest concentration resulting in a significant decrease in inoculums viability (>90%)	Cosentino et al. (2009)
	Lowest concentration resulting in inhibition of growth of microorganism at parallel growth on agar media	Kunicka-Styczynska et al. (2009)
	Lowest concentration which made clearly visible inhibition zone (expressed as microliters of EOs per volume unit of atmosphere above the organism growing on the agar surface)	Nedorostova *et al.* (2009)
	Lowest concentration of oil preventing visible growth of microorganisms	Tyagi *et al.* (2013)
Minimum fungicidal concentration (MFC)	Lowest concentration at which no growth was observed after sub-culturing into fresh media.	Tyagi *et al.* (2013)
	MFC is the lowest concentration of EOs/preservatives at which there was no revival of growth of the inhibited fungal inoculum on treatment-free PDA plates because of permanent inhibition.	Prakash *et al.* (2012)
Minimum bactericidal concentration (MBC)	Concentration where 99.9% or more of the initial inoculum is killed	Carson *et al.* (1995a)
	Lowest concentration at which no growth is observed after subculturing into fresh broth	Cosentino *et al.* (1999)
Bacteriostatic concentration	Lowest concentration at which bacteria fail to grow in broth but are cultured when broth is plated onto agar	Smith-Palmer *et al.* (1998)
Bactericidal concentration	Lowest concentration at which bacteria fail to grow in broth and are not cultured when broth is plated onto agar	Smith-Palmer *et al.* (1998)

To date, only a few studies have investigated the efficacy of essential oil in vapour on selected fungi (Inouye *et al.*, 1998; Inouye *et al.*, 2000; Edris and Farrag, 2003; Matan *et al.*, 2006; Tunc *et al.*, 2007) food-borne bacteria, and yeast (Lopez *et al.*, 2005; Lopez *et al.*, 2007a; Goni *et al.*, 2009; Nedorostova *et al.*, 2009, Tyagi *et al.*, 2013).

The majority of studies in this area have utilized the micro-atmosphere method assay (also known as the 'reverse Petri plate' or fumigation method) which is well established in the pharmaceutical field (Kalemba and Kunicka-Styczynska, 2003, Inouye *et al.,* 1998, Inouye *et al.,* 2006, Cavanagh, 2007), however the methodology and reporting of volatile activity against fungi is inconsistent (Kalemba and Kunicka-Styczynska, 2003, Burt, 2004). Figure 2-4 summarises the common methods to measure antimicrobial or antifungal activity (Kalemba and Kunicka-Styczynska, 2003).

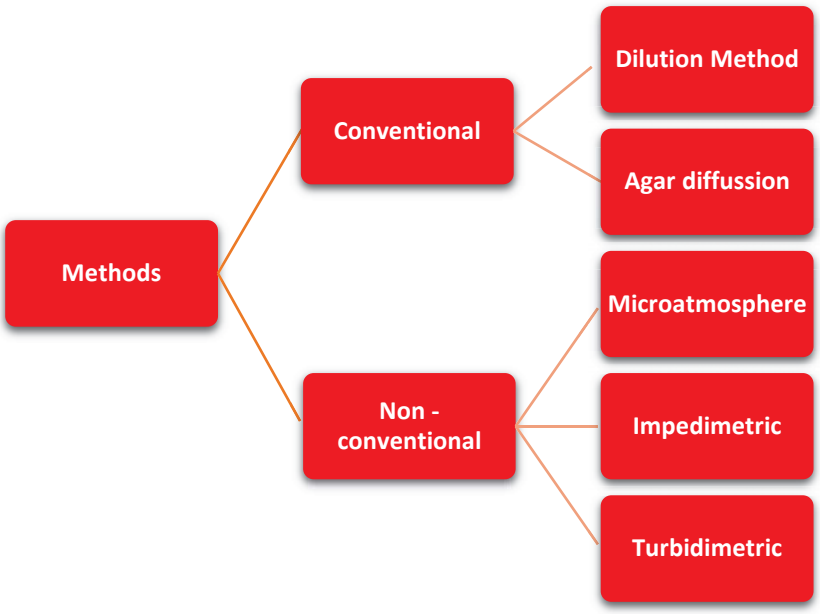


Figure 2-4: Common methods to measure antimicrobial or antifungal activity.

2.3.3.1 Conventional Methods

The two categories of common conventional methods used to measure antimicrobial activity are the agar diffusion methods and the dilution methods.

2.3.3.1.1 The Agar Diffusion Method

The agar diffusion method is widely used for antimicrobial activity assessment activity. However, the weakness is it is not always repeatable (Kalodera *et al*. 1997). This method uses Petri dishes 5-12 cm diameter (standard size 9 cm) filled with 10 to 20 mL of agar broth which is set and then surface is inoculated with the test microorganism. The essential oil is incorporated either on a paper disc or into a well, made in the agar medium. A series of Petri dishes with the same amount of essential oil solution at different concentrations are prepared. The paper disc is sometimes immersed in the oil solution. The plates are stored for a specified period to allow the essential oils to diffuse into the agar, before being incubated (Hinou *et al*. 1989). The effectiveness of the essential oil is calculated by the size of the zone of microorganism growth inhibition around the disc or well and the results can be presented as ***0, +, ++, etc***. (Kalemba and Kunicka-Styczynska, 2003).

According to Kubo *et al.* (1995) and Griffin *et al.* (1999); the diffusion method is considered unsuitable for essential oils because the possibilities of volatiles components being evaporated with the dispersing solvent during the incubation time. Moreover, poorly soluble components do not diffuse well in the agar broth. However, it is still a commonly used technique because it is easy to perform and requires only small amounts of essential oils. As a result it is used as a pre-screening method for a large number of essential oils as the most active ones may be selected for further analysis by other refined methods (Smith-Palmer *et al*. 1998).

2.3.3.1.2 The Dilution Method

The dilution method is mostly applied for bacteria and for fungi and some modifications with liquid broth are used. Normally, Petri dishes or tubes are used for agar broth cultures and conical flasks filled with 100 mL medium are used to cultivate liquid broth cultures (Deans *et al*., 1994). The broths containing a known amount of added antimicrobial are sequentially diluted prior to inoculation and incubation. For example Matan (2008) used this technique to determine antifungal activities of anise and citrus oils against moulds on rubber wood. The author added 50 μL of the essential oils at a concentration 20 -200μL mL^{-1} to 5 mL of yeast extract sucrose broth tubes containing 10^7 spores mL^{-1} (counted using a haemocytometer method). Vegetable oils were used as control. The tubes were then incubated in an incubation shaker at 30°C for 3 days. The inhibitory growth index in the liquid is calculated by the changes in mould's biomass compared to the control culture (Deans *et al*. 1994). The results can be presented in two ways:

- The growth inhibition index, defined as the percentage ratio of the control growth culture without essential oil
- Minimal inhibitory concentration – MIC or minimal lethal concentration (MLC). The highest dilution that showed no visible growth was regarded as minimum inhibitory concentration (MIC). The cells from tubes showing no growth were subcultured onto suitable agar plate media to determine if the inhibition was reversible or permanent. The minimal lethal concentration (MLC) was determined as the highest dilution (lowest concentration) at which no growth occurred on the plate (Rasooli *et al*., 2006).

The weakness of this method is the heterogeneous way of results presentation which makes it difficult to compare the results of different authors. A similar problem is posed by the variety of

essential oil concentration ranges and the ways concentration is expressed (Kalemba and Kunicka-Styczynska, 2003).

2.3.3.2 Non- Conventional Methods

Several non-conventional MIC measurement techniques have also been applied.

2.3.3.2.1 Micro Atmosphere Method

The micro atmosphere method is widely used and is also known as the Reverse Petri dish method. This method is a slightly modified agar disc diffusion method and considered perfectly suitable for the estimation of essential oil activity in the vapour phase. It is used to define the activity of essential oils which are to be employed as atmospheric preservatives. In this technique, a filter paper disc, moistened with essential oil is attached to the lid of an inoculated, agar filled Petri dish, which is then inverted and incubated. The results are presented as the diameter of the microorganism growth inhibition region or as the essential oil minimal inhibitory concentration which inhibits the total growth of the microorganism (Delespaul *et al*., 2000).

Recently, Kloucek *et al*., (2012) modified the most commonly used disc volatilization method by using a four-section Petri dish (PD, 90 mm diameter), with a large filter paper disc (85 mm diameter) evenly impregnated with 69 types of essential oils and medium containing lid. Their findings showed that in comparison with the normal disc volatilization method, this screening method significantly reduced labour and the material needed. Furthermore, the composition of headspace is more uniform than in the case of 6 mm disc, where the different volatility of particular compounds could influence the results.

2.3.3.2.2 Turbidimetry

The turbidimetry method uses equipment to scan small amounts of growth medium by reading the Optical Density (OD). The liquid cultures containing essential oils at different concentrations are placed in 96-100 flat bottom well polystyrene microtiter test plates (type ELISA, Petra Plastic, etc.). After incubation, the plates are vigorously shaken on a vibrating platform and immediately scanned with a multi-scan photometer (Hili *et al.*, 1997; Ponce *et al.*, 2003). The weakness of this method is that the turbidity of the oil-water emulsion can interfere with the endpoint reading and thus, indicators are sometimes used (Kalemba and Kunicka-Styczynska, 2003).

2.3.3.2.3 Bioimpedimetric Method

The bioimpedimetric method is based on the correlation between the altered electrical parameters of a growth culture, strictly linked to the metabolic activity of the tested microorganism, and the number of cells regarded as colony forming unit per mL (CFU/mL). The monitoring system's procedures results in the detection time (DT), defined as the time required by the growing culture to reach the threshold quantity (usually 10^6 CFU/mL).

The essential oil activity against the microorganism can be expressed as either the delay in the detection time of the culture with the essential oil addition compared to that of the negative control culture or the cell number decease (Wan *et al.*, 1998; Marino *et al.*, 1999). The commercially available impedance monitoring systems are equipped with multiple units allowing from 32 to 512 tests to be performed at the same time, which makes the experiment quick and reliable. The wells of the unit differ in capacity from 2 to 100 mL and are tightly covered by plastic lids preventing the essential oils from evaporating. The other advantages of the impedimetric assessment of essential oil bioactivity are the shorter times for the

experiment; two or three times faster depending on the type of microorganism (Rule, 1997; Kunicka-Styczynska *et al.*, 2009).

2.3.3.3 Application to Vapour Phase Measurement

The antifungal activity of essential oil volatiles was first reported by Maruzzella and Liguori (1958), and specific antifungal activity associated with these volatiles has focused on inhibition of either food spoilage or post-harvest plant pathogens (Nielsen and Rios, 2000). Methodology and reporting of volatile activity against fungi is inconsistent, and the majority of studies in this area have utilised the 'reverse Petri plate' (micro atmosphere) or fumigation method (Cavanagh, 2007).

Vapour based active compounds are known to be absorbed by foods and plastic materials and can be lost from the system during the incubation process (Gocho, 1991a; Gocho, 1991b; Inouye *et al.*, 1998; Nedorostova *et al.*, 2009). Because of this, knowledge of transport phenomenon such as the diffusion and partition coefficients of active compounds (Savary *et al.*, 2006; Juteau-Vigier *et al.*, 2007; Savary *et al.*, 2007; Cayot *et al.*, 2008; Deleris *et al.*, 2008; Deleris *et al.*, 2010) in foods and plastic components that influence the composition and its rate of change of the gas phase are required, as reported by several authors (Peyches-Bach *et al.*, 2009; Cerisuelo *et al.*, 2012).

The reporting of effective volatile concentration in relation to airspace, evaporation speed, exposure times, microbial strains and definitive source of the volatile antimicrobial activity is variable. There are also method variations in terms of Petri dish sizes, filter paper diameter, and choice of media. For example, exposure time varies between reports from up to 42 days, to

exposure times of only a few minutes to hours (Inouye *et al.*, 2000; Nielsen and Rios, 2000; Guynot *et al.*, 2005).

Table 2-7 shows the methods used to determine antimicrobial activity in vapour contact techniques while Table 2.8 summarizes the data of minimum inhibitory concentration reported in the literature. Table 2-9 shows activities of the major components of sweet basil, peppermint, and clove and cinnamon essential oils in the vapour phase against selected bacteria and fungi.

Table 2-7: Some of the method used to determine antimicrobial activities with vapour contact assay.

Antimicrobial Agent	Purpose	Amount added	Carrier/container	Test type media	Target microorganism	References
Tea tree oil	To determine antifungal activity and mode of action towards *Botrytis cinerea*	8, 32,56 and 80 µL (0.1, 0.4, 0.7 and 1.0 mL/L_{air})	• Standard Glass Petri Dish • 20 mm diameter Filter paper • Sealed with Parafilm at edges	Petri dishes incubated at 25°C for 5 days	*Botrytis cinerea*	Shao *et al.* (2013)
Origanum, Lavender and Rosemary	To determine antifungal activities of the essential oils of various plants against tomato grey mould	The concentrations tested were 0.4 to 25.6 µg/ml. To obtain final concentrations of 0.05 to 1.6 µm/ml air.	• Standard glass Petri dishes (90 × 20 mm, which offer 80 ml air spaces after the addition of 20 mL agar media) were used. • 10 mm diameter, Whatman no.1) • sealed with a para film	Petri dishes incubated at 22°C for 7 days	*Botrytis cinerea*	Soylu *et al.* (2010)
Allium sativum *Armoracia rusticana* *Ocimum basilicum var. grant verte* *Origanum majorana* *Origanum vulgare* *Satureja montana* *Thymus pulegioides* *Thymus serpyllum* *Thymus vulgaris*	To determine antimicrobial properties of the selected essential oils	(32, 16, 8, 4, 2, 1, 0.5, 0.25 µL)	• sterile blank filter disks (ø = 6 mm) • Standard Petri Dish – 90 mm diameter • Sealed with vinyl tape • a little amount of agar placed on the lid.	The Petri dishes were incubated at 37 °C for 18–24 h.	*Escherichia coli,* *Listeria monocytogenes,* *Pseudomonas aeruginosa,* *Salmonella enteritidis,* *Staphylococcus aureu*	Nedorostova *et al*. (2009)

Continued from previous page

Antimicrobial Agent	Purpose	Amount added	Carrier/container	Test type media	Target microorganism	References
Clove oil Cinnamon oil	To determine antifungal activity	Undiluted 20 mg/ Plate	• Filter paper (Whatman 1) 12 mm diameter • (Standard Petri dish / 82 mm)	Potato dextrose Agar (PDA). Incubated 7 days, room temperature	*Aspergillus niger, Alternaria alternata Colletotrichum gloeosporioides, Lasiodiplodia theobromae, Phomopsis viticola and Rhizopus stolonifer*	Sukatta *et al.* (2008)
Carvacrol	To control fungal decay during table grape storage	0.1, 0.4, 1 or 2 mL of carvacrol impregnated in four gauzes which given 0, 0.05, 0.2, 0.5 and 1.0 mL/L of carvacrol in 2L transparent packages.	Gauze placed in 2 Litre Package	Plates with PDA medium culture were inoculated with 50 µL stock (375 spores). Then 4 plates were placed in 2L transparent packages. Observed after incubation 4 and 7 days at 25°C	*B. cinerea* CECT21000	Martinez-Romero *et al.* (2007)
Trans-Cynnamal-dehyde Carvacrol	To measure the time to kill the bacteria at the tested concentration at 24 hours	25 and 175 mg/Lair	• Filter paper, size not mentioned • Standard Petri Dish – 90 mm diameter • sterile adhesive tape	Incubation 24 hrs., 37 °C, 100µL bacterial suspension , 10^6 CFU/mL	*E.coli, S. aureus*	Becerril *et al.* (2007)

Continued from previous page

Antimicrobial Agent	Purpose	Amount added	Carrier/container	Test type media	Target microorganism	References
Carvacrol, Eugenol Menthol	Determination MIC	Increasing quantities (2·5, 5, 10, 15, 20, 30 mg/plate) of aroma compounds were dissolved in 500-μl of ethyl acetate and deposited homogeneously on sterilized filter papers	• Filter paper, 90mm diameter • Standard Petri Dish – 90 mm diameter	Potato Dextrose Agar 10^4 spores inoculated in the dish centre The incubation conditions of the petri dish 14 days at 22°C for the fungi.	*Botrytis cinerea*	Ben Arfa *et al.* (2006)
Cinnamon Clove	Determine MIC by GCMS	Serial dilutions down to 1% (v/v). then 10µL was placed on the filter paper	• sterile blank filter disks (ø = 25 mm) • Standard Petri Dish – 90 mm diameter • Sealed with vinyl tape	Sabouraud chloramphenicol agar, 37.5°C, 7 days incubation	*A. flavus* *P. islandicumb*	Lopez et al. (2005)
Linalool Eugenol Sweet basil oil Peppermint oil	evaluation of the antifungal activity of essential oils and their individual constituents from the vapour phase	10, 15, 20, 30 and 40 µL/400 mL air space	• filter paper (110 mm diameter) • a Petri dish (140 mm) • Sealed with vinyl tape	PDA media. 9mm disc of tested fungi placed in the dish centre. Incubation 6 days at 25 C	*Sclerotinia sclerotiorum, Rhizopus stolonifer Mucor* sp.	Edris and Farrag (2003)
Citron oil Lavender oil Tea tree oil Perilla oil Lemongrass oil Thyme oil	To measure the absorption of antimicrobial to mycelia and agar. To determine elongation rate of hypha	0 – 100mg/L_{air}	• Filter paper, size not mentioned • Nunc dish – 90 mm diameter • Sealed with vinyl tape	RPMI 1640 agar. (Exposure time : 5 – 30 minutes), 30°C	*Aspergillus fumigatus*	Inouye *et al.* (2000)

Table 2-8 : Selected [a]MICs of essential oil components tested in vitro against food borne and airborne pathogens (adapted from Burt 2004 and Hyldgaard *et al.*(2012)).

Essential oil component	Species of bacteria	MIC, approximate range (mg/L)[b] (ppm)	References
α-Terpineol	*Escherichia coli*	450– >900	Kim *et al.* (1995a), Cosentino *et al.* (1999)
	Salmonella typhimurium	225	
	Staphylococcus aureus	900	
	Listeria monocytogenes	>909	
	Bacillus cereus		
Carvacrol	*E. coli*	225– 5000	Kim *et al.* (1995a), Cosentino *et al.* (1999), Ultee *et al.* (2000b)
	S. typhimurium	225– 250	
	Staph. aureus	175– 450	
	L. monocytogenes	375– 500	Bassole *et al.* (2010)
	B. cereus		Ahmad *et al.*(2011)
Citral	*E. coli*	188– 900	Kim *et al.* (1995b), Cosentino *et al.* (1999), Pol and Smid (1999)
	S. typhimurium	500	
	Staph. aureus	500	
	L. monocytogenes	500	
Eugenol	*E. coli*	100	Kim *et al.* (1995a)
	S. typhimurium	500	
	L. monocytogenes	>1000	
Limonene	*E. Coli*	1840	Di Pasqua *et al.* (2006, 2007)
	Pseudomonas flouresecents	1680	Espine *et al.(*2011)
	S. aureus		
Geraniol	*E. coli*	500	Kim *et al.* (1995a)
	S. typhimurium	500	
	L. monocytogenes	1000	
Perillaldehyde	*E. coli*	500	Kim *et al.* (1995a)
	S. typhimurium	500	
	L. monocytogenes	1000	
Thymol	*E. coli*	225– 450	Cosentino *et al.* (1999), Nazer *et al.* (2005),
	S. typhimurium	56	
	Staph. aureus	140– 225	Ahmad *et al.*(2011)
	L. monocytogenes	450	
	B. cereus	450	

[a] Essential oil components present in plants used in cooking were selected, and MICs for a selection of important foodborne pathogens cited.

[b] In the references MICs have been reported in the units mg mL^{-1}, % (v/v), μgmL^{-1}, μmL^{-1} and mmol L^{-1}.

For ease of comparison, these have been converted to mg/L (ppm), whereby it was assumed that essential oils have the same density as water

Table 2-9: Examples of reported vapour phase MICs for essential oils or active compounds.

Essential Oil / Main constituent	Microbe	Reported MIC / MFI	References
Allyl isothiocianate	*Penicillium commune, P. roqueforti, Aspergillus flavus*	1.8 – 3.5 mg/L	Nielsen and Rios (2000)
Linalool	*Rhizopus stolonifer*	49.2 mg/L #57.4 µL/L	Edris and Farrag (2003)
Menthol	*Rhizopus stolonifer*	22.1 mg/L #24.8µL/L	Edris and Farrag (2003)
Clove	*Aspergillus flavus*	18.3 mg/L #17.5 µL/L	Lopez *et al*. (2005)
Cinnamon	*Aspergillus flavus*	13.76 mg/L #13.1 µL/L	Lopez *et al.* (2005)
Carvacrol	**Trichophyton mentagrophytes*	1.6 mg/L	Inouye *et al.* (2006)
Thymol	**Trichophyton mentagrophytes*	1.6 mg/L	Inouye *et al.* (2006)
Carvacrol	*Botrytis cinerea*	62.5 mg/Lair	Ben Arfa *et al. (*2006)
Clove	*Staphylococcus aureus*	27.0 mg/L	Goni *et al.* (2009)
Cinnamon	*Staphylococcus aureus*	36.0 mg/ L	Goni *et al.* (2009)
Limonene	*Aspergillus flavus*	8000 mg/L	Jose Velazquez-Nunez *et al.* (2013)
Black mustard	*Aspergillus niger, Aspergillus ochraceous, or Penicillium citrinum*	**41.1 µL/L	Mejia-Garibay *et al.* (2015)

*MFI – minimum fungi inhibition. Unit = (weight (mg) or volume (µL) of antimicrobial agent/ volume of air (L) in the headspace). MIC was defined lowest concentration resulting inhibiting growth of microorganism at parallel growth on agar media. For ease of comparison, these have been converted to mg/ L (ppm), whereby it was assumed that essential oils have the same density as water. #Original value reported by the authors. **MIC inhibits 10 days delays

From Table 2-8, the minimum inhibitory concentration against foodborne pathogen for carvacrol and thymol is in the range 175-5000 mg/L and 56 – 450 mg/L respectively, and it is likely that thymol has stronger antimicrobial activity compared to carvacrol.

According to Nedorostova *et al.* (2009), many investigators have reported that the direct contact assay method faces many problems because of the high hydrophobicity and volatility of the essential oils. Their explanation was that in diffusion assays, the essential components are partitioned through the agar according to their affinity with water. The addition of emulsifiers or solvents such as Tween 80 or ethanol in dilution methods to overcome low water solubility may alter the antimicrobial activity of essential oils. Because of this weakness, the disc volatilization method was used by Nedorostova *et al.* (2009) to determine antimicrobial properties in vapour phase against foodborne bacteria.

There have been several efforts by investigators (Ward *et al.*, 1998; Edris and Farrag, 2003; Inouye *et al.*, 2006; Lopez *et al.*, 2007b; Goni *et al.*, 2009) to utilize the volatile nature of essential oils to determine the antimicrobial and antifungal activity. Inouye *et al.* (2006) applied a vapour contact method by impregnating the active compounds on filter paper to determine vapour activity of 72 essential oils against *Trichophyton mentagrophytes.* Some of the findings of the study are summarized in Table 2-10.

Various researchers have reported the different factors that contribute to the measurement of minimum inhibitory concentration. The presence of specific functional groups can reduce the MIC, for example, phenolic compounds and aldehydes as the major components were found to be the most potent vapour compounds, followed by alcohols, ketones, esters, ether/oxides and hydrocarbons (Ultee *et al.,* 2002; Kalemba and Kunicka-Styczynska, 2003; Inouye *et al*., 2006).

Table 2-10: Box vapours assay and agar diffusion assay of essential oils constituents against *T. mentagrophytes TIMM2789*

Major component of Oil	Functional Chemical Group	Average MFD Box vapour assay (mg/L air)	Agar diffusion Assay ID ± SD (mm)*
Phenol	Carvacrol	15.6	80 ± 0
	Thymol	15.6	41 ± 2
Aldehyde	Cinnamaldehyde	15.6	56 ± 4
	Citral	31.3	60 ± 5
Alcohol	Linalool	125	11 ± 2
	Terpinen-4-ol	125	11± 1
Ketone	Camphor	250	0
	(-)-Carvone	125	Spur
Ester	Linalyl acetate	250	25 ± 3
Ether/oxide	1,8 – Cineole	500	13 ±1
Hydrocarbon	Limonene	1000	53±3

Minimum fungicidal dose (MFD) (μg oil/mL air); *Inhibitory diameter (ID) including size of disc ±SD; 0, no inhibition zone. MFD was defined as minimum fungicidal dose per unit space that killed more than 99.9% of the original inoculums. Adapted from Inouye (2006).

It is apparent in analysing the data in Tables 2-8 to 2-10, that the numbers reported using some methods are very misleading. As discussed above it is common practise to report MIC values as the amount of essential oil component added to the system divided by the headspace volume. In many cases the reported MIC values are much higher than the saturated headspace concentrations for these compounds. For example MIC values for limonene are given as 1840, 1680 $mg.L^{-1}$ (Table 2-8), 8000 $mg.L^{-1}$ (Table 2-9) and 1000 $mg.L^{-1}$ (Table 2-10), while the saturated concentration in air at 20°C is 9 $mg.L^{-1}$ (EPA, 2012). Similarly values for the MIC of citral, camphor and terpineol and some for carvacrol are well in excess of the saturated vapour pressure for these compounds. It is likely that some of the vapour is absorbed into the food/media and in some cases into the dish or lost from the system. If the saturated vapour

pressure is low, then it is possible the headspace concentration remains at this vapour pressure until the reservoir (filter paper) is eventually exhausted due to these absorption processes.

Inouye *et al.* (2000) point outs that it is difficult to differentiate the direct effect of vapour on fungi from the indirect effect of vapour working after absorption in the medium during a long incubation period. Moreover, in their report, Inouye *et al*. (2000), highlighted that Gocho (1991a; 1991b) insisted that the antimicrobial activity of vapour was measured after absorption to the medium. Additionally, from their investigation, Inouye *et al.* (2000) concluded that essential oils inhibited the apical growth of *Aspergillus fumigates* by the direct accumulation of their vapours on mycelia, accompanied by the contribution from those absorbed into the agar. Inouye *et al.* (2000) also mentioned that the activity of essential oils in the gas phase should offer more promise in preventing fungal infection than bacterial infection. As discussed above however, it is likely that a three-way equilibrium exists locally at the surface and the partitioning between all three phases is important.

2.3.4 Synergistic Effects

A key limitation of volatile release systems is the potential for modification of the sensory properties of the fruit. For instance, slow reddening and perception of green taste notes have been observed when using hexanal to extend shelf life in tomatoes (Utto *et al.,* 2008). Similarly, Wolford (1998) reported red spot on sliced apple kept under a hexanal enriched atmosphere. Modification of flavour was also reported for strawberries (Hamilton *et al*., 1996) and grapes (Archbold *et al*., 1997). One approach to overcome this constraint is to use the synergistic effect of combining multiple volatiles at reduced concentrations and hence avoid sensory changes.

In the previous section on existing data for MICs of antimicrobials, mixtures were sometimes used. It is possible that these components can be working, synergistically, additively or antagonistically. Barry (1976) in Davidson and Parish (1989) defined an;

- Additive effect as "*the combined effect is equal to the sum of the effects observed with the two agents tested separately or equal to that of the most active agent in combination.*" Or in other words, an additive effect occurs when the combination of antimicrobial has a combined effect equal to the sum of the individual components (Hyldgaard *et al*., 2012).
- Synergistic effect as "th*e effect observed with a combination that is greater than the sum of the effects observed with the two agents independently*." or else a blend of two antimicrobial compounds has an antimicrobial activity that is greater than the sum of the individual components. While Periago *et al.* (2001) and Nazer *et al.* (2005) mentioned that synergy is generally described as when a "combination *of two compounds is more effective than each compound alone or when the observed inhibition of the combination is higher than the one predicted by adding the inhibitions created by the different compounds alone*".
- Antagonism occurs when a blend of antimicrobial compounds has a combined effect less than when applied separately (Davidson and Parish, 1989).

Several researchers used a checkerboard method to quantify the synergies between two components. For instance, Sukatta *et al.* (2008) employed this method to quantify the synergistic effect of the antifungal activity of clove and cinnamon oils at a combination ratio of 3:7, respectively towards decay fungi of grapes. Pei *et al.* (2009) reported synergistic effects occurred on evaluation of the combined antibacterial action of thymol/carvacrol, thymol/eugenol, carvacrol/eugenol and cinnamaldehyde against *E. coli* (Refer to Table 2-11). In

contrast Rivas *et al*. (2010) reported that the combination of thymol/carvacrol against *E. coli* in a model broth in a rumen system indicated an additive effect.

The fact that a high concentration of these compounds is needed to inhibit spoilage bacteria can result in undesirable flavours to food is the main drawback of the use of individual aromatic compounds alone (Nazer *et al*., 2005). The combination of anti-microbial components that exhibit a synergistic effect has the potential to reduce the sensory detection to a level acceptable to consumers (Nazer *et al.*, 2005; Goni *et al.*, 2009). The practical implications of these observations are important when using essential oil components in food systems since the use of the lower concentration needed to yield a similar antibacterial activity will mean a reduction in the flavour notes detectable in food products.

The ratio of combined compounds and percentage of effective reduction in concentration by synergy as compared with the individual components is shown in Table 2-12.

Table 2-11: Combination of components and their antimicrobial interactions against several microorganisms using the checkerboard method.

Pair combination	Organism	Interaction	References
α-pinene/ Limonene	*Saccharomyces cerevisiae*	Synergism (FIC = 0.375)	Tserennadmid *et al.* (2011)
α-pinene/ Linalool	*Saccharomyces cerevisiae*	Additive (FIC= 0.75)	Tserennadmid *et al*. (2011)
Linalool/ Terpene-4-ol	*Saccharomyces cerevisiae*	Additive (FIC = 0.75)	Tserennadmid e*t al.* (2011)
Carvacrol/linalool	*Listeria monocytogenes*	Synergism FIC≤0.5	Bassole *et al.* (2010)
Eugenol/linalool	*Enterobacter aerogenes*	Synergism FIC≤0.5	Bassole *et al.* (2010)
Eugenol/Menthol	*E. coli, P. aeruginosa*	Synergism FIC≤0.5	Bassole *et al.* (2010)
Thymol/carvacrol	*Escherichia Coli*	Synergisms FIC=0.75	Pei *et al.* (2009)
Thymol/carvacrol	*S. aureus, Bacillus cereus, E coli*	Antagonisms (FIC = 4)	Gallucci *et al* (2009)
Thymol/carvacrol	*E. coli*	Additive (FIC = 0.75)	Rivas *et al* (2010)
Thymol/Eugenol	*E. coli*	Synergism FIC=0.75	Pei *et al.,* (2009)
Carvarol/Eugenol	*E. coli*	Synergism FIC=0.75	Pei *et al.* (2009)
Carvarol/Eugenol	*S. aureus, B. cereus, E. coli*	Antagonisms (FIC = 4)	Gallucci *et al* (2009)
Carvacrol/myrcene	*S.aureus, B. cereus, E.coli*	Antagonism (FIC >2)	Gallucci *et al* (2009)
Menthol/Geraniol	*S. aureus, B. cereus*	Synergism FIC $\leq$ 0.5	Gallucci *et al*. (2009)
Menthol/Thymol	*S. aureus, B. cereus*	Synergism FIC≤0.5	Gallucci *et al.* (2009)
Cinnamaldehyde/ Carvacrol	*E. coli,*	Synergism FIC=0.75	Pei *et al.* (2009)

Fraction Inhibition Concentration (FIC).

Table 2-12: Ratio of combined compounds and percentage of effective reduction concentration by synergy as compared with the individual components (Bassole *et al.,* 2012).

Pair synergistic combinations	Organism	Ratio of combined compounds	Reduction of effective concentration (%)	References
Cinnamaldehyde/Thymol	*E. coli*	1:1	25	Pei *et al.* (2009)
Cinnamaldehyde/Thymol	*E. coli*	1:4 or 1:8	50	
Thymol/ Cravacrol	*E. coli*	1:1	25	
Thymol/Eugenol	*E. coli*	1:4	50	
Carvacrol /Eugenol	*E. coli*	1:4 or 1:8	25	
Geraniol/Menthol	*S. aureus*		50	Gallucci *et al.* (2009)
Thymol/Eugenol	*B. cereus*		25	
Eugenol/geraniol	*B. cereus*		35	
Thymol/menthol	*B. cereus*		65	
Geraniol/Menthol	*B. cereus*		94	
Cinnamaldehyde/Thymol	*S. thypinurium*	1:1	25	Zhou *et al*. (2007)
Cinnamaldehyde/ Carvacrol	*S. thypinurium*	1:1	25	
Thymol/Carvacrol	*S. thypinurium*	1:1	50	
Thymol/Carvacrol	*S.aureus*	2:3	25	Didry *et al*. (1993)
Thymol/Carvacrol	*E. coli*	2:3	25	
Thymol/Carvacrol	*Klebsiella pneumoniae*	2:3	50	
Thymol /carvacrol	*Streptococcus pneumoniae*	2:3	25	

2.3.4.1 Antimicrobial Interaction Measurement (Methods for Identifying if Synergy Exists)

In the food industry, combinations of antimicrobials are used, for example, the blend of potassium sorbate and sulphur dioxide in sparkling wines. However often the interactions are not well characterized (Davidson and Parish, 1989).

To measure the interaction between two components, the MICs of the individual and the various combinations of two components are required. The methods to determine the types of interactions between combined antimicrobial agents are extensively studied (Barry, 1976;

Krogstad and Moellering, 1986) and the correlations between antimicrobial synergism methods are well established in the literature; mainly for pharmaceutical applications (Berenbaum, 1984; Berenbaum, 1988; Berenbaum, 1989). However, the application of this method in food preservative combination has not been comprehensively explored (Davidson and Parish, 1989) and there is no commonly accepted methodology to quantify synergistic activity (Dufour *et al.,* 2003). Even today, after more than three decades, the methods developed by Barry (1976) are usually implemented (Hyldgaard *et al*., 2012).

The *Checkerboard, Graphical, Epsilometer test (Etest)* and *Time Kill Method* are the most widely used procedures, and the principles are described in the literature (Davidson and Parish, 1989; White *et al.*, 1996; Bonapace *et al.*, 2000; Orhan *et al.*, 2005; Bassole and Juliani, 2012). According to White *et al.* (1996), the checkerboard method is mostly preferred because the Time-kill curve methods are time-consuming and labour-intensive. Bonapace *et al.* (2000) stated that the Etest method appears promising although further testing should be performed with the addition of antimicrobial agents, since the agreement between the E-test method to Checkerboard and Time Kill method are only 71% and 51%, respectively.

The combined effect of a mixture is analyzed by using measurements of the MIC to calculate the Fractional Inhibition Concentration Index (FIC_{index}). Most researchers add FICs for individual agents in a combination to produce an FIC Index (Barry, 1976). For instance, for two antimicrobials agent, A and B, the FIC for each antimicrobial agent is the MIC of combination antimicrobial (A+B) divided by MIC of individual components respectively. Finally, the FIC index is calculated by the summation of FIC_A and FIC_B. The method transforms an MIC to a fractional inhibitory concentration as shown below (Barry, 1976, Davidson and Parish, 1986, Hyldgaard *et al.,* 2012)**:**

- FIC_A = (MIC of active compound A in presence of B)/ MIC of A
- FIC_B = (MIC of active compound B in presence of A)/ MIC of B
- $FIC_{index} = FIC_A + FIC_B$

Each checkerboard test produces many combinations, and by gathering it, the FIC values of the most effective combinations are employed.

Theoretically, the general interpretation of FIC is:

- $FIC_{index} > 1$ indicates an antagonistic effect
- $FIC_{index} = 1$ indicates an additive effect
- $FIC_{index} < 1$ indicates a synergistic effect

However, deciding whether the FIC indicates a significant effect is not clearly established. The FIC definition was suggested to be restricted to synergistic for FIC is <0.5, additive for FIC 0.5 – 4.0 and antagonistic for FIC >4.0 (Odds, 2003).

Table 2.13 shows examples of fractional inhibitory concentrations employed by researchers. For example, Didry *et al.* (1993) and Gallucci *et al.* (2009) in their works to determine antimicrobial activity of essential oils components alone or in combination against several bacteria, defined that FIC≤0.5 represented total synergy effects, partial synergy at 0.5 <FIC≤0.75, indifference at 0.75 <FIC≤2.00 and at FIC index > 2 indicated antagonism effects. The values of the FIC index used for the definition of the nature of the interaction differ between publications and makes a comparison between studies difficult. Examples are presented in Table 2-13.

Table 2-13: Examples of various fractional inhibitory concentration (FIC) indexes used to determine the type of interactions.

FIC Index				References
Synergy	Addition	Indifference	Antagonism	
∗≤0.75	-	0.75 - 2.00	>2.00	*Didry *et al.* (1993), *Gallucci *et al.* (2009)
≤0.50	0.5.00 – 4.00	0.50 - 4.00	>4.00	Lopez *et al.* (2007b)
<1.00	1.00	-	>1.00	Sukatta *et al.* (2008)
<1.00	1.00	1.00 - 2.00	>2.00	Pei *et al.* (2009)
≤0.50	0.50 – 1.00	-	>4.00	Goni *et al.* (2009)
≤0.50	0.50 – 1.00	1.00 - 4.00	>4.00	Gutierrez *et al.* (2008; 2009a), Tserennadmid *et al.* (2011)
≤0.50	0.5 – 0.4	-	>4.00	Bag and Chattopadhyay, (2015)

*0.5 ≤ synergy total, 0.5 <FIC≤0.75 synergy partial

Krogstad and Mollering (1986) and Hall *et al*., (1983) mentioned that the results of the checkerboard method can be represented graphically by plotting the FIC values on a graph known as an isobologram. On the x- and y-intercepts, the half maximal effective concentrations are plotted or MIC values of the two agents when used alone. Additive effects will produce straight lines while synergistic and antagonistic effects cause different shaped curves as shown in Figure 2-5.

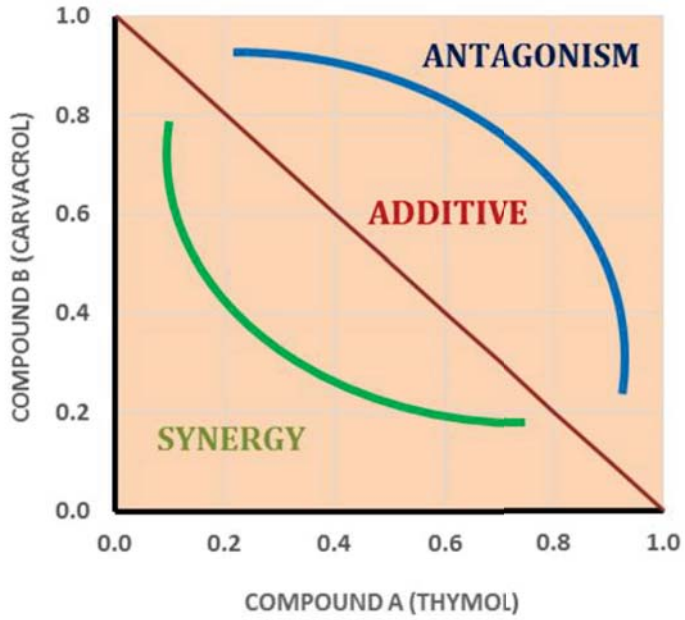


Figure 2-5: **Isobolograms displaying the three types of results possible with combinations of antimicrobials (adapted from Davidson and Parish, 1986)**

For example, Tables 2-14 and 2-15 shows the synergistic effect of the components either in the form of essential oil and its major component alone. It is well known that essential oils consist of mixtures of terpenes, terpenoids, and aldehydes. Individual essential oils may contain complex mixtures of compounds. Interactions between these terpenes may lead to additive, synergistic or antagonistic effects. The synergistic rationale for using combinations of products seems to be the obtaining of a dynamic product that has multiple modes of action, respecting the principle that the action of the combined product is greater than the sum of individual chemical components (Delaquis *et al.*, 2002; Gallucci *et al.*, 2009).

Table 2-14: MICs (mg/L) of essential oil in vapour phase effective against selected bacteria. The MIC is expressed as microliters of essential oils per unit of the atmosphere above the organism growing on the agar surface.

	Essential Oil dose (mg $_{EO}$/ L $_{headspace}$) [a]								
Microorganism	Clove (*Sygium aromaticum*)	Cinnamon (*Cinnamomum sativum*)	Clove-Cinnamon (1:1)	Oregano (*Origanum vulgare*)	Marjoram (*Origanum Majorana*)	Thyme (*Thymus Vulgaris*)	Sweet Basil	Oregano-Thyme	References
Gram-positive									
S. aureus	26.2	34.9	-	-	-	-	-	-	Lopez *et al.* (2005)
				500		1000		200:250	Stojkovic *et al.* (2013)
	-	-	-	17	530	17	530	-	Nedorostova *et al.* (2009)
	136	36	27:27	-	-	-	-	-	Goni *et al.* (2009)
		20	-	20	-	-	-	-	Becerril *et al.* (2007)
L. Monocytogenes	17.5	34.9	-	-	-	-	-	-	Lopez *et al.* (2005)
	54	18	45:45	-	-	-	-	-	Goni *et al.* (2009)
	-	-	-	66	-	260	-	-	Nedorostova *et al*. (2009)
E. Faecalis	87.3	52.4	-	-	-	-	-	-	Lopez *et al.* (2005)
	90	54	45:45	-	-	-	-	-	Goni *et al.* (2009)
B. cereus	17.5	17.5	-	-	-	-	-	-	Lopez *et al*. (2005)
	18	18	18:18	-	-	-	-	-	Goni *et al.* (2009)

Continued from previous pages.

Microorganism	Essential Oil dose (mg $_{EO}$/ L $_{headspace}$) [a]								References
	Clove (*Sygium aromaticum*)	Cinnamon (*Cinnamomum sativum*)	Clove: Cinnamon (1:1)	Oregano (*Origanum vulgare*)	Marjoram (*Origanum Majorana*)	Thyme (*Thymus Vulgaris*)	Sweet Basil	Oregano: Thyme	
Gram Negative									
S.thyphimurium				1000		1000		500: 50	Stojkovic et al. (2013)
E. Coli	26.2	17.5	45:45	-	-	-	-	-	Lopez *et al.* (2005)
	-	5	-	5	-	-	-	-	Becerril *et al.* (2007)
				66	260	33			Nedorostova *et al*. (2009)
	27	18	-	-	-	-	-	-	Lopez *et al.* (2007b)
Y. Enterolitica	8.73	17.5	19:19	-	-	-	-	-	Lopez *et al.* (2005)
	18	9	-	-	-	-	-	-	Goni *et al.,* (2009)
S. choleraesuis	54	136	68:68	-	-	-	-	-	Goni *et al.* (2009)
P. aruginosa	*	*	-	-	-	-	-	-	Lopez *et al.* (2005)
			*	-	-	-	-	-	Goni *et al.* (2009)
S. enteritis	-	-	-	130	-	33	-	-	Nedorostova *et al. (*2009)

(Colon (:) in the numbers mean the ratio of the combination).

Continued from previous pages.

Microorganism	Essential Oil dose (mg $_{EO}$/ L $_{headspace}$) [a]								References
	Clove (*Sygium aromaticum*)	Cinnamon (*Cinnamomum sativum*)	Clove-Cinnamon (1:1)	Oregano (*Origanum vulgare*)	Marjoram (*Origanum Majorana*)	Thyme (*Thymus Vulgaris*)	Sweet Basil	Peppermint	
Yeast									
C. albicans	13.1	13.1	-	-	-	-	-	-	(Lopez *et al.,* 2005)
Fungi									
A. flavus	17.5	13.1	-	-	-	-	-	-	(Lopez *et al.,* 2005)
P. islandicum	8.73	9	-	-	-	-	-	-	(Lopez *et al.,* 2005)
R. stolonifer	-	-	-	-	-	-	75	75	(Edris and Farrag, 2003)
Mucor sp.	-	-	-	-	-	-	75	75	(Edris and Farrag, 2003)
Sclerotinia sclerotiourum	-	-	-	-	-	-	75	75	(Edris and Farrag, 2003)

'*' no inhibition was observed.

[a] In the references MICs have been reported in the units µL/L, µL/400cm^3 and mg/L, and µL/cm^3. For ease of comparison, these have been converted to mg/L by the assumption that the t essential oils have the same density as water.

Table 2-15: **MICs (mg/L_{air}) of major components of sweet basil, peppermint, and clove and cinnamon essential oil in vapour phase effective against selected bacteria and fungi. The MICs is expressed as milligram (mg) of essential oils major components per unit of the atmosphere (L) above the organism growing on the agar surface.**

	Dose (mg_{mc} / $L_{headspace}$)								
Microorganism	Linalool	Eugenol	Combination Linalool: Eugenol	Menthone	Menthol	Combination Menthone: Menthol	Carvacrol	Cinnamaldehyde	References
Bacteria									
S. aureus	-	-	-	-	-	-	10	20	Becerril *et al.*, (2007)
E. Coli	-	-	-	-	-	-	< 5	10	Becerril *et al.*, (2007)
Fungi									
R. stolonifer	57	No inhibition observed at 12mg/L	43 : 9	No inhibition observed at 37mg/L	22	18:15	-	-	Edris and Farrag, (2003)
Mucor sp.	57	No inhibition observed at 12mg/L	43:9	No inhibition observed at 37mg/L	22	18:15	-	-	Edris and Farrag, (2003)
Sclerotinia sclerotiourum	No inhibition observed at 57mg/L	No inhibition observed at 12mg/L	57:12	No inhibition observed at 37mg/L	22	28:22	-	-	Edris and Farrag, (2003)

There are a few reported studies of what governs synergy and antagonism among essential oil constituents (Ultee *et al*., 1999; Burt, 2004; Bakkali *et al*., 2008). Krosgstad and Moellering (1986) in Davidson and Parish (1989) stated that synergistic interactions are generally due to

- sequential inhibition of a common biochemical pathway,
- inhibition of protective enzymes that degrade antimicrobials, and
- interaction of cell wall-active agents to enhance uptake of another antimicrobial.

These mechanisms also have been reported by Burt (2004), Bassole and Juliani, (2012) and Hyldgaard *et al.* (2012).

David and Parish (1989) claimed that another possible mechanism for the observed synergistic effect was due to a different mode of action for the active antimicrobial agents, attacking two different sites on the cell respectively that indirectly depend on each other. A few studies reported the effect of antagonism. Nevertheless, it has been hypothesized that this effect occurs when

- combining bacteriostatic and bactericidal antimicrobials
- antimicrobials have the same site of action
- Antimicrobials interact with each other.

2.4 Conclusion

This review provides an overview of the use of natural antimicrobial compounds in active packaging system with emphasis on applications in food products. Essential oils are natural resources that can be exploited as active agents in controlled release active packaging systems since their antimicrobial activities are well documented in the literature.

Analysis of the literature results highlighted several key points that require consideration. Firstly if the mechanism of the inhibition is considered, it is the concentration of essential oil compound in the microorganism membrane that is important. Because the growth of interest is normally at the product surface (where oxygen is available), there will be a three way local equilibrium between the concentration of active compound in the microorganism membrane and the surrounding product and air spaces. Because of this equilibrium, the MIC could be reported as any of these concentrations, as long as equilibrium has been achieved. In the fields of medicine and pharmacology the use of octanol-water partition coefficients are used to quantify this, although their use in the food spoilage literature is absent.

In vapour release active packaging systems, the headspace concentration required to inhibit growth is the most convenient measure to consider. This is not the same as the MIC normally reported (amount of oil added divided by the headspace volume), which can be much higher than can physically be achieved and is misleading. Most of the reported literature MIC values can not be used for antimicrobial packaging design.

In most experimental vapour phase systems, it appears that the dynamics of vapour release and absoption are important and it is unlikely that the headspace concentration is ever constant, making reporting of a MIC difficult. As a result, it would be useful to characterise the dynamics of headspace concentration changing with time, during common MIC

measurement practises. Because of the relatively low saturated vapour pressures of most essential oils being considered for antimicrobial applications, the partitioning between the product and air, as well as the saturated vapour pressures of the volatiles should also be considered.

The review also outlined principles and approaches to measuring the MICs of targeted microorganism. However due to the factors discussed above, the accuracy of the techniques applied to measure MICs against targeted microorganism in the headspace are questionable. In the traditional techniques, the concentration of active compound vapour in the headspace may not be stable and could be lost during the treatment. The sorption of volatile by the culture media and in the system must also be considered. The review also identified that the combination of major constituents of some essential oils, such as thymol and carvacrol, may provide synergistic activities to avoid sensory impact.

CHAPTER 3

SELECTION OF A MINIMUM INHIBITORY CONCENTRATION (MIC) STANDARD SYSTEM

3.1 Introduction

The main aim of the preliminary work covered in this chapter was to understand and to become familiar with the microbiological preparation techniques to determine the minimum inhibitory concentration (MIC) for a target organism. MIC is one of the chief parameters required to develop a model based design controlled release active packaging system that uses essential oils to enhance the shelf life of foods. A number of issues were highlighted in the literature review, that call into question some of the reported results, methodologies used and the suitability to use these systems for targets for active packaging design. Despite these concerns, application of the reversed petri dish method (one of the most common vapour phase techniques as widely reported in the literature) was carried out as a starting point, to provide a basis for the wider work on accurate MIC measurement.

For this purpose, the antifungal activity was evaluated against a model pathogen, *Botrytis cinerea,* one of the common spoilage fungi of fresh fruits. Thymol and carvacrol were chosen in these studies because these volatiles are well known to have excellent antibacterial

properties as reported in numerous literature data (Didry *et al.*, 1993; Kim *et al.*, 1995a; Ultee *et al.*, 1999; Lambert *et al.*, 2001; Bagamboula *et al.*, 2004; Ben Arfa *et al.*, 2006; Veldhuizen *et al.*, 2006; Chalier *et al.*, 2007). In this section, only preliminary results for antifungal activity were demonstrated.

3.2 Experimental Section

3.2.1 Screening of Antifungal on Minimum Inhibitory Concentration

From the literature, there are several methods that have been demonstrated to measure antimicrobial or antifungal activity (Hsu *et al.,* 2007).

For the design of active packaging systems, the vapour contact method is considered most appropriate to measure the MICs against target organism. This is because the antimicrobial components can be incorporated into the carrier such as a sachet or the packaging itself. The emission of antimicrobial vapour from the carrier into the package headspace potentially can be controlled in order to get the desired outcome. A range of concentrations of active compounds with specific ratio combination in the gas phase will be introduced to the Potato Dextrose Agar (PDA) surface agar (as the food system model). The concentration of the active vapours can be quantified by using Gas Chromatography Mass Spectrophotometer (GCMS) technique. The antifungal activity in the gas phase can be measured by determining the growth-inhibiting zone on the agar medium inoculated with test fungi after exposure to vapours arising from active compounds as demonstrated by Hsu *et al.* (2007). The key objective of this section was to screen the antifungal chosen to verify their effectiveness to the selected spoilage fungus and media.

3.2.2 Materials and Methods

3.2.2.1 Chemical Information

Thyme oil, carvacrol and thymol, were purchased from Sigma-Aldrich, New Zealand. Due to its solid state at ambient temperature, thymol was dissolved in ethyl acetate (1:1). Clove oil was purchased from Aromasense, New Zealand. All information has been summarised in Appendix 1.

3.2.2.2 Preparation of Strain and Spores Suspension.

Isolated *Botrytis cinerea* were obtained from the School of Engineering and Advanced Technology Micro Lab, Massey University and cultured on Potato Dextrose Agar medium (PDA) medium (Diffco, Becton Dickinson & Company, USA) plates and incubated for five days at 25 °C. A *Botrytis cinerea* suspension was made by flooding the surface of the plates with 10 mL of sterile 0.1% buffered peptone water, gently rubbing the surface with a glass L-rod, then transferring the solution into a 10 mL glass bottle. Serial dilutions were made using more peptone water until a suspension containing 10^5 spores/mL was obtained. Spore counts were carried out using haemocytometer (Thoma cell) under an optical microscope at 400X magnification.

3.2.2.3 Samples Preparation

The antifungal activity test was carried out using the solid diffusion assay method as described by Lopez *et al.* (2005).

Potato Dextrose Agar medium (PDA: Diffco, Becton Dickinson & Company, USA) was used as a basal medium for the growth of fungi. Solidified PDA medium was prepared in 9.0 cm glass. Petri dishes containing ~20 mL PDA that resulted in a headspace of approximately 75cm^3 in the Petri dish and the calculated surface area was 63.62 cm^2. The PDA was inoculated with

0.1 mL of peptone water suspension containing 10^6 CFU/mL *Botrytis cinerea* by spreading on the solidified PDA with glass L-rod spreader.

Three µL of undiluted active compounds (except thymol 6 µL, 50 % (v/v)) were added to a sterile blank filter disk (5 mm) using a digital pipette (Transfepette®, 2 -20 µL, Germany), which was placed on the top of the culture media. A control sample disk containing ethyl acetate free of volatile compounds was tested to assure that ethyl acetate did not show any bacterial activity. Glass Petri dishes were then sealed by using paraffin film tape (Brand: Parafilm M).

The incubation period was 18 days at 25°C. The mycelium of the fungi in the control reached the edges of the dish after 3 days of incubation. The antifungal index was calculated as shown below.

The antifungal index is given as the mean of three replicates. The inhibition zone was measured up to 18 day's incubation. Volatile compounds added (3 µL~20 mL PDA), except thymol (6 µL; (50% (v/v).

Determination Antifungal Index

The measurement of the antifungal index was adapted from the method given by Hsu *et al.,* (2007).

$$\mathbf{AI = (1 - D_a/D_b) \times 100} \quad \text{..... Eq. 3-1}$$

Where

AI = Antifungal Index (%)

D_a is the diameter of the growth zone in the experimental dish (mm)

D_b is the diameter of the growth zone in the control dish (mm)

3.2.3 Results and Discussion

Botrytis cinerea appeared two days after inoculation in control samples without antimicrobial compounds. The macroscopic appearance of the mycelium of the *Botrytis cinerea* was cotton white.

The results of the antifungal activity of thymol, carvacrol, thyme oil and clove oil against *Botrytis cinerea* are represented in Table 3-1. Figure 3-1 shows photographs of the effect of each volatile compounds on the growth of *Botrytis cinerea* after 3 days incubation.

Table 3-1: Antifungal investigations of active compounds using the agar diffusion method after three days at 25 °C

	Thyme Oil	Clove Oil	Thymol	Carvacrol
*Antifungal index (%)	100.0± 0.0	49.3 ±0.5	100.0±0.0	100.0±0.0
Inhibition Zone (cm^2)	4.9±0.1	3.5±0.1	63.6±0.5	9.6±0.1

Results are mean ± SE (n = 3).

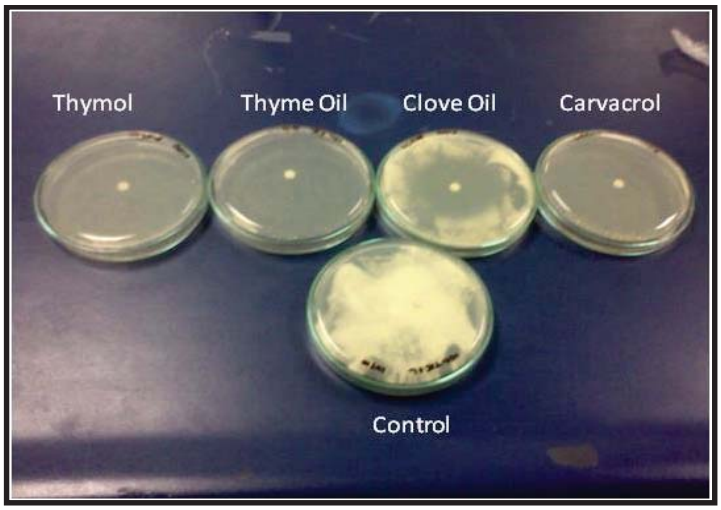


Figure 3-1: Photograph illustrating the effect of volatile compounds on *Botrytis cinerea growth*. (Incubation period: 3 days, temperature: 25° C)

As shown in Table 3-1, the results indicated that after 3 days incubation, clove oil at 3 µL did not completely inhibit the growth of *Botrytis cinerea*; however it did reduce the growth. The presence of eugenol, a key component of clove oil, which is known to exhibit antimicrobial properties (Inouye *et al*., 2006) may contribute to the suppression of the fungal growth. Thyme oil, thymol and carvacrol at 3 µL were found to be 100 % effective against the tested fungi after 3 days incubation. Following these measurements, these samples were incubated

to 18 days under the same conditions to see the consequences of further exposure to the compounds (Figure 3-2).

Figure 3-2 demonstrates the effect of each volatile compound against *Botrytis cinerea* after 6, 10 and 18 days incubation. The results indicate that carvacrol; a major constituent of thyme oil showed stronger inhibition properties compared to thyme oil. The inhibition zone caused by carvacrol is 9.6 cm^2, compared to thyme oil which was 4.9 cm^2 after 18 days incubation. These results indicate that carvacrol, the major components of Thyme oil likely to be responsible for the antifungal properties of thyme oil as reported by several investigators. (Marino *et al.*, 1999; Guynot *et al.*, 2003; Inouye *et al.*, 2006).

In these preliminary studies, the concentration of thymol obtained to inhibit the growth of *Botrytis cinerea* after three days incubation was 52.6 µg/mL. The calculation was made by assuming that all the active compound added vapourised from the filter paper into the petri dish air space. It should be noted that the saturated air phase concentration of thymol at 20°C is $1.35x10^{-4}$ ug/mL (EPA 2012), showing that this interpretation is not correct. Inouye (2006) reported that the minimum fungicidal concentration of thymol towards *Trichophyton mentagrophytes TIMM2789* was 1.56 µg/mL air (1.56 ppm and also much greater than the saturation air concentration).

According to Lambert *et al*. (2001), the partition coefficient of active compounds in cell membranes is a crucial determinant of their effectiveness. Thus quantitative variations in the activity of essential oils are expected against different bacteria. The next experiment, estimates the carvacrol MIC for further evaluation in the vapour phase, using a method similar to Lopez *et al*. (2005) with slight modification. The aim of this study is to identify volatile compound concentrations that deliver effective inhibition of fungal growth.

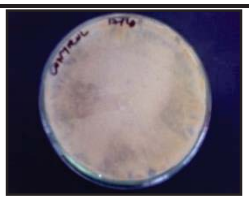
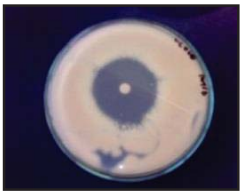
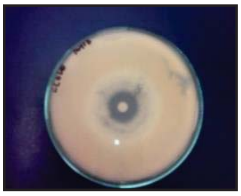
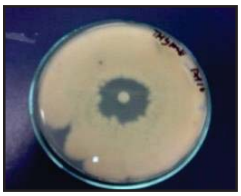
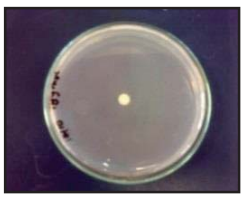
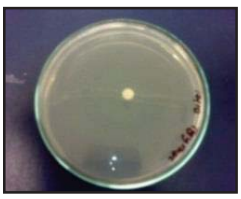
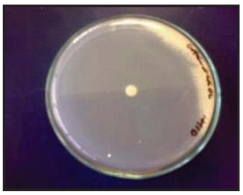
	Incubation period (Days)		
	6	10	18
Control			
Clove Oil			
Thyme Oil			
Thymol			
Carvacrol			

Figure 3.2: **Photograph illustrating the effects of volatile compounds on the growth of *Botrytis cinerea* after up to 18 days incubation, (Temperature: 25°C).**

3.2.4 Summary Findings

Thymol and carvacrol showed antifungal activity, and there is potential to control the growth of spoilage fungi in a closed system thus verifying observations from other authors (Didry *et al.*, 1993; Kim *et al.*, 1995a; Ultee *et al.*, 1999; Lambert *et al.*, 2001; Bagamboula *et al.*, 2004; Ben Arfa *et al.*, 2006; Veldhuizen *et al.*, 2006; Chalier *et al.*, 2007). The MIC values collected however are not a true reflection of the air phase concentration and cannot be used for active packaging design.

3.3 Determination of Carvacrol Minimum Inhibitory Concentration

As stated earlier, there are many definitions of MICs in the literature. In this section, the MIC was defined as the lowest concentration inhibiting visible growth of the test organism after 5 days incubation. In this section, 5 days incubation period was chosen based on the standard incubation condition (25 °C , 5 days) specified by Internal Commission of Food Mycology (ICFM) (Pitt andHocking, 1997; Batt & Tortorello, 2014).

The concentration was calculated according to Lopez *et al.* (2005) as µL of volatile compounds per unit of the atmosphere (µL/L), above the organism growing on the PDA medium surface. To evaluate the antifungal activity of carvacrol from the vapour phase against *Botrytis cinerea,* the common inverted Polystyrene Petri dish vapour contact (reverse Petri plate) method was chosen. This method was selected because it is reported to be suitable to define the activity of essential oil in the vapour phase (Kalemba and Kunicka-Styczynska, 2003) and provides a baseline for future analysis of MIC measurement methodologies.

3.3.1 Materials and Methods

3.3.1.1 Sample Preparation.

The modified vapour diffusion test used in this study has already been described in detail by Lopez *et al*., (2005). Briefly, the solidified agar medium was inoculated with 100 μL of a 0.1% buffered peptone solution containing 10^5 colony forming units/mL (CFUs/mL) of the microorganism under study. Different volume (0.0, 0.05, 0.1, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 µL) of carvacrol were added to 20 mm diameter sterile filter paper disk and placed on a sterilised microscope slide to prevent interaction between the compound and the plastic Petri dish as shown in Figure 3.3. Because the minimum practical volume that could be pipetted was 5 µL, the carvacrol was diluted using Ethyl acetate to concentration 0.0, 0.01, 0.02, 0.1, 0.4, 0.6, 0.8 and 1 µL carvacrol / µL (carvacrol + ethyl acetate).

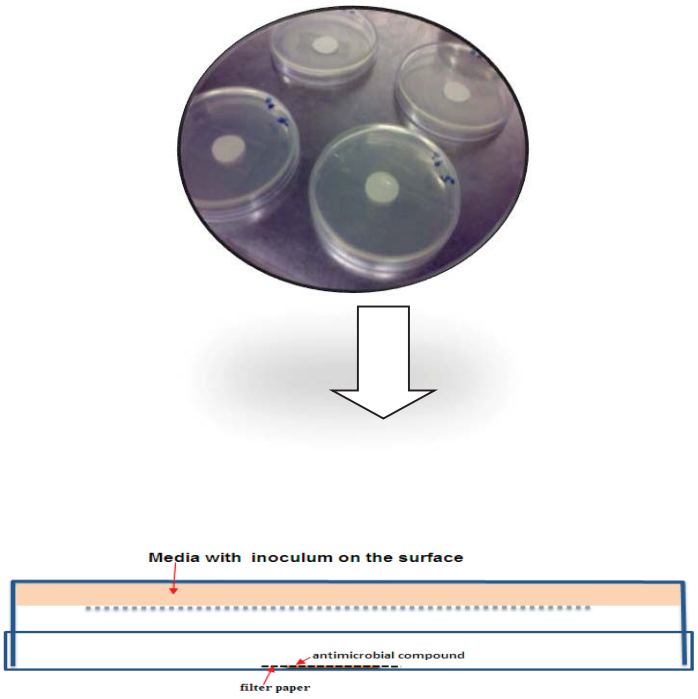


Figure 3-3: **Illustration of Petri dish setup to determine the minimum inhibitory concentration in the vapour phase in this work based on the common micro-atmosphere method (Inverted Petri dish method).**

The Polystyrene Petri dish was then sealed with Para film as demonstrated in Figure 3-3. To ensure that ethyl acetate did not contribute to the antimicrobial activity, controls with 5µL of ethyl acetate were added to every experimental set. After the incubation period, the minimal inhibitory concentration (MIC, expressed as microliters of carvacrol per litre of the atmosphere above the agar surface) was calculated. All tests were performed in triplicate.

3.3.2 Results and Discussion

Generally, in this method, a paper disc moistened with pure essential oils or its major constituents is attached to the lid of polystyrene Petri dish, which is then sealed with Para film or vinyl tape, inverted and incubated. Previous researchers mostly use Petri dishes with internal diameters of 85 and 90 mm, however other researchers have worked with 140mm diameter polystyrene Petri dishes (Edris and Farrag, 2003) and smaller diameter dish was 60 mm (Nunc dish) (Inouye *et al*., 2000). The results of antifungal/antimicrobial activity are presented as the diameter of the microorganism inhibition zone (Didry *et al.*, 1993; Sukatta *et al.*, 2008) or minimal inhibitory concentration which inhibits the total growth of microorganism (Edris and Farrag, 2003; Ben Arfa *et al.*, 2007; Martinez-Romero *et al.*, 2007; Nostro *et al.*, 2007; Nedorostova *et al.*, 2009; Nostro *et al.*, 2009). The dosage of active compound by vapour contact was expressed by added weight or volume divided by unit headspace of the container.

In the literature MICs have been reported using various including units of µg added compound/mL headspace, µL added compound/L headspace, µL added compound/400 mL headspace and mg added compound/L headspace. In order to compare the results, the different units were changed to mg added compound/L and µL added compound/L headspace (ppm) as shown in Table 2-9 in Chapter 2.

In this study, the antimicrobial activity of carvacrol through vapour phase against *Botrytis cinerea* was qualitatively and quantitatively assessed by the absence and presence of the growth of the tested species. In all cases, the head headspace volume was 75 mL. Figure 3.4 demonstrates the result of carvacrol vapour against *Botrytis cinerea* after 5 days incubation. The growth of *Botrytis cinerea* was observed at carvacrol concentrations up to 0.5 µL/ 75 mL ($6x10^{-3}$ µL/mL Plate E). After 6 days incubation period, the presence of *Botrytis cinerea* at concentration 1.0 µL/ 75 mL ($1.3e^{-2}$ µL/mL Plate F) was evident (Figure 3-5) while the other plates (G, H, I, J) showed no signs of growth. The experiment was extended to 10 days under the same conditions, and no sign of growth was seen at the level of $2.7x10^{-2}$µL/mL air and above (The photographic results are not shown). Note that all concentrations are greater than saturation at 20°C $8.3x10^{-5}$ µL/mL (EPA 2012).

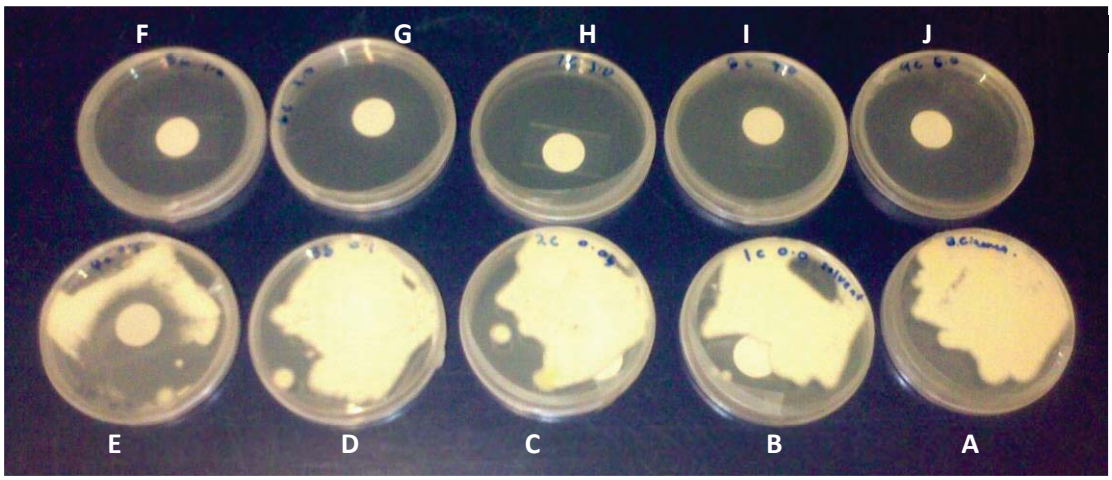


Figure 3.4: **Photograph illustrating the effect of carvacrol volatile on the growth of *Botrytis cinerea* after 5 days incubation (temperature: 25°C). Letters A-J denotes different added carvacrol additions as defined in Table 3.2.**

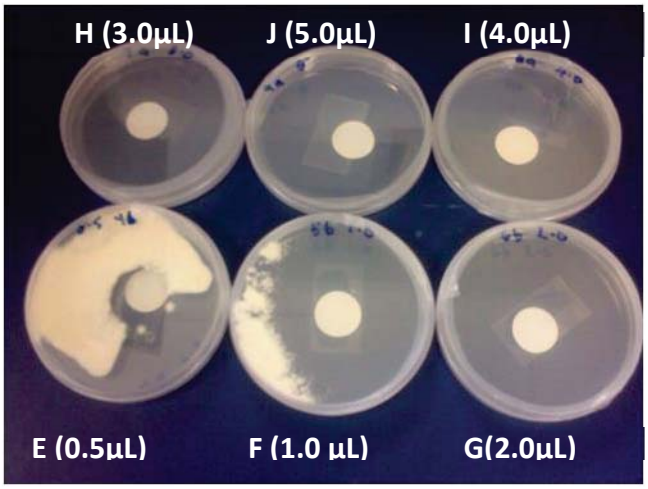


Figure 3.5: **Photograph illustrating the effect of carvacrol volatile at concentrations on the growth of *Botrytis cinerea* after 6 days incubation (temperature: 25°C). Letters E-J denote different added carvacrol additions as defined in Table 3.2.**

Where partial inhibition was observed (e.g. Plate E in Figures 3.4 and 3.5), there was a clear zone near the filter paper. This suggests the concentration at the surface of the agar is higher near the filter paper and that the air space may not be at a uniform concentration.

Table 3-2 summarizes the observations of the test and Table 3-3 indicates the level of minimum inhibitory concentration of carvacrol against *Botrytis cinerea* at 25 °C. This result was similar to data reported by Ben Arfa *et al*. (2006). The minimal inhibition dose (MID) for carvacrol against *Botrytis cinerea* was 5 mg per Petri dish (62.5 mg/L, 80 mL headspace, 64 µL/L). In this case, the MID was defined as the minimal dose of antimicrobial required to completely inhibit fungi growth for 15 days at an incubation temperature of 25 °C.

Even though the results obtained in this work agree well with those reported in the literature, the question arises as to the way the data reported. As shown in Table 3.3, the relative vapour pressure (P/Psat) is greater than 100% for all experiments other than the control. Clearly this is not possible and the assumption that all the added carvacrol volatile moves into the gas phase is not true. Reporting of MIC values in this way is not sensible, and they do not provide

useful targets for active packaging designs. It is clear that the volatile concentration in the headspace must be lower. It could be held at the saturated vapour pressure for different lengths of time depending on the volume added. Because no inhibition was observed in the lower added volume experiments (0.05, 0.1uL added compound), it is evident that losses from the headspace also occur.

Table 3-2: Results after 10 days incubation at 25 °C.

Label	Description	Presence of growth (n = 3)	Observation
A	Nil solvent and antimicrobial agent	+ve, +ve, +ve	Growth of *Botrytis cinerea* was observed after 2 days of incubation
B	Nil antimicrobial agent, 50 µL solvents (ethyl acetate)	+ve, +ve, +ve	Growth of *Botrytis cinerea* was observed after 2 days of incubation
C	Carvacrol 0.05 µL	+ve, +ve, +ve	Growth of *Botrytis cinerea* was observed after 2 days of incubation
D	Carvacrol 0.1 µL	+ve, +ve, +ve	Growth of *Botrytis cinerea* was observed after 2 days of incubation
E	Carvacrol 0.5 µL	+ve, +ve, +ve	Growth of *Botrytis cinerea* was observed after 5 days of incubation
F	Carvacrol 1.0 µL	+ve, +ve, +ve	Growth of *Botrytis cinerea* was observed after 6 days of incubation
G	Carvacrol 2.0 µL	-ve -ve, -ve	No Growth of *Botrytis cinerea* was observed after 10 days of incubation
H	Carvacrol 3.0 µL	-ve, -ve, -ve	No Growth of *Botrytis cinerea* was observed after 10 days of incubation
I	Carvacrol 4.0 µL	-ve, -ve, -ve	No Growth of *Botrytis cinerea* was observed after 10 days of incubation
J	Carvacrol 5.0 µL	-ve, -ve, -ve	No Growth of *Botrytis cinerea* was observed after 10 days of incubation

'+ve' indicates growth of *Botrytis cinerea,* '-ve' indicates No growth of *Botrytis cinerea.*

Table 3-3: **Minimum inhibitory concentration (MIC) of carvacrol against *Botrytis cinerea* incubated at 25 °C tested by micro-atmosphere method.**

Filter paper µL/75mL air	µL/L	mg/L	mmol/m^3_{air}	*$P_{crv}/P_{sat, crv}$	Sign of microbe growth after incubation time (days)	Lag days before growth
0.0	0.0	0.0	0.0	0.0	2.0	0.0
0.05	0.7	0.7	4.3	3.0	2.0	0.0
0.1	1.3	1.3	8.7	6.2	2.0	0.0
0.5	6.7	6.5	43.3	30.7	5.0	3.0
1.0	13.3	13.0	87.0	61.6	6.0	4.0
2.0	26.7	26.1	173.0	123.0	>10.0*	> 8.0
3.0	40.0	39.1	260.0	184.0	>10.0*	> 8.0
4.0	53.3	52.2	347.0	246.0	>10.0*	> 8.0
5.0	66.7	65.3	433.0	307.0	>10.0*	> 8.0

*No sign of growth after 10 days incubation. These data were calculated by on assumption that all the antimicrobial agent vapourised in the headspace. *Using $P_{sat, crv}$ = 3.5 Pa from Van Roon (2002)

Several researchers have stated that there was a loss of active compound vapour during the incubation process (Gocho, 1991a; Gocho, 1991b; Inouye *et al.*, 1998; Nedorostova *et al.*, 2009). To minimise the loss, Nedorostova *et al.* (2009) modified the micro-atmosphere method by pouring the agar media into polystyrene Petri dishes with a small amount on its cover to serve as a sealant to prevented adsorption of essential oils onto the plastic material of Petri dish cover. Conversely, some investigators (Lopez *et al*., 2005) explained that hermetic sealing is not needed in Petri dish in order to represent the worst case scenario in the system, where the possibility leaking of the active components to the atmosphere will increase the potential for microorganism growth.

3.3.3 Conclusion

The key idea of this experiment was to determine minimum inhibitory concentration for carvacrol via the vapour phase. The data presented showed that the minimum inhibitory concentration of carvacrol against *Botrytis cinerea* varies depending upon the definition of MIC chosen. The results obtained were within the range as reported in the literature. It is also clear however that the headspace concentrations cannot be what is normally reported using this method (P > Psat) for all experiments which are not possible. However, in this method, it is important to highlight factors that likely influenced the results of MIC. For example, the reporting of effective concentration in relation to airspace, volatile evaporation speed, exposure times, microbial strains and definitive sources of the volatile antimicrobial are evident as well as the variation in a method in terms of Petri dish sizes, filter paper diameter, and choice of media. In addition, the vapours leaking through the un-hermetic closure system, the absorption into the media and the container as well as the incubation temperature all affect the partial vapour pressure of the active compound. These factors must be considered as it is likely that the vapour phase concentration changes dynamically during the incubation period.

CHAPTER 4

CHARACTERISING THE MINIMUM INHIBITORY CONCENTRATION (MIC) MEASUREMENT SYSTEM

4.1 Introduction

In Chapter 3, preliminary screening was conducted to verify the effectiveness of the selected antifungal agent to the selected spoilage fungi and media system. This clearly showed that MICs of the natural antimicrobial, carvacrol were within the range as reported in the literature. These measurements and reported MIC's are very high and exceed the saturation concentration of the volatile in the air. The actual volatile concentration in the headspace is likely to vary due to a release from the carrier, absorption into the media or Petri dish and potential losses to the ambient. For this reason, it is important to characterize what was happening in the Petri dish system during the incubation period. In order to demonstrate the mechanisms that are influencing the active compound concentration in the polystyrene Petri dish system, an experimental device was developed to allow measurement of the concentration of volatiles in the headspace. The key objective of this chapter was to

characterize the dynamics of volatile concentration changes in the headspace during a standard MIC measurement using the reverse Petri dish method.

4.2 Characterization of the Headspace Dynamics for Carvacrol Vapour Release in Polystyrene Petri Dishes

4.2.1 Materials and Methods

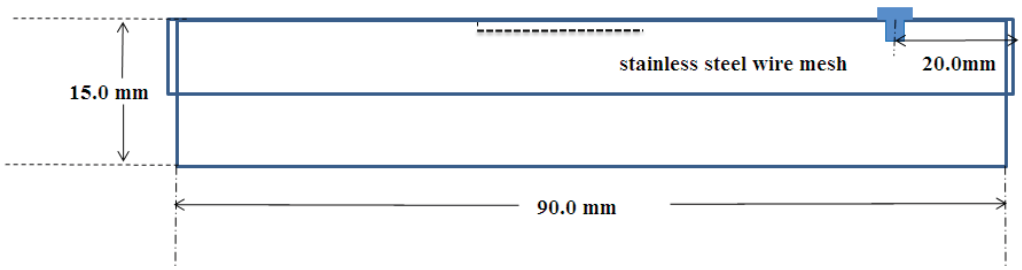
4.2.1.1 Chemicals

Potato Dextrose Agar (PDA) was used as a model food system was purchased from Diffco, Becton Dickinson & Company, (USA). Carvacrol and polystyrene Petri dishes (P5731, Excel Scientific D-910) were purchased from Sigma-Aldrich, New Zealand. Parafilm-M was purchased from (Pechiney Plastic Packaging, Chicago, Illinois)

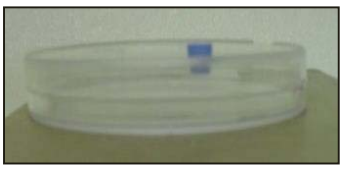
4.2.1.2 Experimental Device

Polystyrene Petri Dish and Aluminium Dish

The lid of the Polystyrene Petri dish, (90 mm diameter x 15 mm high, internal dimension) was modified by adhering (Loxeal Engineering Adhesives, 43) a rectangular 15 mm x 25 mm stainless steel wire mesh to the centre of the lid to hold the filter paper disk to provide a reservoir for the essential oil. A 3 mm diameter hole; was drilled 20 mm from the edge of the polystyrene Petri dish lid and hermetically stopped with a Teflon plug septum (Phenomenex, USA), to allow gas sampling. Teflon is known to be impermeable to essential oils volatile compounds (Utto, 2008). The modified Petri dish can be seen in Figure 4-1.



(a)



(b) (c)

Figure 4-1: **Images of the experimental setup to sample the headspace generated by the active compound in the Petri dishes. a) Schematic diagram, b) and c) photographs of example dishes.**

Figure 4-2 illustrates the design of the aluminium dishes which were fabricated to determine headspace dynamics. The aluminium dish was designed to enable the measurement of the headspace gaseous concentrations without the issue related to absorption into the polystyrene dish. The internal height and diameter of the aluminium dishes were identical to the internal dimensions of commercial polystyrene Petri dish. Stainless steel mesh was used to hold the filter paper reservoirs in the same way as described above, and Teflon plug septa were used to allow gas sampling. Six screws, distributed evenly around the top of the lids were used to clamp the lid to the body.

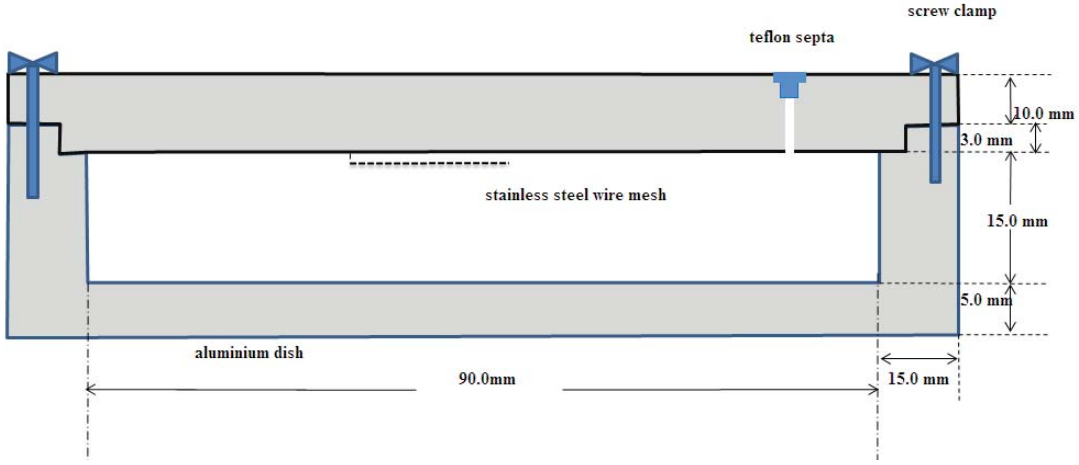


Figure 4-2: Schematic representation of the experimental aluminium dishes

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4.2.1.3 Media and Sample Preparation

Four dish configurations were used, either empty or filled with PDA. Each combination was prepared in duplicate. The dish configurations were:

(a) - Polystyrene Petri dish unsealed.

(b) - Polystyrene Petri dish wrapped with plastic paraffin film at the edge.

(c) - Polystyrene Petri dish wrapped with aluminium foil at the edge.

(d) - Aluminium dish.

Configuration (a) is the worst case scenario where leakage and absorption can take place.

Configuration (b) is the most commonly used system.

Configuration (c) should prevent leaks more effectively as foil is a better gas barrier.

Configuration (d) will remove both leaks and absorption into the polystyrene dish

For those filled dish samples, sterilised potato dextrose agar medium was prepared, and ~20 mL agar was poured aseptically into the respective dishes and solidified. This resulted in an approximately 75 mL of air space after the addition of the PDA media.

(a) Polystyrene Petri dish

(b) Polystyrene Petri dish wrapped with plastic paraffin film at the edge

(c) Polystyrene Petri dish wrapped with aluminium foil at the edge

(d) Aluminium dish

Figure 4-3: The dish configurations.

4.2.1.4 Headspace Dynamics Characterisation

All devices used were equilibrated at 25 °C overnight prior to the testing. Five microliters of carvacrol were injected with a Hamilton glass micro syringe (10 µL maximum capacity, Hamilton Co., USA) onto to the paper disc (filter paper 20 mm diameter, brand Whatman 54, hardened, qualitative fast) which was already slotted into the wire mesh in the lid of the dish. After the carvacrol was impregnated on the filter papers, the lids were fitted, and the samples were stored in a controlled temperature room at 25 °C. The concentration profiles of carvacrol vapour in the headspace were evaluated for a week. The GCMS system cycle needed half an hour to be ready for each sampling; therefore, the sampling was carried in such a way that the measurements could be carried out alternately with the duplicate samples. The carvacrol concentration was measured twice daily for a week. For the day, 0 gas samplings, these were performed 30 minutes after carvacrol was introduced to the filter

paper disk and the sampling repeated every hour for 6 hours (alternating sampling since there were two samples).

4.2.1.5 Headspace Measurement

In this study, the headspace volatiles sampling method was similar to that used by Utto *et al.* (2008), with direct sampling using an airtight syringe. The vapour concentrations were quantified using gas chromatography combined with mass spectrometry (GCMS-QP2010S, Shimadzu) using EPA Method 8260b (EPA, 1996).

Standard solutions were prepared by dissolving carvacrol (>98%, GC Grade, SAFC supplied by Sigma-Aldrich, New Zealand) in ethanol (>99%, Anchol GC grade, supplied by Thermo Fisher Scientific, New Zealand). Carvacrol standard curves for GCMS analyses were carried out with a split/splitless injector and a Shimadzu autosampler AOC-20s and autoinjector AOC-20i. A Zebron-ZB-5 capillary column was used (30 m x 0.25 mm I.D., 0.25 µm film thickness). The temperature program was set between 60 to 200 °C heating at 20°C/min and then ramped at 25°C/min to 280°C and finally held for 3 minutes. The injection temperature was set at 250 °C, which is higher than the boiling point of carvacrol (237 °C). The injection volume was 1.0 µL, and the inlet pressure was set at 49.5 kPa. Helium was used as a carrier gas at a linear velocity of 36.1 cm/s. The column flow was 0.98 ml/min. The split ratio was set at ratio 1:5. The interface temperature was set at 250 °C, ionization energy at 70 eV, scanned at a mass range 40–350 *m/z*; and the solvent cut time was set at 3 min. With this programme, the retention time for carvacrol was determined at 5.87 minutes (Figure 4.4).

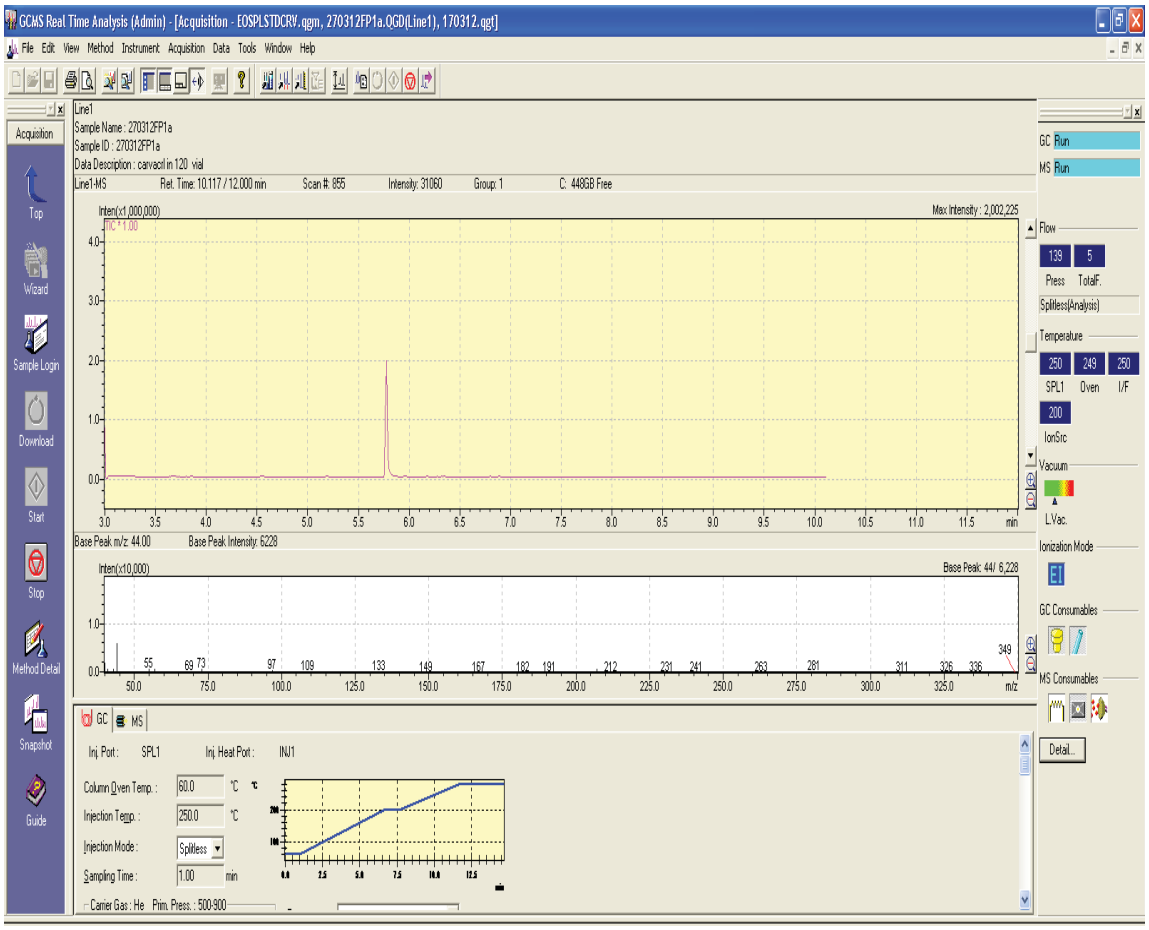


Figure 4-4: Snapshot of carvacrol concentration using GCMS –QP2010 Series software.

The carvacrol concentration in the gas phase was measured by taking 500 µL out of 75 to 95mL of the sample headspace, using an airtight syringe (Hamilton Gastight, Hamilton Co., US) through the septum fitted on the dish lid. This represents ~ 0.5% change in the amount of carvacrol in the headspace and will have minimal impact on MIC calculations.

These samples were injected manually into the GCMS with a splitless injector. The programme setup was similar to that described above except the injector was operated in

the splitless mode. The split-less program was used due to the low carvacrol vapour concentration in the headspace.

The concentration (mol.m^{-3}) of gas in the polystyrene Petri dish headspace was calculated by using Eq. 4-1 as demonstrated by Utto (2008).

$$C^i = \frac{K_{GC}^i A_{GC}^i}{Vol_{inj}}$$

Eq. 4-1

Where

C^i	=	Concentration of volatile organic compound (VOC) *i*, (mol.m^{-3})
K_{GC}^i	=	Detector response or slope (mol. Area $^{-1}$) of a standard curve of VOC *i*.
A_{GC}^i	=	Area of gas chromatogram peak from the injected volume of sample (Area).
Vol_{inj}	=	Injection volume of sample (m^3)

Van Roon *et al.,* (2002) reported, the experimental saturated vapour pressure (*Psat*) of carvacrol at 25 °C is 3.5 Pa, allowing the vapour concentration in the headspace to be given as the relative vapour pressure, *Pv/Psat.*

4.2.1.6 Statistical Analysis

The significance of differences between means among treatments or storage times was calculated by 2-way ANOVA using time as a covariate. Post-hoc Bonferroni analysis was carried out to identify which dish treatments were significantly different. Statistical analysis was performed using Minitab version 16 (2010, Minitab Inc., USA). In this study, data was considered to be significantly different at ($P \leq 0.05$).

4.2.2 Results and Discussion

Experimental results are shown in Figure 4-5. Table 4-1 summarises the ANOVA results showing that dishes containing PDA responded significantly different to empty dishes, keeping the headspace volatile concentration lower.

Table 4-1: Analysis of variance for carvacrol concentration in the dishes headspace with time as a covariate.

Source	DF	SS	MS	F	P
Time	1	0.9074	0.9074	28.09	0.000
[a]Dishes	3	5.0436	1.6812	52.04	0.000*
[b]Full	1	2.6183	2.6183	81.05	0.000*
Error	250	8.0767	0.0323		
Total	255				

[a] Represent dish configuration, [b] Represent dishes filled with PDA or empty.
*Significant difference at P $\leq$0.05.

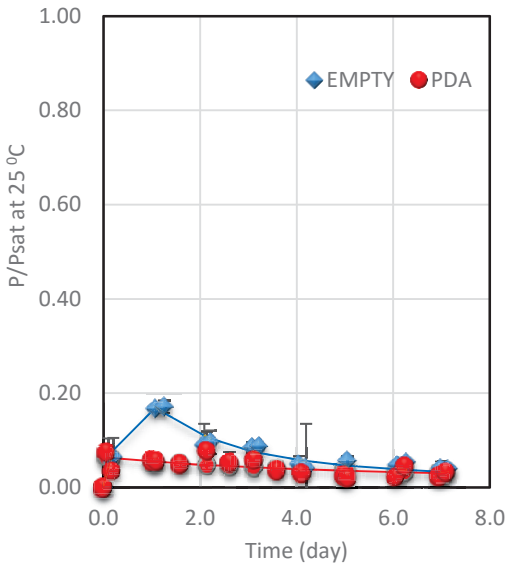
Table 4-2: Grouping Information Using Bonferroni Method and 95.0% Confidence Level

Dish configuration	N	Mean	Grouping
(a)	68	0.0	A
(b)	68	0.1	A
(c)	52	0.2	B
(d)	68	0.4	C

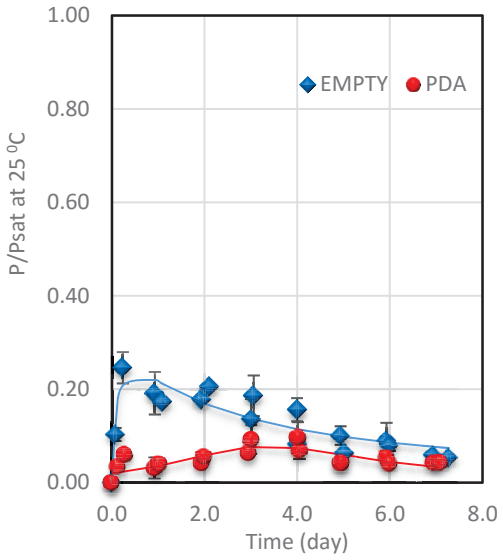
Means that do not share a grouping letter are significantly different

Bonferroni analysis (Table 4-2) showed that the empty aluminium dish (d) and polystyrene Petri dish sealed with aluminium foil (c) were significantly different to each other and the other dishes. There were no significant differences between the unsealed polystyrene dish (a) and the Parafilm sealed Petri dish (b) at $P \leq 0.05$. These results suggest that the aluminium foil was more effective at sealing the dish than the Parafilm.

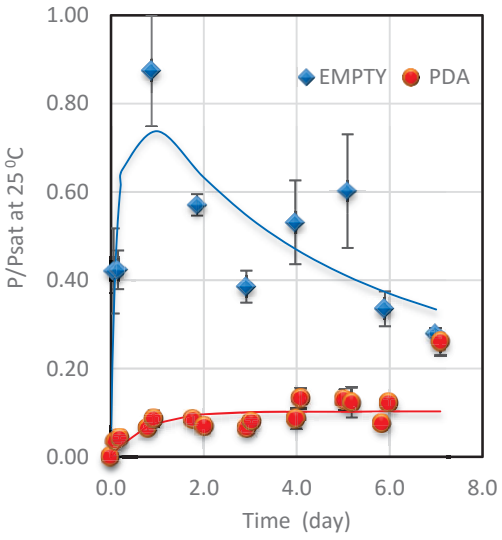
In the reverse Petri plate method, the whole oil or volatile component is assumed to evaporate into the atmosphere of a closed Petri dish. The release profiles in the headspace in experiments that reproduce the standard method are shown in Figure 4-5(a). Interestingly, even though the carvacrol supply was in excess, (5µL/75 mL air corresponding to > 300 times saturated air), the amount of carvacrol in the headspace was very low, with or without the PDA being present, suggesting either volatile release from the filter paper was slow, or it is lost from the headspace to other parts of the system. These results suggest that the literature values reported are overstated and much lower headspace concentrates can inhibit growth.



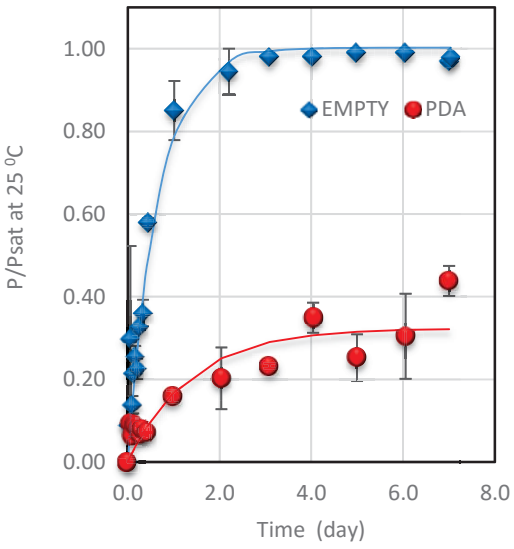
(a) Polystyrene Petri dish



(b) Polystyrene Petri dish wrapped with Parafilm at the edge.



(c) Polystyrene Petri dish wrapped with aluminium foil at the edge



(d) Hermetically sealed aluminium dish

Figure 4-5: **The profiles of the headspace carvacrol concentration change in the dishes over time (days) with and without PDA (Vertical bars represent the average ± standard error of duplicate measurement).**

Sealing of the Petri dish with vinyl tape (Edris and Farrag, 2003) or sterile adhesive tape (Lopez *et al.*, 2007b; Goni *et al.*, 2009) is commonly practised by researchers to determine antibacterial activity in vapour systems. The release profile in the Parafilm sealed dish (Figure 4-5(b)) showed a similar trend as for the unsealed dish (Figure 4-5(a)) although the concentration of carvacrol is slightly higher. This demonstrates that significant vapour losses do occur in the unsealed dish and that the Parafilm can partially reduce these losses.

In contrast, the polystyrene Petri dishes sealed with the aluminium foil (Figure 4-5 (c)), showed significant increase in headspace concentration. This can be explained in that the vapour was able to slowly absorb into and diffuse through the Parafilm whereas the polystyrene Petri dish sealed with aluminium foil gave a good barrier with very low vapour permeability. The relatively high peak vapour pressure in the empty foil sealed dish shows that losses are an important cause of vapour loss at the start of the incubation periods. The gradual reduction on further storage suggests absorption into the plastic dish material is slow but significant.

The experimental data results for all the Petri dish configurations studied showed the partial pressure of carvacrol in all systems failed to achieve a saturated state, and the concentration in the headspace depleted gradually. It was of interest, therefore, to measure the carvacrol release behaviour in a system that was hermetically sealed and had negligible carvacrol absorption capacity (Figure 4-5 (d)). In comparison with the previous results, in the hermetically closed system aluminium dish, the volatile compound reached saturation equilibrium after two days storage. This reinforces the explanation that the low carvacrol vapour pressure in empty polystyrene Petri dishes as exhibited in Figure 4-5 (a), (b) and (c), were likely due to losses to the ambient atmosphere and the absorption of carvacrol into the polystyrene dish material. In the literature, no data is available for the partition coefficient

between carvacrol and polystyrene. However Cerisuelo *et al.* (2012) reported that the carvacrol partition coefficient constant in air/polypropylene system is ~10^5. This value suggests a strong affinity for absorption of carvacrol in the polystyrene dish is likely. The low diffusion coefficient of volatiles in polystyrene (10^{-14} $m^2.s^{-1}$, Cayot *et al.,* 2008), would explain why an initial increase is observed in the sealed dish, despite the high absorption capacity of the plastic, and then the subsequent slow reduction in vapour pressure.

In an ideal closed system (in this case, empty aluminium dish) the active compound added to the carrier (filter paper) will evaporate (desorb) from the carrier into the surrounding headspace until equilibrium is achieved. At this point, the saturation of airspace takes place, and the concentration of vapours becomes constant (Edris and Farrag, 2003).

In the same system, when the agar media (PDA) was added in the Petri dish, the absorption capacity and diffusion rate of volatile into the media (10^{-9}-$10^{-10}$$m^2.s^{-1}$, Cayot *et al.,* 2008), strongly influenced the release profile of the active compound in the dish headspace. If the quantity of active compound added was not enough to saturate the PDA with the volatile compound, a saturated headspace could not be achieved. Figure 4-5 (d) shows that a large uptake of carvacrol occurred into the PDA (about 65% of carvacrol uptake). The low concentration of carvacrol in the headspace was expected due to the absorption of carvacrol in the agar media in addition to absorption by the plastic in the Petri dish and leaking to the environment. The air/water partition coefficient, for linalool was $3.84x10^{-3}$ in water (11.0 Pa, 25°C), (Sadafian and Crouzet, 1987) and carvacrol was $9.14x10^{-5}$. These indicate a high absorption capacity of carvacrol in PDA as PDA consists of 97% of water.

4.2.3 Summary Findings

Minimum inhibitory concentrations (MIC) measured using the inverse Petri plate method are considered the premium standard to evaluate the resistance of organisms to antimicrobial agents. From these results, it can be concluded that absorption of carvacrol vapour by the PDA strongly influenced the headspace dynamics and was the main reason for the low volatile concentrations in the headspace in the reverse Petri plate MIC measurement method. The relative partial pressure of the carvacrol was much lower (0.02 to 0.35) than the saturated vapour pressure except in the empty sealed aluminium dish. These results suggest the MICs reported by investigators are much higher than the actual headspace concentrations in the system and thus overstated. In order to determine reliable minimum inhibitory concentrations, a method that can provide a consistent concentration in the system headspace must be developed.

The interaction of essential oil volatiles and the cell culture substrate (in this case PDA) is a key factor influencing the interpretation of MIC measurements and requires further investigation. The relatively slow response of the vapour release (even in the empty aluminium dish) suggests that the interaction between the volatile and the filter paper could also be an influence on the headspace dynamics. For this reason, these two factors are investigated below.

4.3 Determination of the Desorption Isotherm for Carvacrol and Filter paper

Section 4.2 discussed the measurement of the carvacrol vapour concentration in the standard Petri dish headspace. The release of carvacrol from the filter paper and the absorption of the volatile into the PDA medium influenced the concentration of carvacrol vapour in the headspace. Carvacrol release from the filter paper will be dependent on the

equilibrium vapour pressure at the surface of the filter paper. As such, the isotherm sorption of the filter paper may provide some explanation for the relatively slow release from the filter paper. Sorption isotherms can be measured for adsorption of vapour onto the solid phase (adsorption isotherm) or for desorption from the solid phase into the air phase (desorption isotherm). These can be significantly different and hysteresis can occur when cycling between adsorption and desorption. In the reverse Petri dish MIC measurement method, the vapour release (desorption) from the filter paper is of most relevance. There have been no reports of sorption isotherms for carvacrol on filter paper. Due to this, an experiment was performed based on the method of Lu *et al*., (2004), with some modifications.

4.3.1 Materials and Method

Carvacrol purity > 97% was obtained from Sigma-Aldrich. Filter Paper 20 mm diameter (Grade 54, pore size 20-25 μ) was obtained from Whatman. The sorption isotherms relate the concentration of carvacrol in the adsorbed state versus its gas phase concentrations or partial pressure.

The desorption isotherms were determined by using the constant volume method at 25 ± 1 °C. One initial carvacrol volume was used for the single solute system. Each set of bottles was accompanied by one blank to check for any volatilization, adsorption onto the walls of the bottles, and biodegradation of the adsorbate. Filter papers were accurately weighed (±0.000 mg, brand Sartorius M2P). The 120 mL glass Wheaton bottles were filled with different masses of filter paper (refer to Table 4-1). To obtain the desired initial concentration in the bottle, 5.00 μL of carvacrol was injected into the filter paper in the bottle with an electronic micropipette (Transferpette, range 2- 20 μL, Germany) which provide an initial amount of 3.25×10^{-5} mol. The bottles were immediately tightly sealed with rubber butyl Teflon lined

stoppers and crimped with aluminum caps using an EZ Wheaton crimper (Millville, N. Jersey). At a pressure of one atmosphere, each bottle contains an air volume of 117 mL (actual volume of the bottle). The bottles were shaken at 150 rpm, 25.0 °C (Infos HT Multritron) for one hour to ensure proper contact of the adsorbent with the filter paper.

The preparation of samples was carried out at the room temperature. No pre-drying of the filter paper was done in this method to represent the same conditions as the characterization of headspace in the standard Petri dish as described in Section 4.2.1.4. All of the bottles were equilibrated in a room that had a constant temperature of 25.0 ± 1.0 °C overnight (24 hours). After a 24-hour equilibration period, the blank bottle was sampled, and the headspace concentration was analyzed. The measured values were recognized as the initial concentration for each set of bottles. Through initial testing, it was found that 24 hours were sufficient for attaining equilibrium. Gas samples were taken and analyzed to determine the equilibrium gas phase concentrations.

(a) weighing (b) shaker (c) equilibrated samples

Figure 4.6: Sample preparation to determine the filter paper isotherm of carvacrol

Table 4-3: Dataset for the carvacrol isotherm

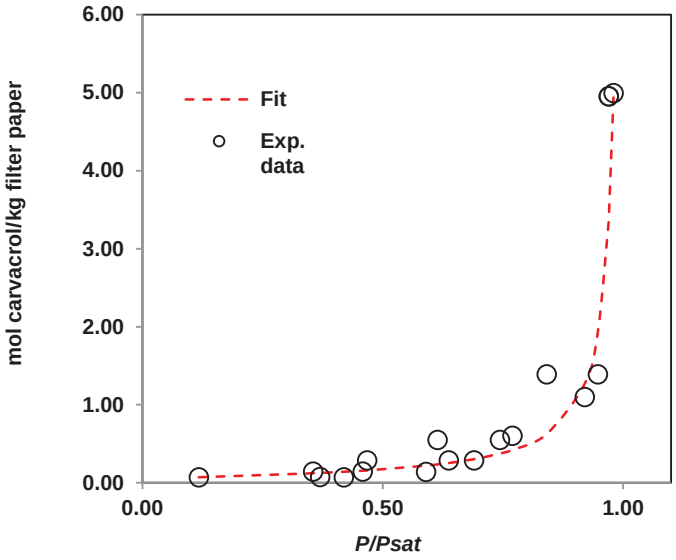
Bottles	Weight of filter paper (mg)	No of pieces	Weight Carvacrol (μL)/Filter paper (mg)
Set A			
1a	0.000	0	inf
2a	26.381	1	0.190
3a	58.956	2	0.085
4a	112.514	4	0.044
5a	223.414	8	0.022
6a	450.366	16	0.011
Set B			
1b	0.000	0	inf
2b	29.532	1	0.169
3b	53.909	2	0.093
4b	112.67	4	0.044
5b	228.15	8	0.022
6b	443.092	16	0.011

Analytical Procedures

The headspace concentration at equilibrium was measured with Gas Chromatography-Mass Spectrometers. The method was similar as described in Section 4.2.1.5, GCMS method.

4.3.2 Results and Discussion

Figure 4-7 represents the experimental data of the equilibrium desorption isotherm for carvacrol on the filter paper. The equilibrium desorption isotherm profiles appeared to be Type III isotherm.



Coefficient Data:

Harris Model:

$$y = \frac{1}{a + bx^c}$$

a = 24.9941 kg/mol

b = -24.9820 kg/mol

c = 0.37868

Figure 4-7: **Experimental data and the curve-fitted equilibrium carvacrol concentration in the filter paper against carvacrol relative vapour pressure in the bottle headspace.**

In this study, the Harris equation was fitted to the experimental data with *CurveExpert Professional 1.5* software. The equation is expressed as

$$y = \frac{1}{a + bx^c} \qquad \textbf{Eq. 4-2}$$

With the correlation coefficient R^2 of 0.96, where the y axis represents the absorbed carvacrol concentration on filter paper (M_c) and x is expressed as carvacrol relative vapour pressure($P/Psat$).

$$M_c = \frac{1}{a + b(P/Psat)^c}$$ **Eq. 4-3**

This can be rearranged as follows to predict *P* as a function of (M_c)

$$a + b(P/Psat)^c = \frac{1}{M_c}$$ **Eq. 4-1**

$$b(P/Psat)^c = \frac{1}{M_c} - a$$ **Eq. 4-4**

$$(P/Psat) = \left(\frac{1}{b.M_c} - \frac{a}{b}\right)^{\frac{1}{c}}$$ **Eq. 4-5**

$$P = Psat.\left(\frac{1}{b}\left(\frac{1}{M_c} - a\right)\right)^{\frac{1}{c}}$$ **Eq. 4-6**

This can be converted to a molar carvacrol gas phase concentration using the ideal gas law.

$$C_0 = \frac{n}{V} = \frac{P}{RT}$$ **Eq. 4-7**

$$C_0 = \frac{P}{RT} = \frac{Psat}{RT}.\left(\frac{1}{b}\left(\frac{1}{M_c} - a\right)\right)^{\frac{1}{c}}$$ **Eq. 4-8**

Where M_c represents the equilibrium loading of mol carvacrol in kg filter paper, *a (kg/mol), b (kg/mol)* and *c* are constant parameters and $C_0(\mathrm{mol/m^3\ air})$ is the initial carvacrol concentration, *R* is ideal gas constant and *T* is temperature (K).

4.3.3 Summary Findings

A Harris isotherm equation was used to describe sorption capacity dependence on the carvacrol concentration on the filter paper. The final equation to predict the isotherm of carvacrol in the filter paper was shown in equation Eq. 4-7. The equation Eq. 4-7was derived based on the ideal gas law to determine the release of carvacrol from filter paper in the standard Petri dish method.

The shape of the isotherm explains some of the release behavior in the reverse petri dish method. In the release profiles (Figure 4-5 - a, b, c and d), an initial rapid increase that subsequently reduces is observed. The isotherm shape supports this as at the onset of the experiments; the amount of carvacrol in the filter paper is high and, therefore, the equilibrium vapour pressure is high. This allows the release to match or be greater than absorption in the plastic dish. After the concentration of carvacrol on the filter paper surface reduces to about 1 mol/m^3, the equilibrium vapour pressure dramatically reduces. Therefore, the release rate will decrease.

This behavior would occur earlier if larger filter paper disks were used for the same applied volume of essential oil liquid. If diffusion within the filter paper were also to be partially limiting, then this together with the equilibrium behavior of the carvacrol and filter paper could explain the relatively low release rate in the empty aluminum dish. Because the air phase, initially has no volatiles present, the surface of the filter paper may dry and internal diffusion limitations may slow vapour. The interaction between the volatile and filter paper suggest that for reliable MIC measurements, the use of the filter paper should be avoided.

4.4 Partition Coefficient

The equilibrium distribution of carvacrol vapour between different phases (air and PDA medium) is an important aspect of measuring the carvacrol vapour concentration in the headspace. One way to investigate the retention or release of carvacrol compound in the PDA medium is to measure the gas/PDA medium partition coefficient. Therefore, experimental work to determine the partition coefficient between these phases was carried out. The static headspace (headspace analysis) direct method was used in this work.

The partition coefficient ($K_{g/pda}$) is the ratio of the concentration of the analyte in the gas phase(C_g) to the concentration of the analyte in the sample phase (C_{PDA}) at equilibrium, and is shown in Eq.4-9.

$$K_{g/PDA} = \frac{C_g}{C_{PDA}} \qquad \textbf{Eq. 4-9}$$

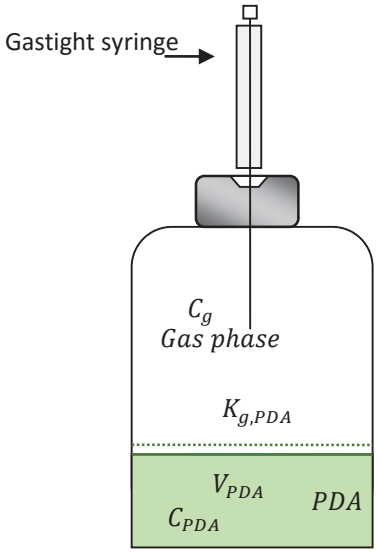


Figure 4-8: Illustration of sample and the phase in the Wheaton bottle as they relate to the partition coefficient

This study characterised the partitioning between carvacrol or thymol and PDA, the model food system used in evaluating the reverse petri dish MIC method. The illustration of the sample and the phases in the Wheaton bottle is shown in Figure 4-8.

4.4.1 Materials and Methods

Carvacrol and thymol were bought from Sigma-Aldrich New Zealand. Potato Dextrose Agar (PDA) was used as a model food system and was purchased from Diffco, Becton Dickinson & Company, (USA).

Wheaton bottles (120 mL) and their lids (aluminium cap and rubber butyl Teflon lined stopper caps were purchased from Millville, N. Jersey) were exposed to UV light (German Sciences, Biohazard-Protection, UV Light Intensity, ASTM. No 1807.23) overnight to sterilise them. Potato dextrose agar (Diffco) was prepared aseptically following the manufacturer's instructions. Approximately 20 mL of molten (~ 50 °C) potato dextrose agar (PDA) was blended with various amounts of carvacrol and thymol to a final concentration (ranging from 0.0 – 8.0 mol/m^3_{PDA}) respectively, and then poured into the Wheaton glass bottles. Each bottle was hermetically sealed with an aluminium cap with the Teflon /rubber butyl septa using an EZ Wheaton crimper (Millville, N. Jersey). Samples were prepared in quadruplicate. The solidified PDA was stored in the controlled temperature room at 25°C overnight without shaking**.**

The headspace of the samples in Wheaton glass bottles were analysed using Gas Chromatography-Mass Spectrometry (Shidmadzu 2010). Vapour phase samples (0.5 mL) were taken with a gas-tight syringe and injected into a Shimadzu 2010 gas chromatograph as described in Section 4.2.1.5. Only one gas sample was removed from each Wheaton bottle as removal of 0.5mL would produce a partial vacuum and change the equilibrium behaviour.

4.4.2 Results and Discussion

Figures 4-9 and 4-10 show the plots of carvacrol and thymol concentration, in each phase (mol/m^3 air) vs. (mol/m^3_{PDA}) respectively. The solid phase of concentration was assumed to not change significantly due to the release into the gas phase. This is possible due to the partitioning between the phases strongly favouring the solid. For example at a concentration $4mol/m^3_{PDA}$, there was 80 μmol present in the 20 mL of PDA. In the gas phase after equilibrium was reached, there was 25 nmol in the 100 mL of headspace. The experimental data showed that the partition coefficient of carvacrol is $5.95x10^{-5}$, and thymol is 2.58×10^{-4}. The concentration changes in the solid phase were, therefore, negligible. The sorption behaviour was linear over the concentration range investigated.

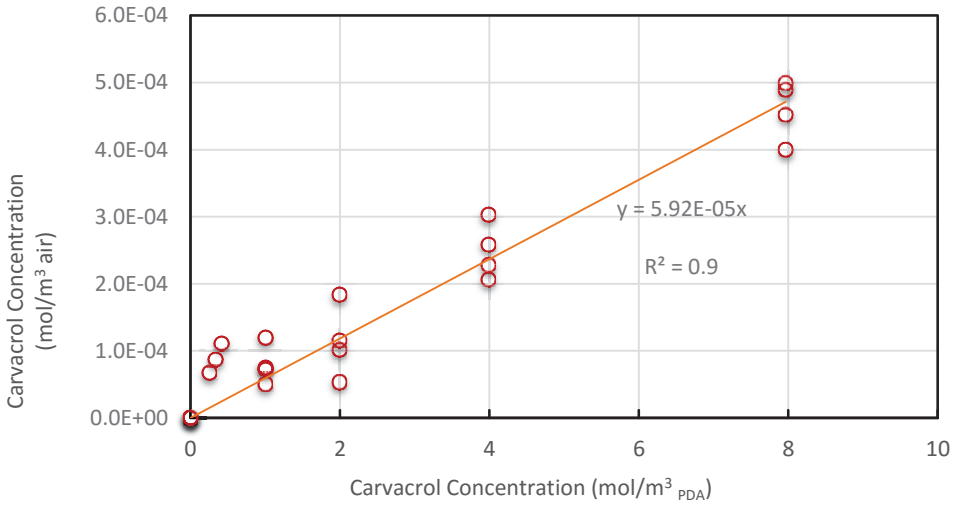


Figure 4-9: **Carvacrol partitioning, (mol/m³ air) vs. (mol/m³$_{PDA}$). Measurement of the gas phase in the sample vials was performed after the samples were stored at 25°C overnight. (Direct Method: Vapour Phase Calibration method)**

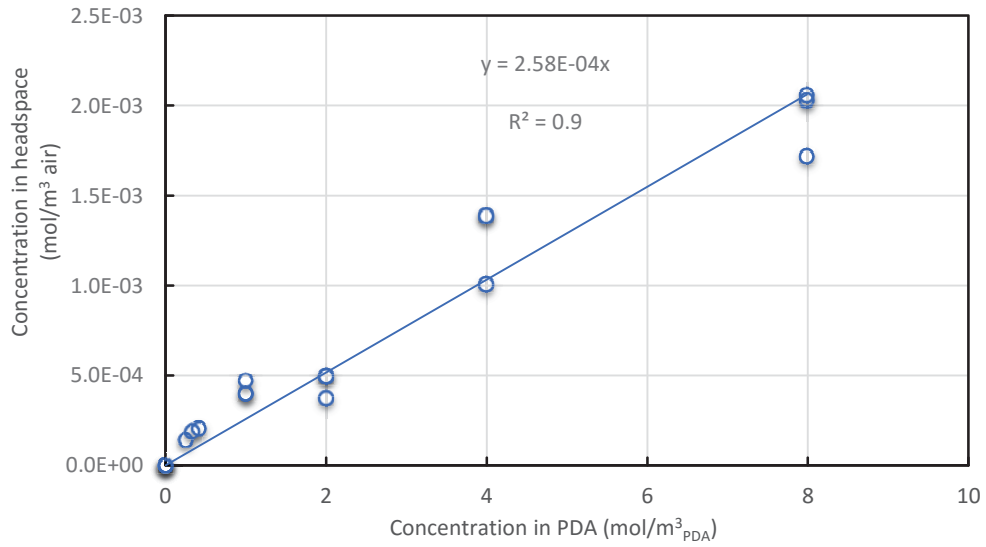


Figure 4-10: **Thymol partitioning (mol/m³ air) vs. (mol/m³ $_{PDA}$). Measurement of the gas phase in the sample vials were performed after the samples were stored at 25°C overnight (Direct Method: Vapour Phase Calibration method).**

Table 4-4 summarises air/water partition coefficients for several essential oil volatiles. From this data, it can be seen that the result measured in this work are much lower than observed for most reported systems. This means for carvacrol and thymol; the partitioning more strongly favours the solid phase. To check these findings, the partitioning of this volatiles for water were predicted using the BOND method in the EPI Suite™ 4.1 software. These provide $K_{A/W}$ values of 9.14×10^{-5} and 1.97×10^{-5} for carvacrol and thymol respectively. These compare reasonably with the measured values of 5.94×10^{-5} and 2.58×10^{-4} measured in this work. It makes sense that similar equilibrium behaviour would be found for potato dextrose agar and water as the moisture content of the PDA was 95%, and it had a water activity of 0.97.

Table 4-4 also shows that the predictions of $K_{A/W}$ for the other volatiles are in good agreement with the experimental results reported in the literature. This gives confidence that the values measured in this work are reasonable.

Table 4-4: Vapour-liquid partition coefficient, Kaw experimental results from the literature as well as quantitative structure-property relationship (QSPR) estimates, K at 25°C.

	Gas-water Partition Experimental data from literature	[a]P_{sat} (Pa)	References	QSPR estimate [b]
2-nonanone	1.54×10^{-2} 6.70×10^{-3} 1.50×10^{-2}	193	(Lloyd *et al.,* 2011) (Da-Mi and Ebeler, 2003) (Buttery *et al.,* 1969)	1.11×10^{-2}
Limonene	1.13 1.70 1.18×10^{-1}	192	(Lloyd *et al.,* 2011) (Welke *et al.,* 1998) (Sadafian and Crouzet, 1987)	1.2
Camphene	2.96	237	(Lloyd *et al.,* 2011)	6.6
Hexanal	1.16×10^{-2} 87	1280	(Jouquand *et al.,* 2004) (Buttery *et al.,* 1969)	8.7×10^{-3}
Ethyl Butanoate	1.80×10^{2} 1.83×10^{2}	2080	(Jouquand *et al.,* 2004) (Lethanh *et al.,* 1993)	1.63×10^{-2}
2-Heptanone	0.57×10^{2}		(Jouquand *et al.,* 2004)	6.90×10^{-3}
2-Octanone	0.62×10^{2}		(Jouquand *et al.,* 2004)	7.69×10^{-3}
Linalool	3.84×10^{-3}	11.1	(Sadafian and Crouzet, 1987)	8.79×10^{-4}
Ethyl hexanoate	$2.82 \times 1 0^{-2}$ 2.95×10^{-3}	240	(Lethanh *et al.,* 1993) (Voutsas *et al.,* 2001)	2.96×10^{-3}
Carvacrol	$*5.95 \times 10^{-5}$	3.09	This work	9.14×10^{-5}
Thymol	$*2.58 \times 10^{-4}$	2.50	This work	1.97×10^{-5}

[a] Estimated via Modified Grain and Antoine method within EPI Suite™ 4.1 software

[b] Estimated via BOND Method, Henry Win v3.20 within EPI Suite™ 4.1 software

*partition coefficient air/PDA

4.5 Conclusion

The characterisation of the headspace dynamics in the reverse petri dish method in this chapter demonstrate it is difficult to obtain reliable MIC values for volatile antimicrobial agents. This is especially true if the values are needed for active packaging design rather than for testing the comparative activity of different compounds. The dynamics associated with release from the filter paper, losses from the dish and absorption into the dish walls and media result in much lower gas phase concentrations than are normally reported with this method.

These mechanisms also result in changing conditions during incubation and explain why differences in methodology such as filter paper area, dish diameter, etc. affected the results. As a result, an alternative method to determine MIC and potential for synergistic behaviour is required. The strong partitioning behaviour toward the solid phase provides a clue on how more stable headspace conditions might be possible to enable better MIC measurement.

CHAPTER 5

MEASURING MINIMUM INHIBITION CONCENTRATION: NEW METHOD DEVELOPMENT

5.1 Introduction

In the previous chapter, it was shown that in the standard reverse Petri dish MIC measurement method, there was the absorption of the antimicrobial agent into the standard polystyrene Petri dish and PDA, and loss into the environment. As consequence, the volatile concentration in the gas phase changes dynamically and is at much lower concentrations that have been assumed by other researchers.

As discussed in the literature review (section 2.3.2), some researchers claim vapour phase contact (release through the air phase) is more effective than direct contact for some systems (e.g. Inouye *et al.,* 2000, Mejia-Garibay *et al.,* 2015 and Shao *et al*., 2013). It is clear from the results reported in Chapter 4 that these measurements are the net result of a number of different rate processes and other factors such as vapour release, saturated vapour pressure, partitioning between phases and diffusion into food/media.

The mechanisms for the antimicrobial activity of essential oil volatiles are dependent on the concentration of the active agent in the cell membrane (see section 2.3.1). At the surface of the growth medium/food, there will be a three-way equilibrium (at least locally) between the air, cell membrane and surrounding media. It makes sense then that using a measurement system that avoids the dynamic behaviour characterised in chapter 4 and is always at this equilibrium will provide the most reliable estimate of the MIC. A MIC value determined in this way will provide appropriate target concentrations for active packaging system design.

In order to measure the minimum inhibitory concentration accurately, a hermetically sealed glass container system was proposed because it has very good barrier properties, and therefore losses to the environment can be prevented and adsorption into the wall will be negligible. The partitioning of the aromatic antimicrobial volatiles between the air and PDA, (e.g. carvacrol, $K_{air/PDA}$= 5.95x10^{-5}) strongly favours the PDA. Therefore, by adding the volatile compound directly to the PDA, after the bottle is sealed, the air phase will rapidly come to equilibrium with it and the concentration will remain constant.

5.2 Closed System MIC Measurement Method Development

Wheaton bottles (120 mL) are glass, thereby preventing significant absorption by the volatile active agents. They can be hermetically sealed using an aluminium foil lid which is crimped to overcome the volatiles losses observed in the standard Petri dish system investigated in Chapter 3. The Wheaton bottles are also transparent, allowing simple visual inspection for growth without breaking the seal. As discussed above, the partitioning of volatiles such as carvacrol and thymol strongly favour the PDA phase within the bottle. Because of this, it was proposed to add active agent liquid to pre-sterilised PDA before filling into the Wheaton bottles. After the bottles are inoculated with culture and sealed, the volatile will be desorbed from the PDA into its air phase until equilibrium is reached. Because the amount of

desorption required to reach this equilibrium is relatively small, the time was taken to reach a steady state will be short.

5.2.1 Selecting the Required PDA Volume

Using carvacrol as an example for this system, the amount of carvacrol required to reach different relative vapour concentrations for different volumes of PDA can be estimated using a simple mass balance.

$$M_{crv} = V_{PDA}C_{PDA} + V_{air}C_{air} \quad \textbf{Eq. 5-1}$$

Where M_{crv} is the mol of active agent (carvacrol) added to the PDA and C_{air} is the concentration of carvacrol in the air in equilibrium with C_{PDA}, the concentration in PDA. This is given by the partition coefficient $K_{air/PDA}$= 5.95x10^{-5}.

$$M_{crv} = V_{PDA}\frac{C_{air}}{K_{air/PDA}} + (V_T - V_{PDA})C_{air} = \frac{\left[V_T - V_{PDA}(1 - \frac{1}{K_{air/PDA}})\right]P_v}{RT} \quad \textbf{Eq. 5-2}$$

Where V_T and V_{PDA} are the total volume and volume of PDA respectively. This can be converted into a volume of pure carvacrol required for aqueous PDA volumes with equation.

$$V_{crv} = M_{crv}.\frac{W_{w,crv}}{\rho_{crv}} \quad \textbf{Eq. 5-3}$$

Where V_{crv} = volume of carvacrol, $W_{w,crv}$ = carvacrol molecular weight, 150.22g/mol and ρ_{crv} = carvacrol density, 0.9786 g/mL at 25 °C (Sigma-Aldrich, Refer to Appendix 1). This is shown graphically in Figure 5-1 below for different PDA volumes at 25°C in 120mL Wheaton bottles. In this analysis it is assumed the volume of PDA is constant. Some moisture will be

lost from the agar to saturate the gas space with water vapour. This amounts to a 0.01% change in the mass of PDA in the Wheaton bottle and can be considered negligible.

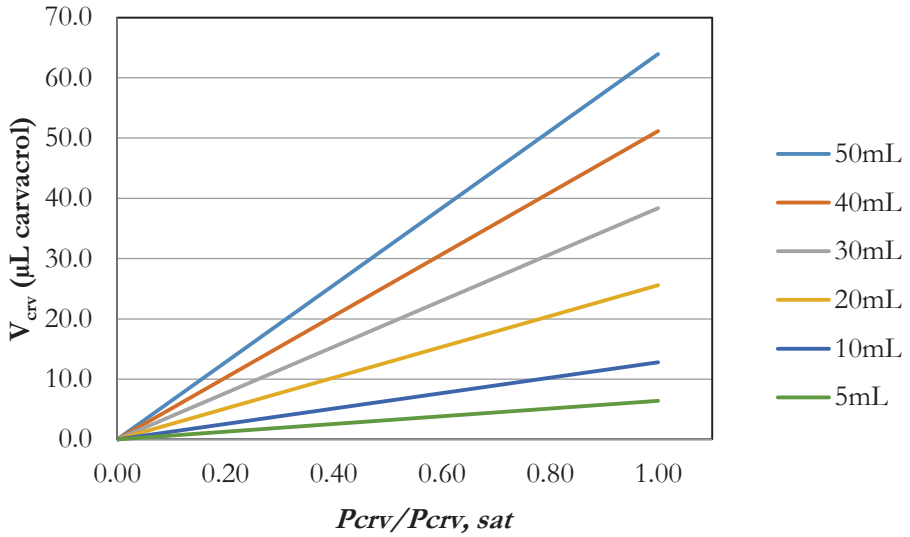


Figure 5-1: **Profiles carvacrol volume (µL) against carvacrol relative vapour pressure at a different volume of PDA in 120 mL Wheaton bottle.**

Some of this added carvacrol will be desorbed into the headspace after sealing to reach equilibrium, thereby changing the concentration in PDA. The change in the PDA concentration due to this effect is small because the partition coefficient strongly favours the PDA as shown in Chapter 4. A mass balance can be carried out to quantify this extent as follows.

$$\frac{C_{PDA}}{C_{PDA,i}} = \frac{C_{PDA}}{M_{crv}/V_{PDA}} = \frac{\frac{1}{K_{air/PDA}} C_{air} V_{PDA}}{V_T - V_{PDA}(1 - \frac{1}{K_{air/PDA}})C_{air}} \qquad \textbf{Eq. 5-4}$$

$$= \frac{\frac{1}{K_{air/PDA}} V_{PDA}}{V_T - \frac{V_{PDA}}{K_{air/PDA}}(K_{air/PDA} - 1)}$$

$$= \frac{V_{PDA}}{K_{air/PDA}V_T - (K_{air/PDA} - 1)V_{PDA}}$$

Eq.5-5 is derived by rearranging Eq. 5-4.

$$\frac{C_{PDA}}{C_{PDA,i}} = \frac{V_{PDA}}{K_{air/PDA}V_T + (1 - K_{air/PDA})V_{PDA}} \quad \textbf{Eq. 5-5}$$

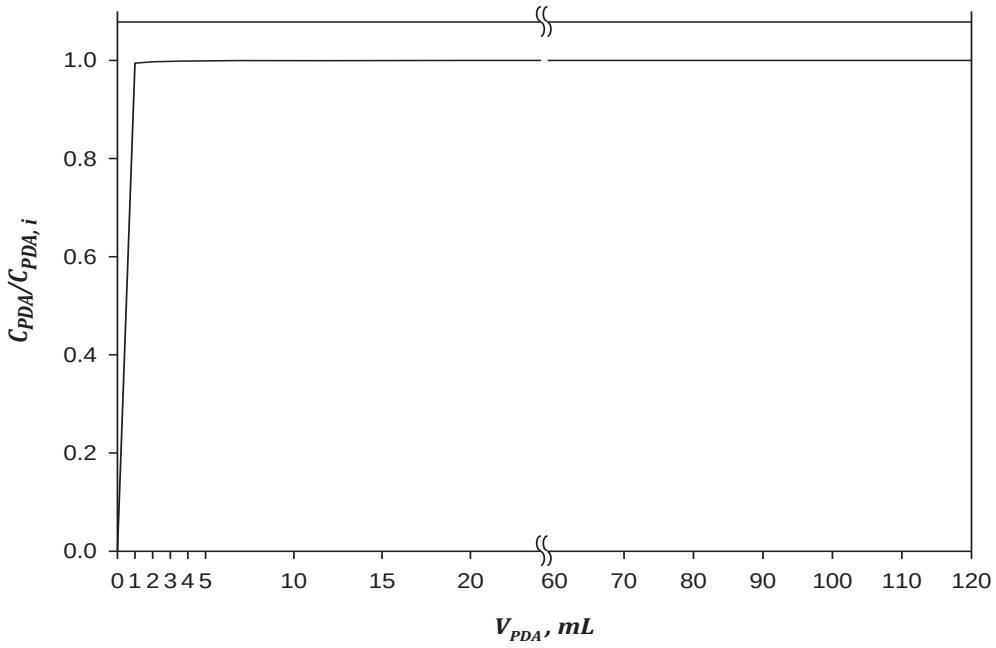


Figure 5-2: Profile fraction carvacrol concentration in PDA drop due to desorption, $C_{PDA}/C_{PDA,i}$ against PDA volume (mL)

From this analysis it can be seen that addition of 20 mL of PDA in the glass bottles can provide enough volatile carvacrol to achieve equilibrium (as shown Figure 5-2) with the air phase without a significant change in the PDA volatile concentration, and hence the rate to reach equilibrium is not likely to be diffusion limited. Twenty mL was chosen because it is practical, commonly used in the standard Petri dish method and provides sufficient nutrients for the

bacteria inoculum. Even though 1 mL (see Figure 5-2) is enough to give the required saturated equilibrium in the headspace, it is more practical to use 20 mL.

By leaving 100 mL of air space in the bottles, it was hoped that there would be enough oxygen to support the growth if inhibition by the active agent did not occur. In this way the absence of growth after incubation could be attributed to the volatile rather than due to depletion of the oxygen supply. This effect was investigated experimentally in the next section.

5.2.2 Testing for Adequate Oxygen Availability

An experiment was carried out to determine if oxygen becomes limiting for the growth of *Botrytis cinerea* inoculated on the surface of 20 mL of potato dextrose agar in 120 mL Wheaton glass bottles selected for the test method. This was to ensure there was sufficient headspace oxygen to ensure rapid growth of *Botrytis cinerea.*

Wheaton bottles (120 mL) and their lids (aluminium cap and rubber butyl Teflon lined stopper purchased from Millville, N. Jersey) were exposed to UV light (German Sciences, Biohazard-Protection, UV Light Intensity, ASTM. No 1807.23) overnight to sterilise them. Potato agar dextrose media (Difco™, Becton Dickinson & Company, USA) was prepared according to the manufacturer's instructions, and 20 mL was aseptically poured into the Wheaton bottles and left to cool down to room temperature under UV exposure for 15 minutes to avoid condensation of water vapour in the bottle. After cooling, the Wheaton bottles were capped with aluminium caps and crimp sealed using an EZ Wheaton crimper (Millville, New Jersey).

Three batches of experiments were carried out in duplicate as shown below:

- Wheaton bottles filled with 20 mL PDA without inoculum,
- Wheaton bottles filled with 20 mL PDA with inoculum,
- Wheaton bottles filled with 20 mL PDA with 100 ppm carvacrol and no inoculum

For the PDA samples with the inoculum, just prior to crimp sealing, the solidified PDA was inoculated with 100µL of bacterial suspension containing 10^5 CFU/mL of the microorganism *Botrytis cinerea*.

5.2.3 Oxygen Measurement

The concentration of oxygen (volume, %) in the vial headspace was measured using a fibre optic oxygen probe XF (Foxy, Ocean Optics, Florida, USA). Prior to the oxygen measurement, a tiny hole was made with a syringe needle (0.5mm) in the rubber Teflon septum attached to the Wheaton bottle sealed cap. The oxygen probe was inserted through the septum hole into the Wheaton bottle headspace. This system consisted of five components as shown in Figure 5-3.

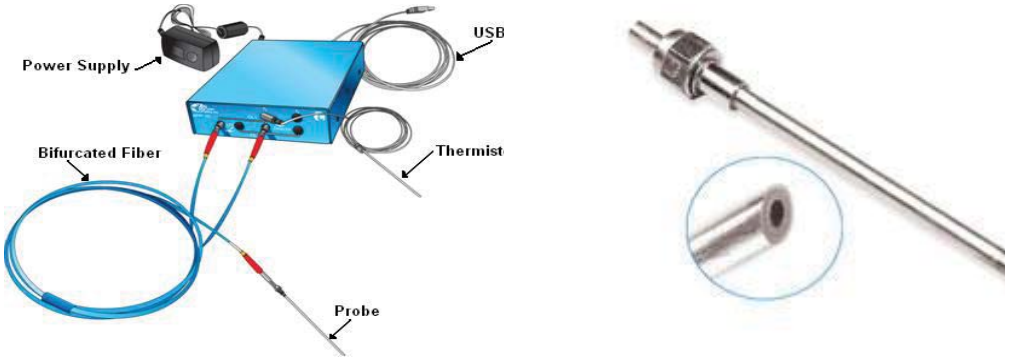
a) Foxy probe

b) Light source

c) Analogue to digital converter

d) Spectrometer

e) Software



(a) (b)

Figure 5-3: (a) Ocean optics oxygen measurement system and (b) Foxy oxygen probe for the analysis

(Sources: http://www.tautheta.com/home.html, http://www.oceanoptics.com/Products/sensorprobes.asp)

Standard Foxy probe tips are covered with a layer of hydrophobic sol-gel material with a ruthenium compound trapped in the sol-gel matrix. The Ruthenium complex coated with dye at the end of the probe is excited by the blue light source, at 475 nm, to one leg of a bifurcated optical fibre to the sensor tip causing photoluminescence. The fluorescence intensity or phase shift is measured by a Tau Theta Instrument Model MFPF -100 spectrometer, attached to the software, and is related to the partial pressure of oxygen. The kinetics of this process follow the Stern–Volmer relationship.

The prepared samples were incubated at 25 ± 1 °C in the incubator for 5 days. Prior to the measurement, the calibration of the probe was carried out with a two-point calibration by exposing the probe in streaming pure nitrogen (0.00% oxygen) and dry air (20.95% oxygen) for two minutes each. Data was taken every 5 seconds. All the experiments were carried out in duplicate, and the results are shown in (Figure 5-4).

The oxygen concentration is found from the measured fluorescence half-life by using Stern-Volmer Equation as defined below:

$$\frac{\tau_0}{\tau} - 1 = kCv \qquad \textbf{Eq. 5-6}$$

Where

τ	Lifetime in the presence of oxygen (µsec)
τ_0	Lifetime in the absence of oxygen
k	constant
Cv	Volumetric Concentration of oxygen (%)

$$\frac{\frac{\tau_0}{\tau} - 1}{k} = Cv \qquad \textbf{Eq. 5-7}$$

By rearranging Eq. 5-7 gives:

$$\frac{\tau_0}{\tau} = kCv + 1$$

Eq. 5-8

From the graph of Lifetime (τ_o/τ) versus volumetric concentration, Cv, the linear graph was ploted and the value of slope (k), with intercept at 1.00 was obtained as demonstrated in Figure 5-4.

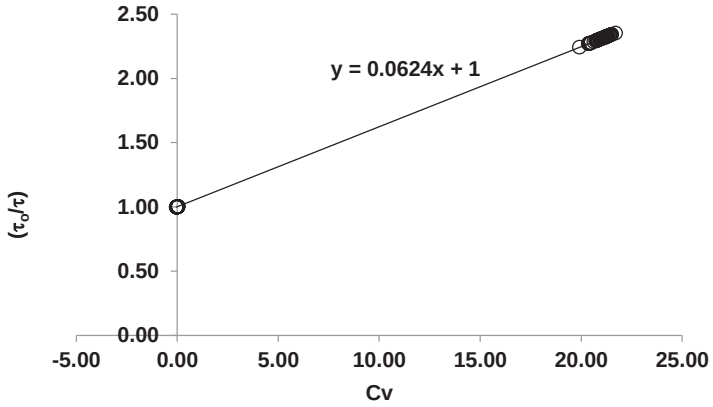


Figure 5-4: Plot of Lifetime against volumetric concentration

Table 5-1 shows the example summary of the results of the oxygen calibration.

Table 5-1: Example summary of the results of the oxygen sensor calibration

Cv (%)	0.0	21.0
τ	3.6950	1.5992
(τ_o/τ)	1.0000	2.3105
k	0.0624	(slope)

Figure 5-5 was plotted using the data that was summarized in Table 5-1 for the calibration experiment.

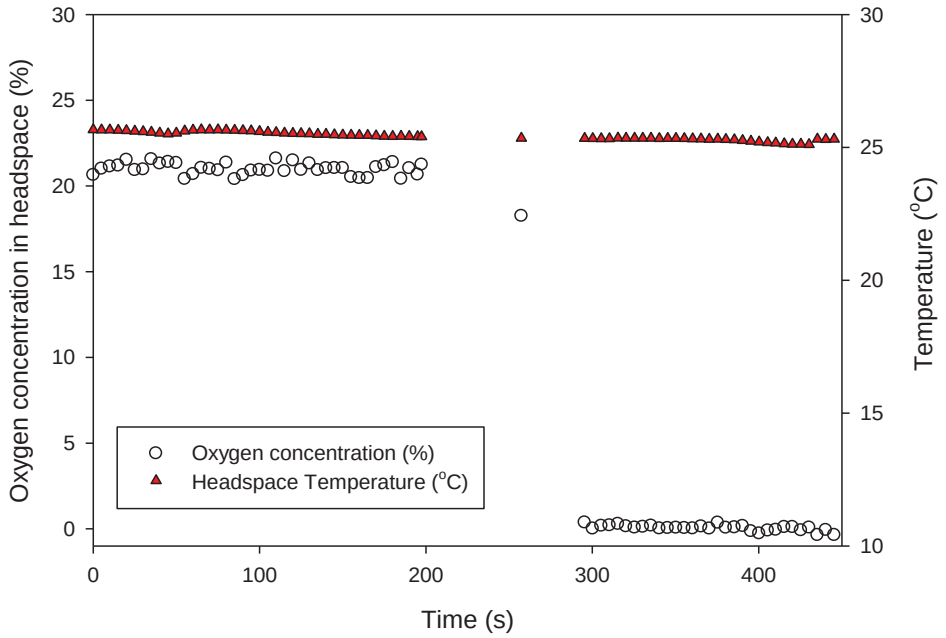


Figure 5-5: **Plot of the two point calibration oxygen versus time in 120 mL Wheaton glass bottle headspace. The profiles show the oxygen concentration in the bottle headspace was up to 21.0% when dry air was introduced and plunged to 0.0% when dry air was replaced by pure nitrogen at time 300 seconds onwards**

5.2.4 Results and Discussion

The results show that the O_2 concentration (Figure 5-6) in the bottle decreased slightly over 1.5 days. There was a gradual reduction in headspace because some of the O_2 was absorbed by the PDA.

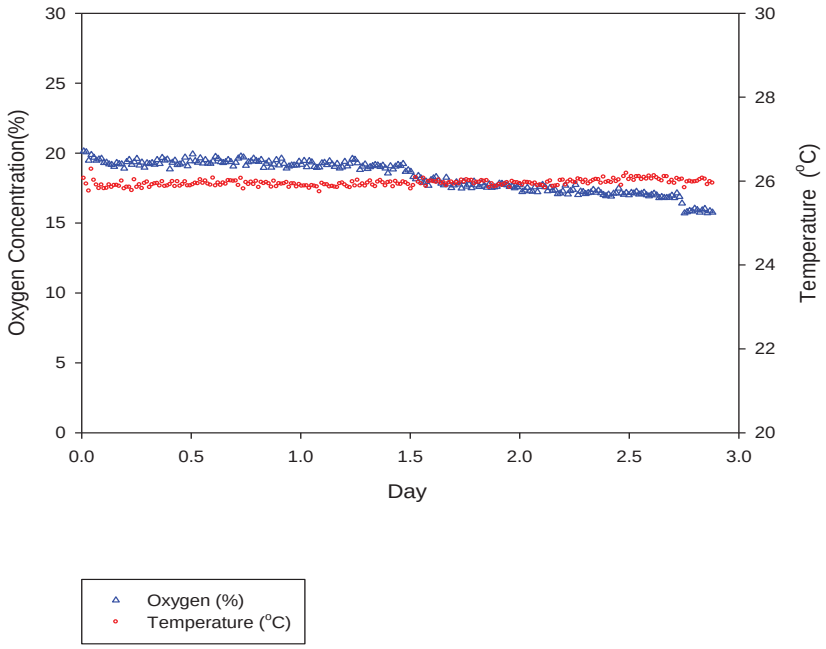


Figure 5-6: **Profiles oxygen concentration (%), temperature (°C) and Lifetime in the glass control Wheaton bottle (100mL headspace in potato dextrose agar incubated at 25 ± 1 °C)**

At atmospheric pressure, the oxygen solubility at 25°C is 8.2 mg/L (Colt, 1984). As the PDA contains more than 95 % of water, it was that estimated 5.13 mmol oxygen would be dissolved in 20 mL PDA. By calculation from the ideal gas laws, the oxygen concentration in 100 mL of headspace air at 25 °C was estimated 0.859 mmol.

Figure 5-7 shows the oxygen consumption of *Botrytis cinerea* inoculated samples in the vial.

The profiles demonstrated that (Kumar, 2012):

Part A: The oxygen concentration remained constant for 0.5 days. This coincides with the lag phase where the fungi are maturing and not yet able to grow.

Part B: The concentration of oxygen dramatically decreased from 21% after 0.5 days to about 10% at day one. This coincides with the exponential phase where the growth is strong, and the consumption of O_2 was high

Part C: The visible growth of *Botrytis cinerea* was observed at this stage. Oxygen concentration was steady at approximately 10% from day 1 to day 2. This coincides with the stationary phase.

Part D: The oxygen concentration gradually declined and was completely depleted in the headspace after 4 days. This coincides with when fungus started to die

Part E: O_2 was totally used.

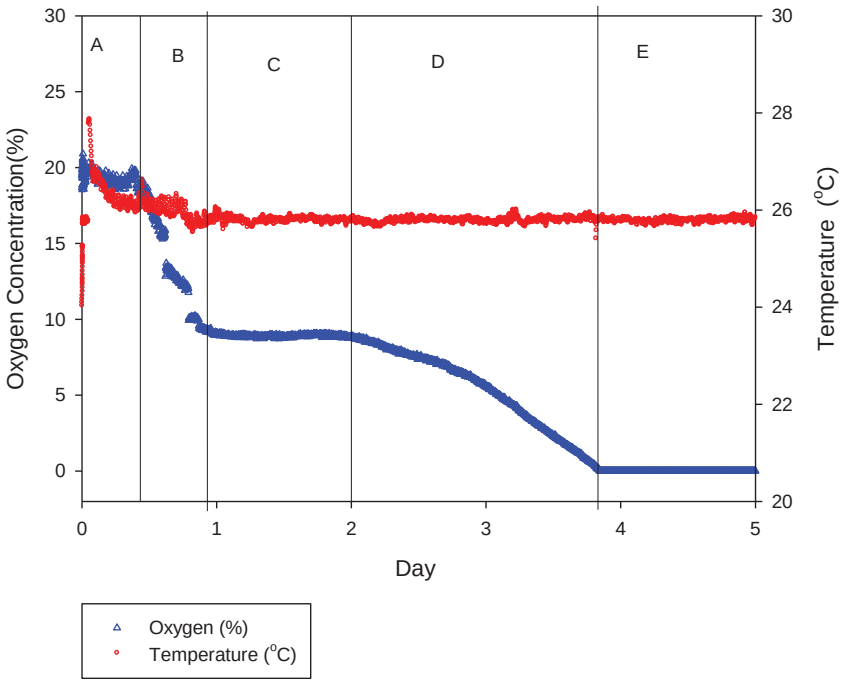
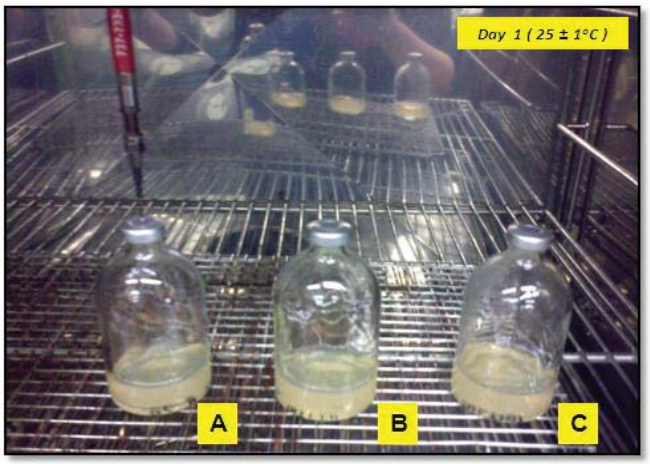
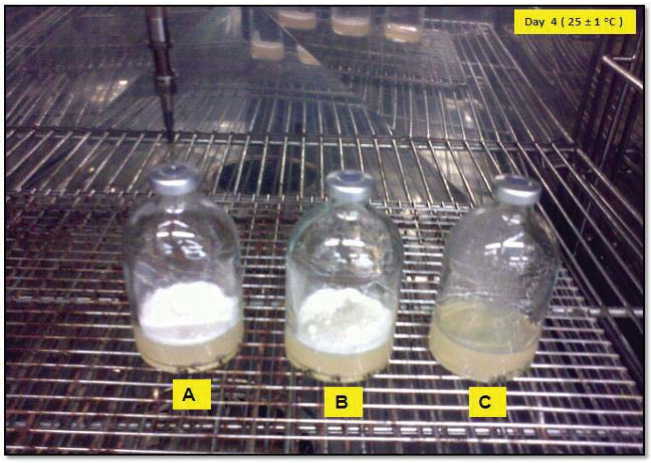


Figure 5-7: **The profiles of the concentration of oxygen (%), temperature (°C) in the Wheaton bottle (100mL headspace) inoculated with *Botrytis cinerea* in potato dextrose agar incubated at 25 ± 1 °C.**



(a)



(b)

Figure 5-8: **Observed growth in Wheaton bottle A and B (PDA inoculated with *Botrytis cinerea*, C (PDA-control) and incubated at 25 ± 1 °C. (a) The photograph was taken after one-day incubation (b) the photograph taken after 4 days incubation.**

There was no visible growth after one-day storage (Figure 5-8 (a)). Figure 5-8 (b) demonstrated that growth of *Botrytis cinerea* did occur in the Wheaton bottles. It was found that the *Botrytis cinerea* at a concentration of 10^6 cfu/mL started to grow after 48 hours

(picture not shown) and after four days, the *Botrytis cinerea* had already covered the surface of the PDA in the bottles.

5.2.5 Summary Findings

From this result, it was concluded that the Wheaton bottle with 100 mL air has adequate oxygen to ensure the rapid growth of *Botrytis cinerea*.

5.3 Determination of Carvacrol Minimum Inhibitory Concentration

In this section, the aim was to determine the minimum inhibitory concentration of carvacrol as an antimicrobial agent against *Botrytis cinerea* with the Wheaton glass bottles based method developed above. The use of sealed bottles eliminates the loss of active compound as discussed in Chapter 4 and maintains a constant gas phase concentration, making the identification and interpretation of MIC much easier.

5.3.1 Material and Methods

Isolated *Botrytis cinerea* was obtained from the School of Engineering and Technology (SEAT), Massey University Micro lab and cultured in potato dextrose agar medium (PDA) (Difco™, Becton Dickinson & Company, USA) and incubated for five days at 25 °C. Spores of *Botrytis cinerea* culture were first harvested by flooding the surface of the plates with about 10 mL of sterile saline solution (NaCl, 8.5 g/L) with 0.1% (v/v) Tween 80. The concentration of the *Botrytis cinerea* suspensions was 10^6 spores/mL as determined using a haemocytometer device under an optical microscope at 400X magnification.

5.3.1.1 Antimicrobial Assay

The experimental system was composed of hermetically closed Wheaton glass bottles (120 mL) and standard polystyrene (Ps) Petri dish (90 mm in diameter). The standard reverse Petri dish plate method was done in parallel to enable comparison between the two methods. The Wheaton bottles with the lids were exposed to UV lights overnight to sterilize them. Sterilized PDA agar with various concentrations of carvacrol (Table 5-2) was poured into Wheaton glass bottles, and Petri dishes. Petri dishes were then sealed by using Para film tape while the Wheaton aluminium bottle lids were crimped on. The tested plates and bottles were incubated at 25 ± 1 °C for 5 - 7 days.

The duration of growth inhibition in terms of days (i.e., lag phase) was defined as the time required to reach observable mycelium minus the time required for the control (without antimicrobial compound to form visible mycelium). Generally, 40 to 48 h were necessary to see the growth of the control culture. All experiments were repeated twice with three triplicate dishes or bottles for a maximum duration of 5 to 7 days. The presence or absence growth was evaluated by naked eye.

5.3.1.2 Experimental Design

25 combinations of various ratios were prepared. The experiments were done in multiple stages. Table 5-2 shows the concentrations of carvacrol considered.

Table 5-2: Concentration of carvacrol ranges studied in MIC determination

No of test	Concentration of carvacrol in PDA (ppm),
First test	0, 150, 300, 600,1200
Second Test	0, 25, 50, 100, 150
Third Test	0, 15, 20, 25, 30
Fourth Test	0, 35, 50, 60

The concentrations were prepared at 0-1200 µg/ mL with ethyl acetate (Edris and Farrag, 2003 and Sukatta *et al.*, 2008). For every compound, MIC was defined as the smallest concentration ensuring a total inhibition of the mycelium growth stored at 25 °C for at least 1 day lag time compared to the control. The MIC was expressed as mol of antimicrobial compounds introduced into the PDA (Davidson and Parish, 1989). Figure 5-9 details a flow diagram for the preparation of the different dilutions of the volatile compounds.

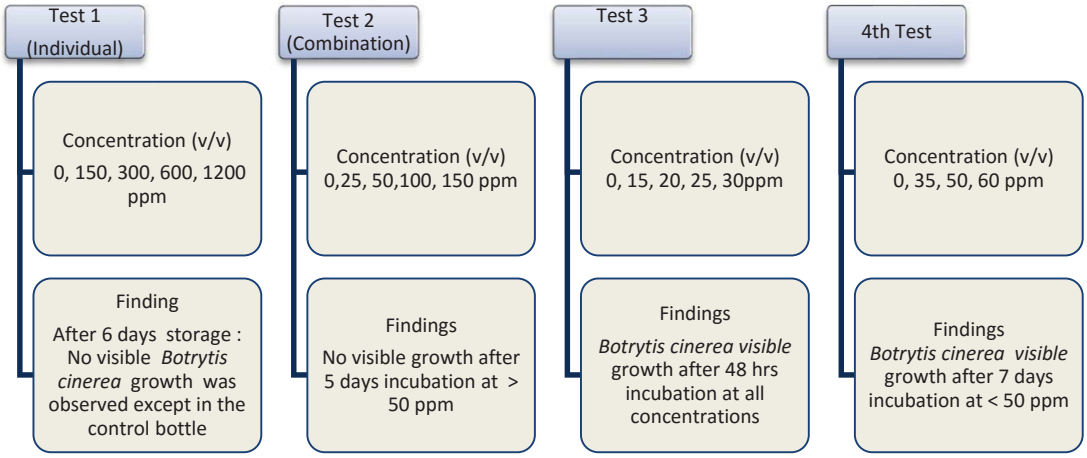


Figure 5-9: Summary diagram of all tests for determination of the minimum inhibitory concentration of the antimicrobial agent, carvacrol against *Botrytis cinerea.*

5.3.2 Results and Discussion

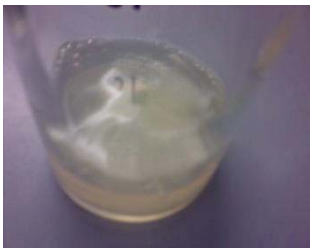
The data for the whole experiment is summarised in Tables 5.3 to 5.7. Five concentrations of carvacrol were prepared (0, 150, 300, 600, 1200 ppm) for test one. Each concentration was prepared in quadruplicate (a, b, c, d). All the bottles were inoculated with *Botrytis cinerea* except the last bottle (d) which served as control. At 0 ppm concentration, bottles 1(c) and 1(d) served as control. There was no microbial growth observed after 5 days incubation in samples 1(c)and 1(d) (without inoculums) indicating that the prepared PDA was sterilized properly and free from microbial contaminants.

Table 5-3: Results of screening of carvacrol MIC determination (Test 1)

	Carvacrol Concentration				Observation Days					
		µL/mL PDA (ppm)	mol crv/m^3 PDA	P/Psat	1	2	3	4	5	Remarks
1	a	0	0.00	0.0%	-	+	+	+	+	with inoculums
	b	0	0.00	0.0%	-	+	+	+	+	with inoculums
	c	0	0.00	0.0%	-	-	-	-	-	without inoculums (c)
	d	0	0.00	0.0%	-	-	-	-	-	without inoculums (c)
2	a	150	1.00	6.6%	-	-	-	-	-	with inoculums
	b	150	1.00	6.6%	-	-	-	-	-	with inoculums
	c	150	1.00	6.6%	-	-	-	-	-	with inoculums
	d	150	1.00	6.6%	-	-	-	-	-	without inoculums (c)
3	a	300	2.00	13.2%	-	-	-	-	-	with inoculums
	b	300	2.00	13.2%	-	-	-	-	-	with inoculums
	c	300	2.00	13.2%	-	-	-	-	-	with inoculums
	d	300	2.00	13.2%	-	-	-	-	-	without inoculums (c)
4	a	600	4.00	26.5%	-	-	-	-	-	with inoculums
	b	600	4.00	26.5%	-	-	-	-	-	with inoculums
	c	600	4.00	26.5%	-	-	-	-	-	with inoculums
	d	600	4.00	26.5%	-	-	-	-	-	without inoculums (c)
5	a	1200	8.00	52.9%	-	-	-	-	-	with inoculums
	b	1200	8.00	52.9%	-	-	-	-	-	with inoculums
	c	1200	8.00	52.9%	-	-	-	-	-	with inoculums
	d	1200	8.00	52.9%	-	-	-	-	-	without inoculums (c)

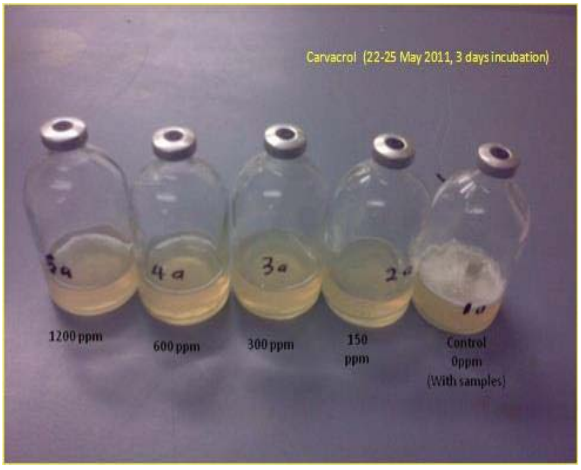
*PDA density =0.99mL/g, '-'no growth observed, '+'growth observed, (c) = control,

As shown in Figure 5-10 (d) there was no growth after 6 days incubation, except at 0 ppm concentration (bottles 1(a) and 1(b)) where the growth was observed after two days incubation.

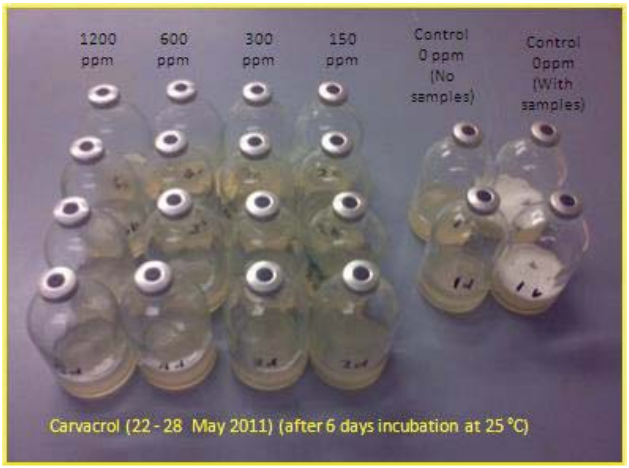


(a) 2 days (control)

(b) 3 days (control)



(c)



(d)

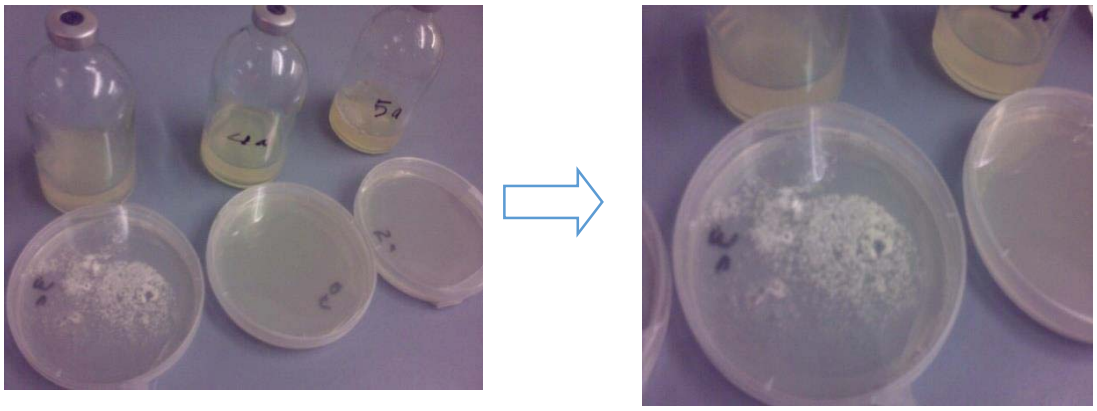
Figure 5-10: The photographic result indicates there was no sign of growth except for the control inoculated with the inoculums after 6 days storage at 25°C (Test 1)

The results of test 2 that focused on the 0 to 150 ppm range where inhibition was observed for 50, 100 and 150 ppm carvacrol are presented in Table 5-4.

Table 5-4: Results of screening of carvacrol MIC determination (Test 2).

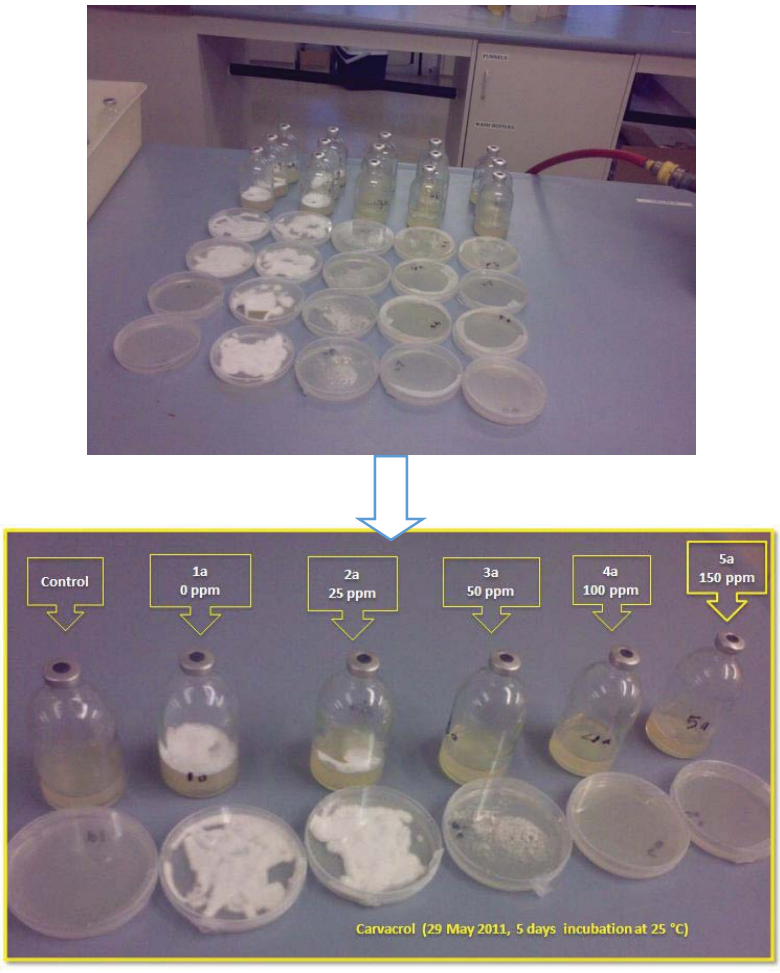
	Carvacrol Concentration				Observation					
					Days					
		µL/mL PDA (ppm)	mol crv/m^3 PDA	P/Psat	1	2	3	4	5	Remarks
1	a	0	0.00	0.0%	-	+	+	+	+	with inoculums
	b	0	0.00	0.0%	-	+	+	+	+	with inoculums
	c	0	0.00	0.0%	-	+	+	+	+	with inoculums
	d	0	0.00	0.0%	-	-	-	-	-	without inoculums (c)
2	a	25	0.16	1.1%	-	+	+	+	+	with inoculums
	b	25	0.16	1.1%	-	+	+	+	+	with inoculums
	c	25	0.16	1.1%	-	+	+	+	+	with inoculums
	d	25	0.16	1.1%	-	-	-	-	-	without inoculums (c)
3	a	50	0.33	2.2%	-	-	-	-	-	with inoculums
	b	50	0.33	2.2%	-	-	-	-	-	with inoculums
	c	50	0.33	2.2%	-	-	-	-	-	with inoculums
	d	50	0.33	2.2%	-	-	-	-	-	without inoculums (c)
4	a	100	0.66	4.4%	-	-	-	-	-	with inoculums
	b	100	0.66	4.4%	-	-	-	-	-	with inoculums
	c	100	0.66	4.4%	-	-	-	-	-	with inoculums
	d	100	0.66	4.4%	-	-	-	-	-	without inoculums (c)
5	a	150	1.00	6.6%	-	-	-	-	-	with inoculums
	b	150	1.00	6.6%	-	-	-	-	-	with inoculums
	c	150	1.00	6.6%	-	-	-	-	-	with inoculums
	d	150	1.00	6.6%	-	-	-	-	-	without inoculums (c)

*PDA density =0.99mL/g, 'X 'no growth observed, '√'growth observed



(a) i **(a) ii**

(a) At 50 ppm concentration, no sign of the growth of *Botrytis cinerea* was observed in 120 mL Wheaton glass bottle compared to Polystyrene dish where the growth was observed after 5 days incubation period.



(b)

Figure 5-11: The photographic results of Test 2 at concentrations 0 – 150 ppm

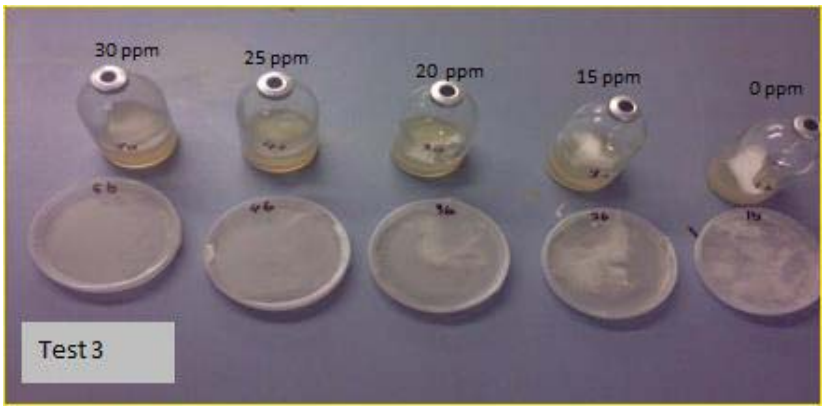
From Table 5-4 and Figure 5-11, it can be seen that after 5 days incubation, visible growth was observed only in the bottles with carvacrol concentrations 0 ppm and 25 ppm where the growth was detected after two days incubation. The growth of *Botrytis cinerea* at 25 ppm was not as much as at 0 ppm after 5 days incubation. This showed there was an antibacterial effect at 25 ppm carvacrol, but not strong enough to inhibit the growth of *Botrytis cinerea*. Figure 5-11 (aii) shows the growth of *Botrytis cinerea* inoculated in Petri dishes at 50 ppm was observed after 5 days incubation but not in 50 ppm bottles. This is likely to be due to the carvacrol in the PDA being lost into the atmosphere during the incubation period and thus reducing the carvacrol concentration in PDA and adjacent headspace.

Table 5-5 shows the results of test 3 that focused on the 0 to 30 ppm range where inhibition was just observed at 30 ppm carvacrol concentration. The results of Test 2 clearly showed that at 25 ppm the concentration of carvacrol was inadequate to inhibit the growth of *B. cinerea.* The conditions at < 25ppm were performed in test 3 to ensure that the result was repeatable.

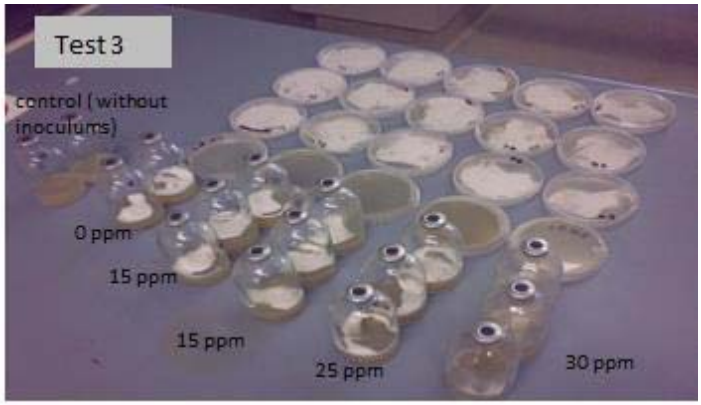
Table 5-5: Results of screening of carvacrol MIC determination (Test 3)

	Carvacrol Concentration				Observation					
					Days					
		µL/L (ppm)	mol crv/m^3 PDA	P/P_{sat}	1	2	3	4	5	Remarks
1	a	0	0.00	0.00%	-	+	+	+	+	with inoculums
	b	0	0.00	0.00%	-	+	+	+	+	with inoculums
	c	0	0.00	0.00%	-	+	+	+	+	with inoculums
	d	0	0.00	0.00%	-	-	-	-	-	without inoculums (c)
2	a	15	0.10	0.66%	-	+	+	+	+	with inoculums
	b	15	0.10	0.66%	-	+	+	+	+	with inoculums
	c	15	0.10	0.66%	-	+	+	+	+	with inoculums
	d	15	0.10	0.66%	-	-	-	-	-	without inoculums (c)
3	a	20	0.13	0.88%	-	+	+	+	+	with inoculums
	b	20	0.13	0.88%	-	+	+	+	+	with inoculums
	c	20	0.13	0.88%	-	+	+	+	+	with inoculums
	d	20	0.13	0.88%	-	-	-	-	-	without inoculums (c)
4	a	25	0.17	1.10%	-	+	+	+	+	with inoculums
	b	25	0.17	1.10%	-	+	+	+	+	with inoculums
	c	25	0.17	1.10%	-	+	+	+	+	with inoculums
	d	25	0.17	1.10%	-	-	-	-	-	without inoculums (c)
5	a	30	0.19	1.30%	-	-	+	+	+	with inoculums
	b	30	0.19	1.30%	-	-	+	+	+	with inoculums
	c	30	0.19	1.30%	-	-	+	+	+	with inoculums
	d	30	0.19	1.30%	-	-	-	-	-	without inoculums (c)

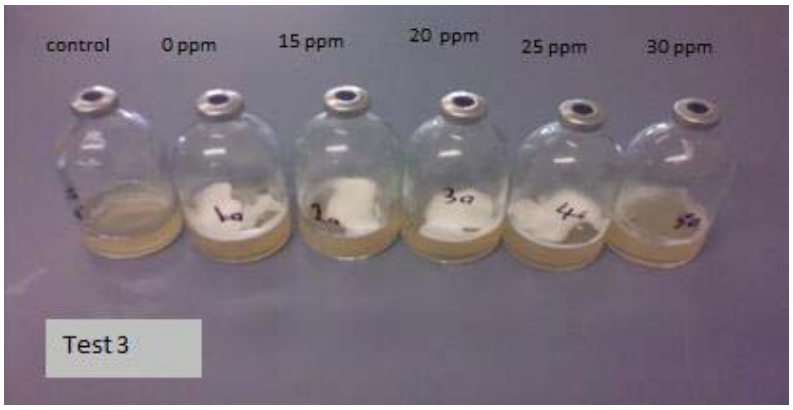
Figure 5-12 shows some of the photographic results of Test 3 at a concentration of 0 to 30 ppm.



(a)



(b)



(c)

Figure 5-12: The photographic results of Test 3 at concentrations of 0 – 30 ppm after 3 days incubation at 25 ± 1ºC

As shown in table Figure 5-12, the visible growth of *Botrytis cinerea* were observed at all concentrations after 3 days incubation. However, the growth of *Botrytis cinerea* were less extensive with increasing carvacrol concentration.

Table 5-6: Results of screening of *Botrytis cinerea* MIC determination (Test 4)

	Carvacrol Concentration				Observation							
					Days							
		µL/mL PDA (ppm)	mol crv/m^3 PDA	P/P_{sat}	1	2	3	4	5	6	7	Remarks
1	a	0	0.00	0.0%	-	+	+	+	+	+	+	with inoculums
	b	0	0.00	0.0%	-	+	+	+	+	+	+	with inoculums
	c	0	0.00	0.0%	-	+	+	+	+	+	+	without inoculums (c)
	d	0	0.00	0.0%	-	-	-	-	-	-	-	without inoculums (c)
2	a	35	0.23	1.5%	-	-	+	+	+	+	+	with inoculums
	b	35	0.23	1.5%	-	-	+	+	+	+	+	with inoculums
	c	35	0.23	1.5%	-	-	+	+	+	+	+	with inoculums
	d	35	0.23	1.5%	-	-	-	-	-	-	-	without inoculums (c)
3	a	50	0.32	2.2%	-	-	-	-	-	+	+	with inoculums
	b	50	0.32	2.2%	-	-	-	-	-	+	+	with inoculums
	c	50	0.32	2.2%	-	-	-	-	-	+	+	with inoculums
	d	50	0.32	2.2%	-	-	-	-	-	-	-	without inoculums (c)
4	a	60	0.41	2.7%	-	-	-	-	-	-	-	with inoculums
	b	60	0.41	2.7%	-	-	-	-	-	-	-	with inoculums
	c	60	0.41	2.7%	-	-	-	-	-	-	-	with inoculums
	d	60	0.41	2.7%	-	-	-	-	-	-	-	without inoculums (c)

(a)

(b)

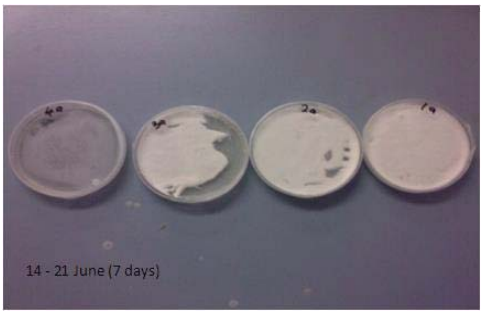
(c)

Figure 5-13: The photographic results of Test 4 in the concentration range 0 – 60 ppm.

Figure 5-14: **Photograph showing at 50 ppm concentration (bottle 3 a,b,c) no sign of growth of *Botrytis cinerea* was observed in 120mL Wheaton glass bottles compared to Petri dishes in which growth was observed after 4 days incubation period (Test 4)**

(a) i

(a)ii



b (i)

b (ii)

Figure 5-15: **Photographic showing at 60 ppm concentration (bottle 4a) no sign of growth of *Botrytis cinerea* was observed in 120mL Wheaton glass bottle compared to Polystyrenes dish where growth was observed after 7 days incubation period (Test 4)**

Table 5-7 shows the summary of the inhibitory growth that focused on the 0 to 1200 ppm range where inhibition was just observed at 35 ppm carvacrol concentration.

Table 5.7: Summary of the inhibitory of the growth of *Botrytis cinerea* at different concentrations of carvacrol incubated at 25 ± 1°C.

			Days incubation (25 ± 1°C)						
Carvacrol Concentration (ppm)	mol/m^3_{PDA}	P/P_{sat}	1	2	3	4	5	6	7
0.0	0.00	0.00 %	0/4	4/4	4/4	4/4	4/4	4/4	4/4
15.0	0.10	0.66%	0/4	4/4	4/4	4/4	4/4	4/4	4/4
20.0	0.13	0.88%	0/4	4/4	4/4	4/4	4/4	4/4	4/4
25.0	0.17	1.10%	0/4	4/4	4/4	4/4	4/4	4/4	4/4
30.0	0.19	1.30%	0/4	4/4	4/4	4/4	4/4	4/4	4/4
35.0	0.23	1.54%	0/4	0/4	4/4	4/4	4/4	4/4	4/4
50.0	0.32	2.20%	0/4	0/4	0/4	0/4	0/4	4/4	4/4
60.0	0.41	2.70%	0/4	0/4	0/4	0/4	0/4	0/4	0/4
100.0	0.65	4.32%	0/4	0/4	0/4	0/4	0/4	0/4	0/4
150.0	0.97	6.48%	0/4	0/4	0/4	0/4	0/4	0/4	0/4
300.0	1.99	12.95%	0/4	0/4	0/4	0/4	0/4	0/4	0/4
600.0	3.98	25.90%	0/4	0/4	0/4	0/4	0/4	0/4	0/4
1200.0	7.97	51.80%	0/4	0/4	0/4	0/4	0/4	0/4	0/4

4/4 visible growth observe of four replicates, 0/4 = no sign of growth of 4 replicates

5.3.3 Summary Findings

In comparison to the control, the introduction of carvacrol to the potato dextrose agar at 35ppm (P/P_{sat} = 1.54%) started to inhibit the growth of *Botrytis cinerea*. At 50 ppm (P/P_{sat} = 2.20%) concentration carvacrol, the growth of *Botrytis cinerea* was delayed for four days in comparison to the control. Similarly, the MIC for more than six days inhibition was 60 ppm (P/P_{sat} = 2. 7%). In Chapter 3 (Refer to Table 3.3), the MIC was 173mmol/m^3_{air}, corresponding to P/P_{sat} equal to 126%, which was not realistic. This observation backs up the investigation that the Petri dish method is inaccurate due to losses and absorption into the dish and media.

5.4 Determination of Thymol Minimum Inhibitory Concentration

In this section, a similar approach was taken to characterise the minimum inhibitory concentration of thymol as an antimicrobial agent against the growth of *Botrytis cinerea* in Wheaton glass bottles.

5.4.1 Materials and Methods

The procedures used were as described in Section 5.3 with the changes that thymol was used as the antimicrobial agent instead of carvacrol.

5.4.2 Experimental Design

For determination of minimum inhibitory concentration thymol against *Botrytis cinerea,* the experimental design is similar to the carvacrol study as described in section 5.3.2, with thymol as the antimicrobial agent in the concentration range from 0 – 150 ppm. The screening was done in multiple stages, and the results are presented as a whole.

5.4.3 Results and Discussion

The summary of the inhibition of the growth of *Botrytis cinerea* is shown in Table 5-8. In comparison to the control, the introduction of thymol to the potato dextrose agar at 25ppm (P/P_{sat} = 0.18%) started to inhibit the growth of *Botrytis cinerea*. At 50 ppm (P/P_{sat} = 0.37%) concentration thymol, the growth of *Botrytis cinerea* was delayed for five days in comparison to the control. Similarly, the MIC for more than six days was 60 ppm (P/P_{sat} = 0.46%). In comparison (refer to Tables 5-7 and 5-8), in order to retard the growth of *Botrytis cinerea* for 4 days, the thymol and carvacrol concentration needed are 37 ppm and 50 ppm, respectively.

Table 5-8: Summary of the inhibitory of the growth of *Botrytis cinerea* at different concentrations of thymol incubated at 25 ± 1°C.

			Days incubation (25 ± 1°C)						
Concentration (ppm)	P/P_{sat}	mol/m^3_{PDA}	1	2	3	4	5	6	7
0.0	0.00%	0.0000	0/4	4/4	4/4	4/4	4/4	4/4	4/4
15.0	0.11%	0.0830	0/4	4/4	4/4	4/4	4/4	4/4	4/4
20.0	0.15%	0.1162	0/4	4/4	4/4	4/4	4/4	4/4	4/4
25.0	0.18%	0.1660	0/4	0/4	4/4	4/4	4/4	4/4	4/4
30.0	0.22%	0.2157	0/4	0/4	0/4	0/4	4/4	4/4	4/4
35.0	0.27%	0.2489	0/4	0/4	0/4	0/4	0/4	4/4	4/4
50.0	0.37%	0.3319	0/4	0/4	0/4	0/4	0/4	0/4	4/4
60.0	0.46%	0.4149	0/4	0/4	0/4	0/4	0/4	0/4	0/4
100.0	0.73%	0.6638	0/4	0/4	0/4	0/4	0/4	0/4	0/4
150.0	1.10%	0.9957	0/4	0/4	0/4	0/4	0/4	0/4	0/4

4/4 visible growth observe of four replicates, 0/4 = no sign of growth of 4 replicates

5.4.4 Summary Findings

In comparison to the control, the introduction of thymol to the potato dextrose agar at 25 ppm started to inhibit the growth of *Botrytis cinerea*. At 50 ppm, thymol delayed the growth of *Botrytis cinerea* for five days compared to the control. The microbial activity of thymol is slightly stronger compared to carvacrol which is in agreement with results reported by several authors (Knobloch *et al.*, 1989; Lambert *et al.*, 2001; Liolios *et al.*, 2009).

5.5 Conclusion

It can be concluded that the new method of measuring the MICs of natural oils apparently solved the problems identified in Chapter 4 and provided reliable and repeatable results. Much lower inhibition concentration values were observed for the natural oils than observed in the Petri dish method and provide a basis for active packaging design or addition directly to food to provide protection against spoilage. The investigation into adapting the method to characterise synergistic effects will be the focus of Chapter 6.

CHAPTER 6

MINIMUM INHIBITORY CONCENTRATION IN MULTIPLE VOLATILE SYSTEMS

6.1 Introduction

Chapter 5 outlined the development and application of a new method of measuring the MIC in volatile systems. As discussed in Chapter 3, researchers have identified synergistic effects when multiple antimicrobial agents are used to control microorganism leading to the greater shelf and lower overall concentrations of each volatile if it were used on its own. This has the potential to reduce the impact of the volatiles on the sensory qualities of foods.

The Petri dish system was characterised in Chapter 4 showed that losses occur into the environment, absorption into the plastic dish and by slow absorption into the PDA. The high partition between the antimicrobial agents and other parts of the system favour PDA and the Polystyrene Petri dish. The system shows only small amounts of antimicrobial agent are available in the headspace and that it changes dynamically over time.

For multiple volatiles, the release from the filter paper (isotherm), the saturated vapour pressure, the partition coefficient, the diffusivity of the different volatiles will all be different. It is likely that different dynamics for each volatile compound will occur. These phenomena may explain some apparent synergies. The method developed in Chapter 4 can be used for multi-component systems to achieve a constant vapour phase for each volatile for the whole incubation period to investigate more accurately whether synergies occur. The aim of this study was to investigate the antifungal effect of binary combinations of carvacrol and thymol as antimicrobial aroma compounds. The efficacy of these combinations was compared to that of these compounds used alone (Chapter 5). The potential of each compound and the binary combinations of compounds as preservatives were evaluated based on MIC measurements.

6.2 Materials and Methods

Carvacrol (purity $\geq$ 98%), thymol (purity $\geq$ 99%), sodium chloride, and Tween 80 were purchased from SAFC and Sigma-Aldrich, New Zealand respectively. Isolated *Botrytis cinerea* from rotten local tomatoes as explained previously.

The experimental system was composed of hermetically closed Wheaton glass bottles (120 mL) as developed in Chapter 5. The Wheaton bottles with aluminium crimped lids were exposed to UV lights overnight to sterilize them. Sterilized PDA agar with various concentrations of carvacrol and thymol were poured into the Wheaton glass bottles and polystyrene Petri dishes. Petri dishes were used for comparison purposes to evaluate the differences growth in between this new method and the standard Petri dish method.

6.2.1 Inhibition of *Botrytis Cinerea* by Individual and Combined Compounds

The minimal inhibitory concentration (MIC) for carvacrol to inhibit fungal growth was determined by the method developed in Chapter 5, section 5.3.1. A number of individual concentrations and combinations at various ratios of carvacrol and thymol were prepared (Refer to Table 6-1, Table 6-2 and Table 6-3).

The concentrations were prepared at 0-62 µg/ mL with ethyl acetate used as a dilution solvent (Edris and Farrag, 2003; Sukatta *et al.*, 2008). For every compound, MIC was defined as the smallest concentration ensuring total inhibition of the mycelium growth when stored at 25 °C for at least 1 day lag time compared to the control. The MIC was expressed in mol of antimicrobial compounds per mL of PDA.

Carvacrol and thymol were added aseptically to 200 mL sterile molten agar (for each combination and alone), at the appropriate volumes to produce final concentrations in the range 0 – 60ppm. At this stage, Tween 80 was not added. The upper limit of the method for carvacrol and thymol was 20000 ppm (ca. 2% v/v), as above this level, Tween 80 did not adequately disperse the terpenoids throughout the growth medium. The solubility of carvacrol in water is 830mg/L as reported by Griffin *et al.* (1999).

The agar and antimicrobial agent mixtures for all combinations were shaken in a Multitron II Incubated Laboratory Shaker, USA (at 180 rpm, temperature 50°C for twenty minutes) and immediately poured into sterilised Wheaton bottles. The agar was then left to set for 30 minutes and then inoculated by pipetting 10 µL of *Botrytis cinerea* culture (1 x 10^6 CFU/mL) onto the bottles and plates and then incubated at 25° C in a constant room temperature for up to 14 days for

repeated individual sets. The observations for combined thymol and carvacrol were evaluated for up to 40 days.

For the degree of synergy analysis, individual inhibitory compounds and their binary combinations were scored out of a possible score of 100%. The method proposed by Davidson and Parish (1989) (as shown in Chapter 2, section 2.3.6.1) was used to transform MIC to Fractional inhibitory concentration (FIC) for carvacrol and thymol as shown below:

$FIC_{carvacrol} = (MIC_{carvacrol}$ in presence of thymol)/ $MIC_{carvacrol}$

$FIC_{thymol} = (MIC_{thymol}$ in presence of carvacrol)/ MIC_{thymol}

$FIC_{index} = FIC_{carvacrol} + FIC_{thymol}$

Interpretation: $FIC_{index} > 1$ antagonistic effect

$FIC_{index} = 1$ additive effect

$FIC_{index} < 1$ synergistic effect

For the purpose of determining the nature of the interaction between two inhibitory compounds, the following criteria were used. If the total score of the combination system was less than or equal to the highest score of either of the two individual compounds, the compounds were determined to have acted indifferently (absence of interaction). If the total score of the combination system was greater than the sum of the total scores of the individual compounds, the compounds were determined to have acted synergistically. If the total score of the combined system lay between these two breakpoints, the compounds were determined to have acted additively.

Two controls were included with each batch of the sample. The first was a negative control involving the presence of the test material but no organism, to check for contamination of the test material. The second was a positive control involving the presence of the microorganisms but the absence of

the antimicrobial. All MIC tests were carried out in three replicates. The MIC was determined as the minimum concentration where at least three of four results showed no growth.

6.2.2 Experimental Design

Two types of experimental design were carried out:-

- Repeated MIC determinations of individual components on their own at various concentrations ranging from 0 to 60 ppm as shown in Table 6-1.

Table 6-1: Concentration of carvacrol and thymol studied in MIC determination for the individual component on their own.

Carvacrol (ppm)	**Thymol** (ppm)
0	0
25	25
30	30
35	35
40	40
50	50
60	60

For combinations of carvacrol and thymol, two sets were prepared. The first set consisted of combinations of both compounds in the range of 40 – 50ppm totals as shown in Table 6-2. The second set consisted of combination of thymol and carvacrol in different ratios (25:75, 50:50, and 75:25) and different total concentrations (20, 30, 40, 50, 60 ppm) as shown in Table 6.2. These experiments were done in multiple stages.

Table 6-2: **Second set of the concentration of six combination ratios studied for MIC determination.**

Total (ppm)	Thymol (ppm)	Carvacrol (ppm)
40	20	20
50	10	40
50	20	30
50	25	25
50	30	20
50	40	10

Table 6-3: **Final set experimental design ratios of thymol and carvacrol in this work**

% (Thymol/Carvacrol)	Total (ppm)	Thymol (ppm)	Carvacrol (ppm)
50:50	20	10	10
	30	15	15
	40	20	20
	50	25	25
	60	30	30
25:75	20	15	5
	30	22	8
	40	30	10
	50	38	12
	60	45	15
75:25	20	5	15
	30	8	22
	40	10	30
	50	12	38
	60	15	45
100:0	20	20	0
	30	30	0
	40	40	0
	50	50	0
	60	60	0
0:100	20	0	20
	30	0	30
	40	0	40
	50	0	50
	60	0	60

Table 6-3 shows the final combinations of thymol and carvacrol with different ratios (25:75, 50:50, and 75:25) and different totals concentrations (20, 30, 40, 50, 60ppm).

Figure 6-1 details a flow diagram for the preparation of the different dilutions of the volatile compounds.

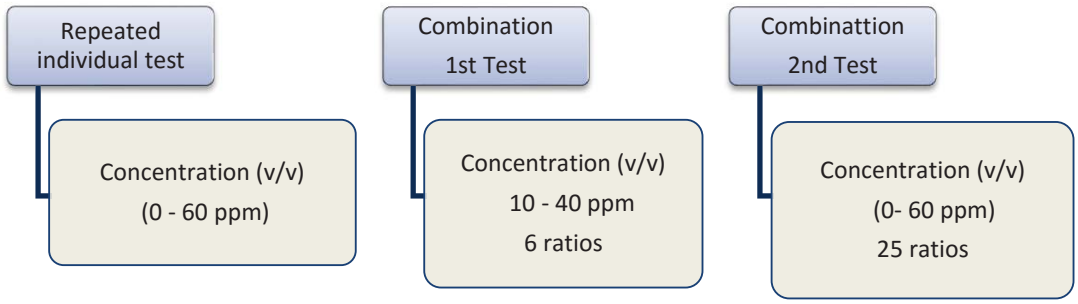


Figure 6-1: **Summary diagram of all tests for determination of minimum inhibitory concentration of the antimicrobial agent, individual and combined carvacrol and thymol against *Botrytis cinerea.***

6.2.3 Statistical Analysis

The significance of the synergistic effect between the two antimicrobial agents was determined by linear regression. Statistical analysis was performed using Minitab version 16 (2010, Minitab Inc., USA). In this study, data sets were considered to be significantly different at $P \leq 0.05$.

6.3 Results and Discussion

6.3.1 Determination of MIC Values of Individual Compounds

Table 6-4 summarises the results of the delayed growth of *Botrytis cinerea* at various concentrations of thymol and carvacrol alone and in combination. The delayed time was measured by subtracting the days required to observe visible growth in the treatment bottle from the number of days visible growth was observed in the control bottle. Table 6-5 shows the results of comparative effects repeated measurement of various antimicrobial concentrations of carvacrol and thymol *on Botrytis cinerea* stored at 25 °C in Potato Dextrose Agar medium. For both carvacrol and thymol, some inhibition of growth (1 day) was observed at 0.24mol/m^3 (37ppm), inhibition for 5 days at 0.32 mol/m^3 (50 ppm) and no growth was observed at concentrations above 0.41mol/m^3 (60 ppm), even after 14 days.

Interestingly, in comparison to Chapter 5, the concentration of carvacrol in the potato dextrose agar started to inhibit the growth of *Botrytis cinerea* at 35ppm (P/P_{sat} = 1.54%) whereas the first result was at 40 ppm (P/P_{sat} = 1 .73%). The results show that the carvacrol started to delay the growth of the *Botrytis cinerea in* the concentration range of 35 to 40 ppm. At 50 ppm (P/P_{sat} = 2.20%) carvacrol concentration, the results from both Chapter 5 and this study showed the growth of *Botrytis cinerea* was delayed for four days. Similarly at 60 ppm, both sets of results showed inhibition of more than 6 days. For this repeated result, the experiment was extended for two weeks whereas in Chapter 5 the evaluation was for one week.

Table 6-4: **Summary of the results of the delayed growth of *Botrytis cinerea* at various concentrations of thymol and carvacrol alone and in combination. The delayed time was measured by subtracting the days for first visible growth in the control bottle from the days visible growth in the treatment bottle.**

Carvacrol				Thymol			
Alone (μL/L) (ppm)	mol/ m^3 PDA	P/P_{sat} (%)	Lag Time (Days)	Alone (μL/mL) (ppm)	mol/ m^3 PDA	P/P_{sat} (%)	Lag Time (Days)
0	0.00	0.00	0	0	0.000	0.00	0
25	0.17	1.10	0	25	0.17	0.18	0
30	0.20	1.32	0	30	0.20	0.22	0
35	0.23	1.53	0	35	0.23	0.25	1
40	0.26	1.73	1	40	0.26	0.29	3
50	0.33	2.21	4	50	0.33	0.37	5
60	0.41	2.72	>12	60	0.42	0.46	>12

In the previous experiment (Chapter 5), the thymol started to inhibit the growth of *Botrytis cinerea* at 25 ppm (I day whereas in this repeated experiment it occurred at 35 ppm (lag 1 days). Similarly, the MIC of more than six days was previously observed at a concentration of 60 ppm *(P/P_{sat}*= 2.7%) for both carvacrol and thymol. From both experiments, it can be concluded that at 60 ppm, both carvacrol and thymol inhibited the *Botrytis cinerea* growth for more than 6 days. These results demonstrate the repeatability of the method at concentrations of more than 60 ppm.

Table 6-5: **Repeated antimicrobial measurement of various antimicrobial concentrations of carvacrol and thymol individually *on Botrytis cinerea* stored at 25 °C Potato Dextrose Agar medium**

	Concentration		Days														Delayed growth (days
	mol/ m^3_{PDA}	ppm (µL/L)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Carvacrol	0.00	0	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0
	0.17	25	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0
	0.20	30	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0
	0.23	35	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0
	0.26	40	---	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	1
	0.33	50	---	---	---	---	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	4
	0.41	60	---	---	---	---	---	---	---	---	---	---	---	---	---	---	>12
Thymol	0.00	0	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0
	0.17	25	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0
	0.20	30	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0
	0.23	35	---	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	1
	0.26	40	---	---	---	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	3
	0.33	50	---	---	---	---	---	--+	+++	+++	+++	+++	+++	+++	+++	+++	5
	0.41	60	---	---	---	---	---	---	---	---	---	---	---	---	---	---	>12

'--- No fungal growth, +++ fungal growth, represent three replicates. Concentration represents mol of antimicrobial compound per m^3 PDA

6.4 Synergy Measurement

Table 6-6 presents the results of the first dataset of 10 different combined concentrations of antimicrobials in the range 40–50 ppm total. From the results, it can be clearly seen that the individual compounds of carvacrol and thymol when at a concentration of 40 ppm delayed the growth of *Botrytis cinerea* for three days. In contrast the combination of 20 ppm carvacrol and 20 ppm thymol delayed growth longer (5 days) than the 40 ppm samples on their own. Samples seem to show more delay if thymol concentrations are higher. This agrees with the results in Chapter 5 where thymol seemed to be a slightly better antimicrobial than carvacrol.

For data set 1 (Table 6.6), the application of the FIC measurement is very difficult as interpretation depends very much on what the MIC is defined as (how many days). For example, for the first row in Table 6-8, there is a delay of 5 days. From the previous results, individually the MIC for 5 days delay was 50 ppm for thymol and 50 ppm for carvacrol. FIC then is 20/50+20/50=0.8. Table 6.8 suggests in synergistic effects in four of the six conditions, while additive effects in the other two. According to some studies, the FIC must be < 0.5 to be significant (Gallucci et al., 2009 and Bassole et al., 2010). While generally the longest delays were observed when the thymol was present at higher concentrations, this data set does not indicate strong evidence of synergistic effects.

The results for Data Set 2 are presented in Table 6.7. The FIC index and associated data is presented in Table 6.9. The longest delays in growth occurred when concentration was greater than 50% in the mix.

Table 6-6: **Dataset 1. Antimicrobial measurement of various combined concentrations of carvacrol and thymol on *Botrytis cinerea* stored at 25 °C on potato dextrose agar medium.**

Total	Thymol	Carvacrol	Day														
	ppm	ppm	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Delay
0	0	0	- - -	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0
40	0	40	- - -	- - -	- - -	- - -	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	3
40	20	20	- - -	- - -	- - -	- - -	- - -	- - -	++-	+++	+++	+++	+++	+++	+++	+++	5
40	40	0	- - -	- - -	- - -	- - -	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	3
50	0	50	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+++	+++	+++	+++	+++	+++	+++	6
50	10	40	- - -	- - -	- - -	+ - -	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	3
50	25	25	- - -	- - -	- - -	- - -	- - -	- - -	- -+	+++	+++	+++	+++	+++	+++	+++	5
50	30	20	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+ - -	+++	12
50	40	10	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+++	+++	11
50	20	30	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+++	+++	+++	10
50	50	0	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+++	+++	11

Table 6-7: **Dataset 2. Antimicrobial measurement of various combined concentrations of carvacrol and thymol on Botrytis cinerea stored at 25 °C potato dextrose agar medium.**

Total combination Concentration, (ppm, µl/L)	Thymol	Carvacrol	1	2	3	4	5	6	7	8	9	10	15	20	25	30	35	Lag Time
			Days															
	***50**	***50***																
20	10.0	10.0	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0.5
30	15.0	15.0	---	---	+--	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	1.5
40	20.0	20.0	---	---	---	---	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	4.0
50	25.0	25.0	---	---	---	---	---	---	---	---	---	+++	+++	+++	++	+++	+++	8.0
60	30.0	30.0	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	>35.0
	***75.0**	***25.0***																
20	15.0	5.0	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0.0
30	22.0	8.0	---	---	+--	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	1.5
40	30.0	10.0	---	---	---	+--	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	2.5
50	38.0	12.0	---	---	---	---	---	---	+-	+++	+++	+++	+++	+++	+++	+++	+++	4.5
60	45.0	15.0	---	---	---	---	---	---	---	---	---	---	---	---	---	+++	+++	27.0
	***25.0**	***75.0***																
20	5.0	15.0	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0.0
30	8.0	22.0	---	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	1.0
40	10.0	30.0	---	---	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	2.0
50	12.0	38.0	---	---	---	---	---	---	+--	+++	+++	+++	+++	+++	+++	+++	+++	4.5
60	15.0	45.0	---	---	---	---	---	---	---	---	---	---	---	---	+++	+++	+++	13.0

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Total combination	Thymol	Carvacrol	1	2	3	4	5	6	7	8	9	10	15	20	25	30	35	Lag time
Concentration, (ppm, v/v)			Days															
	100.0	***0.0***																
20	20.0	0.0	---	---	---	---	---	---	---	---	---	---	+++	+++	+++	+++	+++	0.0
30	30.0	0.0	---	---	+--	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	1.5
40	40.0	0.0	---	---	---	+--	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	2.5
50	50.0	0.0	---	---	---	---	---	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	5.0
60	60.0	0.0	---	---	---	---	---	---	---	---	---	---	---	---	---	+++	+++	29.0
	0.0	***100.0***																
20	0.0	20.0	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0.0
30	0.0	30.0	---	---	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	1.0
40	0.0	40.0	---	---	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	2.0
50	0.0	50.0	---	---	---	---	---	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	5.0
60	0.0	60.0	---	---	---	---	---	---	---	---	---	---	---	---	---	+++	+++	25.0
	[#]*Control*																	
0	[a]0	0	---	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0.0
0	[b]0	0	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	>35.0

[#]Control ([a]PDA with inoculums, [b]PDA without inoculums), +++ (visible fungi growth) and -- - (invisible fungi growth) represent triplicate samples respectively.

Table 6-8: **Fractional inhibitory concentration index of carvacrol and thymol against *Botrytis cinerea* at a total combination 40 and 50 ppm.**

Total (ppm)	Thymol (ppm)	Carvacrol (ppm)	Delay (days)	FIC index
40	20	20	5	0.80
50	10	40	3	1.00
50	20	30	10	0.81
50	25	25	5	1.00
50	30	20	12	0.81
50	40	10	11	0.81

Table 6-9: **Fractional inhibitory concentration index of carvacrol and thymol against *Botrytis cinerea* at various total combination 25 to 60ppm (Applied to dataset 2).**

Total (ppm)	Thymol (ppm)	Carvacrol (ppm)	Delay (ppm)	FIC index
25	5	20	1.0	0.68
25	20	5	1.0	0.68
30	8	22	1.0	0.81
30	10	20	1.0	0.81
30	15	15	1.5	0.75
30	22	8	1.5	0.75
32	20	12	1.0	0.86
35	5	30	3.0	0.89
35	30	5	2.0	0.88
40	10	30	2.0	1.00
40	20	20	4.0	0.80
40	30	10	2.5	0.95
50	12	38	4.5	1.00
50	25	25	8.0	1.00
50	38	12	4.5	1.00
60	15	45	13.0	1.03
60	30	30	35.0	1.00
60	45	15	27.0	1.00

An alternative approach is to define MIC and then determine the FIC. For a given MIC definition, the MIC for the individuals and for the different ratios can be selected. For example for 5 days delay, then the MIC_{Thymol} is 60 ppm and $MIC_{Carvacrol}$ is 60 ppm. For the 50:50 combination the MIC is 50 ppm, leading to the FIC = 25/60+25/60=0.83.

The problem with these approaches is that they depend on the way MIC is defined. In order to be very precise, the results that exactly define the MIC for the definition being used is needed. For example, for carvacrol on its own (Table 5-7), if the definition of MIC is to inhibit growth > 4 days, the results of observations at 5 days is used (MIC = 50ppm) because it was the minimum concentration that delayed growth by at least 4 days. A more precise calculation could be made if experiments at concentrations between 35 and 50ppm were carried out. This would affect the FIC calculation.

In view of this issue, an alternative data analysis approach was adopted. This was achieved by linearising the inhibition time observed for each sample and regressing this against the thymol and carvacrol concentrations. This resulted in a model with a significant thymol/carvacrol interaction term (Eq. 6-1).

$$ln(Delaydays) = (A \times Thymol) + (B \times Carvacrol) + (C \times Thymol \times Carvacrol) + D \quad \textbf{Eq. 6-1}$$

Where *A, B, C* and *D* are constants and Thymol and carvacrol concentrations are expressed in ppm.

If the third term (coefficient *C*) is significant, then it is needed to explain some of the variation in the dataset and as such an interaction or synergy is present.

Table 6-10: Summary of the regression analysis

Predictor	Coef.	SE Coef.	T	P
Constant (A, B,C and D)	-2.3993	0.2071	-11.58	0.000
Thymol	0.08555	0.0060	14.23	0.000
Carvacrol	0.07831	0.0060	13.03	0.000
*Thymol*Carvacrol*	0.00064	0.0003	2.16	0.036

S = 0.472814 R^2 = 0.886, R^2 (adj) = 0.878, *Significant difference at P $\leq$0.05.

Table 6-11: Analysis of variance for carvacrol and thymol

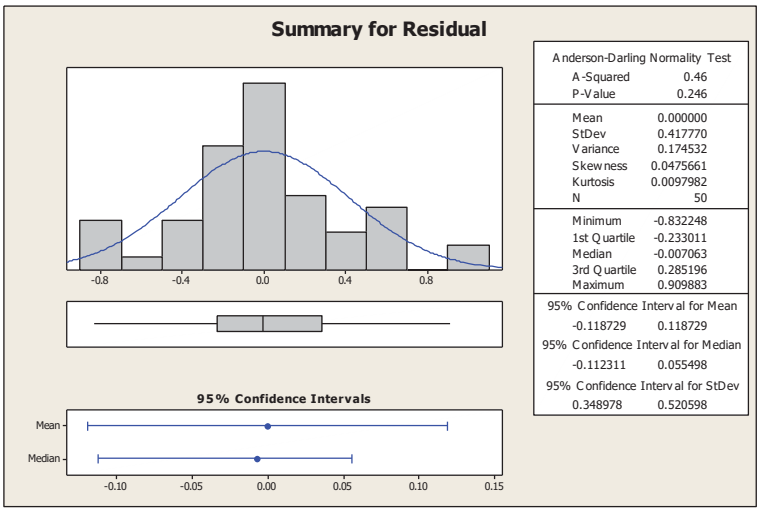
Source	DF	SS	MS	F	P
Regression	3	79.66	26.55	118.78	0.000
Residual Error	46	10.283	0.22		
Lack of Fit	21	8.131	0.39	4.50	0.000
Pure error	25	2.153	0.09		
Total	49	89.940			

*Significant difference at P $\leq$0.05.

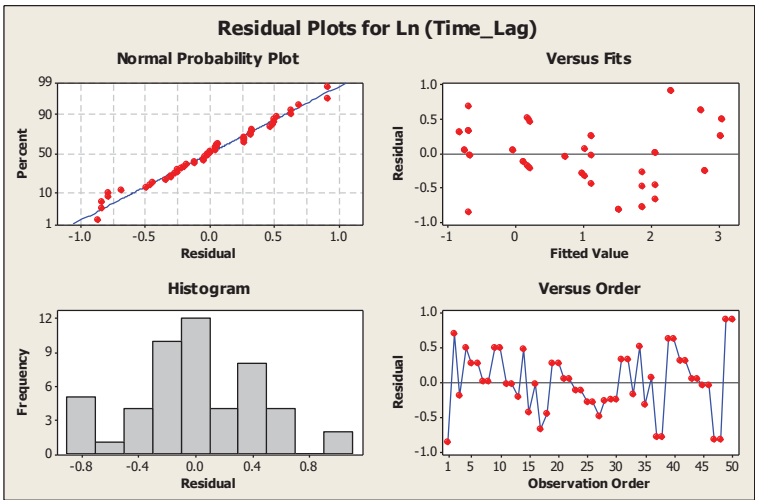
Table 6-12: ANOVA table

Source	DF	SeqSS
Thymol	1	19.882
Carvacrol	1	58.737
Thymol*Carvacrol	1	1.040

The experimental results are shown in Figure 6-2. The resulting regression output is shown in Tables 6-10 and 6-11. Table 6-12 summarizes the ANOVA results showing that P-value for the F test statistic is less than 0.05, providing strong evidence that the regression model is significant and can fit the data. The R^2 is 0.886 which indicates that the explanatory power of this regression is acceptable. Each of the 95 percent confidence intervals for the respective population slope coefficients includes zero. Referring to Table 6-10 and Table 6-11, the mean square error term (MS) is smaller with "Thymol*Carvacrol" included, indicating less deviation between the observed and fitted values. The P value of the combination of thymol and carvacrol (Table 6-10) is less than $P< 0.05$ showing that the combination is significant. This confirmed there was a synergistic effect between these two antimicrobial agents in combination. Examination of the residuals (Figure 6-2) indicates no unusual patterns. The inclusion of the "Thymol", "Carvacrol", and "Thymol*Carvacrol" variables explains 86.7% of the variability of the data.



(a)



(b)

Figure 6-2: **(a) P value more than 0.05 indicates the data is normally distributed. (b)Residuals Plot for Ln (Time Lag) against the variable.**

It should be noted that the regression equation also shows the coefficient for thymol is slightly greater (0.08555) than that for carvacrol (0.07831). This indicates that thymol is slightly more antimicrobial active then carvacrol as observed by the experimental data in this work.

Figure 6-3 shows the experimental results and prediction plot effect of thymol and carvacrol alone on *Botrytis cinerea* growth. This approach has the advantage that all data is used for identification of synergy. This model can be used to define MIC for a whole range of definitions (inhibition times). This provides a more flexible tool for comparing different experimental results (avoiding the problem of different MIC definitions) and a useful tool for active packaging design.

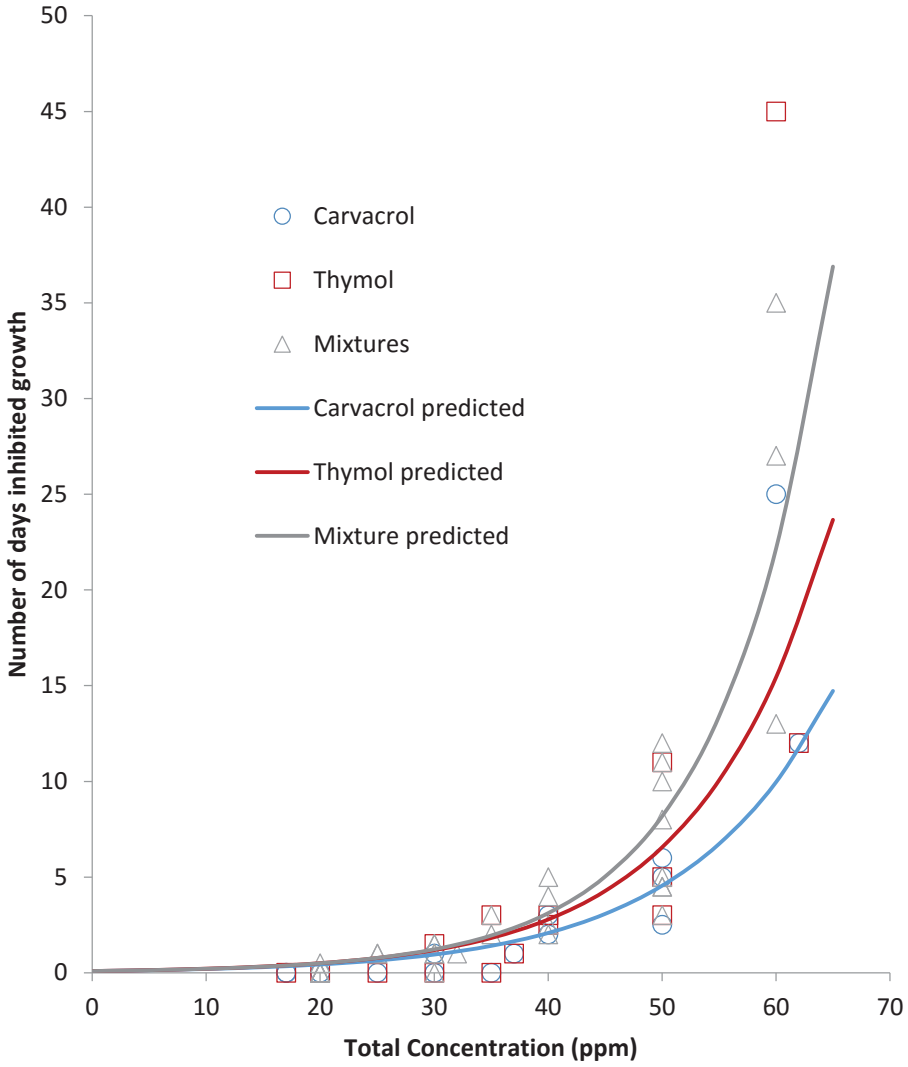


Figure 6-3: **Effect of thymol and carvacrol alone and in combination on *Botrytis cinerea* growth (days) on PDA media stored at 25 °C.**

From the regression model, it is possible to calculate an FIC. By rearranging the regression equation for carvacrol = 0, the MIC (in ppm) for pure carvacrol can be calculated:

$$MIC_{carvacrol} = \left(\frac{\ln(Delaydays) + 2.4}{0.0783}\right) \quad \textbf{Eq. 6-2}$$

Similarly

$$MIC_{thymol} = \left(\frac{\ln(Delaydays) + 2.4}{0.0856}\right) \quad \textbf{Eq. 6-3}$$

If *R* is defined = *Thymol/mixture*, then *(1-R) = carvacrol/mixture* and the regression equation can be rearranged to the form;

$$ln(Delaydays) + 2.4 - (0.0856R + 0.0783(1-R) \times MIC_{mixture}\left(0.000645R(1-R)\right) \times MIC_{mixture}{}^{2} = 0 \quad \textbf{Eq. 6-4}$$

The $MIC_{mixture}$ can be calculated using the quadratic formula.

From this:-

$$FIC = R \times \frac{MIC_{mixture}}{MIC_{thymol}} + (1-R) \times \frac{MIC_{mixture}}{MIC_{carvacrol}} \quad \textbf{Eq. 6-5}$$

This gives FIC values that range between 0.89 - 0.96. The FIC is lower at higher delay times. Because the FIC value is quite close to 1, these results suggest the synergistic effect is relatively small.

6.5 Conclusions

The measurement methodology can be successfully used to investigate synergistic effects for multiple volatile systems. It has the advantage that the volatile concentrations in the solid and gas phase are constant throughout the experiment.

FIC values were calculated for different combinations of volatiles but it was difficult to apply the FIC data analysis from the literature directly. These problems were due to not being able to exactly (without considerable additional experiments) state the MIC for the MIC definition such that the MIC values are known for both mixture and individual components directly. Instead, the FIC index was assessed for each volatile mixture by defining the MIC as inhibiting growth by the time observed. From the data for individual volatiles, it was possible to determine an FIC. These values were variable and ranged from 0.8 to 1, indicating a small degree of synergism.

An alternative data analysis approach was adopted by linearising the inhibition time observed (by taking its log) for each sample and regressing this against the thymol and carvacrol concentrations. This resulted in a simple model with a significant thymol/carvacrol interaction term (P=0.036, Table 6.10). This analysis more clearly demonstrated a synergistic effect, although the FIC values estimated from the model were between 0.89-0.96, suggesting the effect is minor.

As a result, the antimicrobial activity of thymol and carvacrol in the mixture will be largely additive. Sensory tests would be required to determine if the halving of the concentration of each volatile and using them together, would avoid changing taste perception less than using them individually.

The regression-based analysis method has the advantage that all data can be included in the analysis and it provides a tool that is not dependent on arbitrarily selecting a time frame on which to base the definition of MIC.

Although the antimicrobial activity measurement and analysis methods developed in this work offer many advantages over traditional methods, they have the disadvantage that they require the essential oil to be mixed directly with the agar broth before pouring. If solid foods are required to be tested as the food system, such mixing is not possible. As such, control of the concentration in the headspace and at the food surface must be carried out through the gas phase. Due to the partitioning of the volatiles favouring the solids phase, this will be difficult. The next chapter outlines the development of experimental methodologies to try to achieve this.

CHAPTER 7

VOLATILE GENERATION SYSTEM DEVELOPMENT

7.1 Introduction

Chapters 5 and 6 outlined how MIC measurements can be made where the volatile concentrations in the headspace above and in the food is maintained at constant levels. This method takes advantage of the partitioning of the volatile which strongly favours the food phase. This is possible because, in the system used in this work, the liquid oil could be mixed into the liquid agar before it set. In other food systems such as bread, cakes, fruit or vegetable tissue, this is not possible.

In the early work in the thesis (Chapters 3 and 4), it was seen that significant times were required for absorption of the active agents into the agar. Therefore, characterising antimicrobial activity in a solid food system will have to consider these dynamics. The design of controlled release active packaging systems also requires data for absorption rates into foods. Measurements of

MIC for food solids requires the ability to control headspace conditions dynamically to avoid fluctuating levels during the course of the experiments.

With these factors in mind, this chapter outlines the development and initial application of a novel vapour generation system that can be used to produce a gas stream with one or more volatiles present at well controlled and adjustable concentrations.

7.2 System Requirements

To design this system, knowledge is required of how minimum inhibitory concentrations change as a result of volatile composition of different foods, and the absorption and transport behaviour of volatiles in food and packaging components. Chapter 6 demonstrated that synergies between active volatiles (thymol and carvacrol) partially enhanced antimicrobial activity in PDA and lowered total inhibitory concentrations. This activity could be explored for solid foods in the packaging headspace over time. Nevertheless at this stage, the study was to focus on one volatile.

A key requirement for the research was to design a vapour delivery system. The system needed to deliver a volatile at various concentrations in the presence of high water partial pressures. High relative humidity is needed in order to ensure that the inhibition of growth of the spoilage organism (e.g. fungus) is not due to low water activity. Due to the low saturated vapour pressure of many antimicrobial volatile, care is required in the design in order to deliver vapour that can be close to saturation.

The overall scope of this research is broad and the principles and design methodologies developed could be applicable to a variety of volatiles, foods and packaging configurations. To provide a focus for experimental design, an example system was chosen. This system was:

- Volatile: Carvacrol
- Spoilage organism: *Botrytis cinerea*
- Food: Model agar based food

7.2.1 Volatile Properties

Before a reliable vapour generating system could be designed, data for how the vapour pressure changes with temperature were required. The Antoine equation can be used to describe the vapour pressure of volatiles as a function of temperature. The equation is given by:

$$\ln(P_i^S) = A - \frac{B}{(T + C)} \qquad \textbf{Eq. 7-1}$$

Where

(P_i^S) = Saturated vapour pressure (Pa) of volatile i,

T = absolute temperature (Kelvin (K)), and

$A, B\ and\ C$ = constants to be determined, where *C* and *B* must have units of Kelvin.

Linear regression analysis (as suggested by Diaz *et al.* 1999) was used to find these constants from experimental data compiled from Stull (1947) over the temperature range (70 – 237°C), after rearranging the equation into a linear form. This was done by multiplying the Antoine equation by $(T + C)/T$ and rearranging to obtain:

$$\ln P_i^s = A + \frac{(AC - B)}{T} - \frac{C \ln P_i^s}{T} \quad \textbf{Eq. 7-2}$$

In this linear form, $ln\,(P_i^s)$ was the dependent variable, $1/T$ and $\ln(P_i^s)\,/T$ were the independent variables, A is the intercept, $(AC - B)$ is the coefficient for $1/T$ and $-C$ is the coefficient for the $\ln(P_i^s)\ /T$ term.

The regression tool in Excel 2010 software was used to find A, B and C for carvacrol vapour pressure resulting in the coefficients listed in Table 7-1. Additionally, the Antoine equation obtained from Yaws (2007) data is shown after conversion into consistent units and mathematical form. This data is most reliable for the temperature range 150 to 459°C.

Table 7-1: Antoine constants of carvacrol

Antoine constant	Kobe (1941)	Yaws (2007)	*Stull (1947)
A	24.59	21.40	23.76
B	6670.60	3971.0	5830.31
C	-	-108.15	-34.06

* fitted data from Stull (1947).

Table 7-2: Summary of carvacrol vapour pressure reported at 25 ºC in literature and derived from the Antoine equation

Temperature (°C)	Vapour Pressure (Pa)	References
25	6.4	Ben Arfa *et al.,* (2006)
25	3.1	Du *et al.,* (2008)
25	3.9	*Sci. Finder
25	1.7	Yaws (2007)
25	5.3	Stull (1947)
25	3.5	Van Roon *et al.*,(2002)
25	9.2	Kobe (1941)

*SciFinder – vapour pressure calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris (© 1994-2010 ACD/Labs).

The reported vapour pressure of carvacrol found in the literature at low temperatures is quite varied, as summarised in Table 7-2. From Figure 7.1, it can be clearly seen that the fitted carvacrol vapour pressures profiles derived from Stull (1947) and Yaws (2007) gave the same trend at temperatures above 100°C; however, the vapour pressures were lower for the data from Stull (1947) at temperatures below 100°C. The graph profiles derived from Kobe (1941) were a little higher. From the results shown in Figure 7-1, it was likely that the Antoine equation derived from Stull (1947) can be utilised to construct the vapour generating system development. The vapour pressure for thymol has been reported to be similar to carvacrol. The experimentally obtained vapour pressure of carvacrol by Van Roon *et al.,* (2002) was ~ 3.5 Pa at 25 °C, which was quite close to the fitted model obtained from the data of Stull (1947).

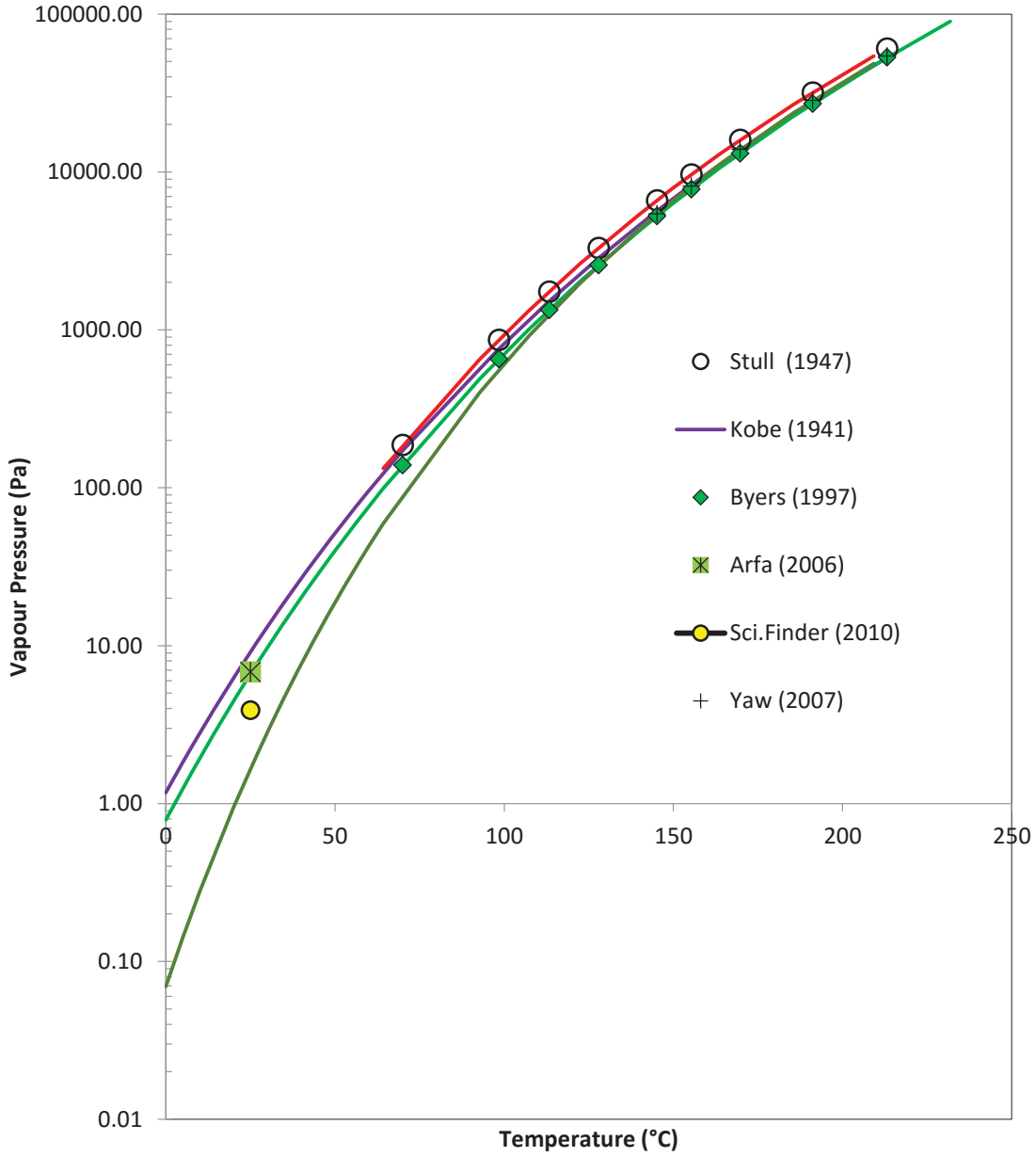


Figure 7-1: **Comparison of carvacrol vapour pressure profiles derived from different sources of data. The experimental data was obtained from Stull (1947) and Yaw (2007) and predicted results (solid line) were derived from the fitted Antoine equations.**

Nevertheless, it was critical to choose one equation that could estimate the vapour pressure at low temperatures. Antoine–Grain's method (Grain, 1990) was also used to estimate the saturated vapour pressure of carvacrol at temperatures less than 70°C. The general form of the Antoine's equation relates the saturated vapour pressure (P_i^S) of active compounds to the temperature (T) as shown in Equation 7-1.

For Antoine–Grain's method the constant $A, B \text{ and } C$ can be calculated from experimental measurements (Diaz *et al.*, 1999), but in this work, these values were estimated using Eq. (7-3) and Eq. (7-4).

$$A = \frac{B}{T_b + C} \quad \textbf{Eq. 7-3}$$

$$B = \frac{\Delta H_{vb}}{\Delta Z_b R T_b^2} \left[(T_b + C)^2 \right] \quad \textbf{Eq. 7-4}$$

Where ΔH_{vb} is the heat of vaporisation at the normal boiling point, ΔZ_b is the compressibility factor, R is the gas constant and T_b is the normal boiling point. Substituting Eqs. (7-3) and (7-4) into Eq. (7-1) yields:

$$ln P_i^S = \frac{\Delta H_{vb}(T_b + C)^2}{\Delta Z_b R T_b^2} \left[\frac{1}{T_b + C} - \frac{1}{T + C} \right] \quad \textbf{Eq. 7-5}$$

The compressibility factor, ΔZ_b, is assumed to have a value of 0.97 (Miller, 1964) $\text{and } \Delta H_{vb}$ is evaluated using a simple method introduced by Fishtine (1963):

$$\frac{\Delta H_{vb}}{T_b} = K_F(8.75 + RlnT_b) \quad \textbf{Eq. 7-6}$$

Where K_F is derived from a consideration of the dipole moments of polar and non-polar molecules (Grain, 1990).

The constant *C* is estimated using Thomson's rule (Thomson, 1959) such that;

$$C = -18 + 0.19T_b \quad \textbf{Eq. 7-7}$$

Table 7-3 summarises the Antoine-Grain constants.

Table 7-3: Antoine-Grain constants for carvacrol.

Antoine-Grain constant	A	B	C	ΔZ_b	[a]K_F	$\Delta \mathrm{H_{vb}}$
A	14.57	8571.65	78.83	0.97	1.15	12383.61

[a](Grain, 1990)

The value of P_i^S for carvacrol at 25°C derived from this equation is 3.46 Pa which is 33% lower than prediction of the extrapolated Antoine equation, (5.2 Pa) and agrees closely with the experimental data by Van Roon *et al.* (2002) (see Figure 7-2).

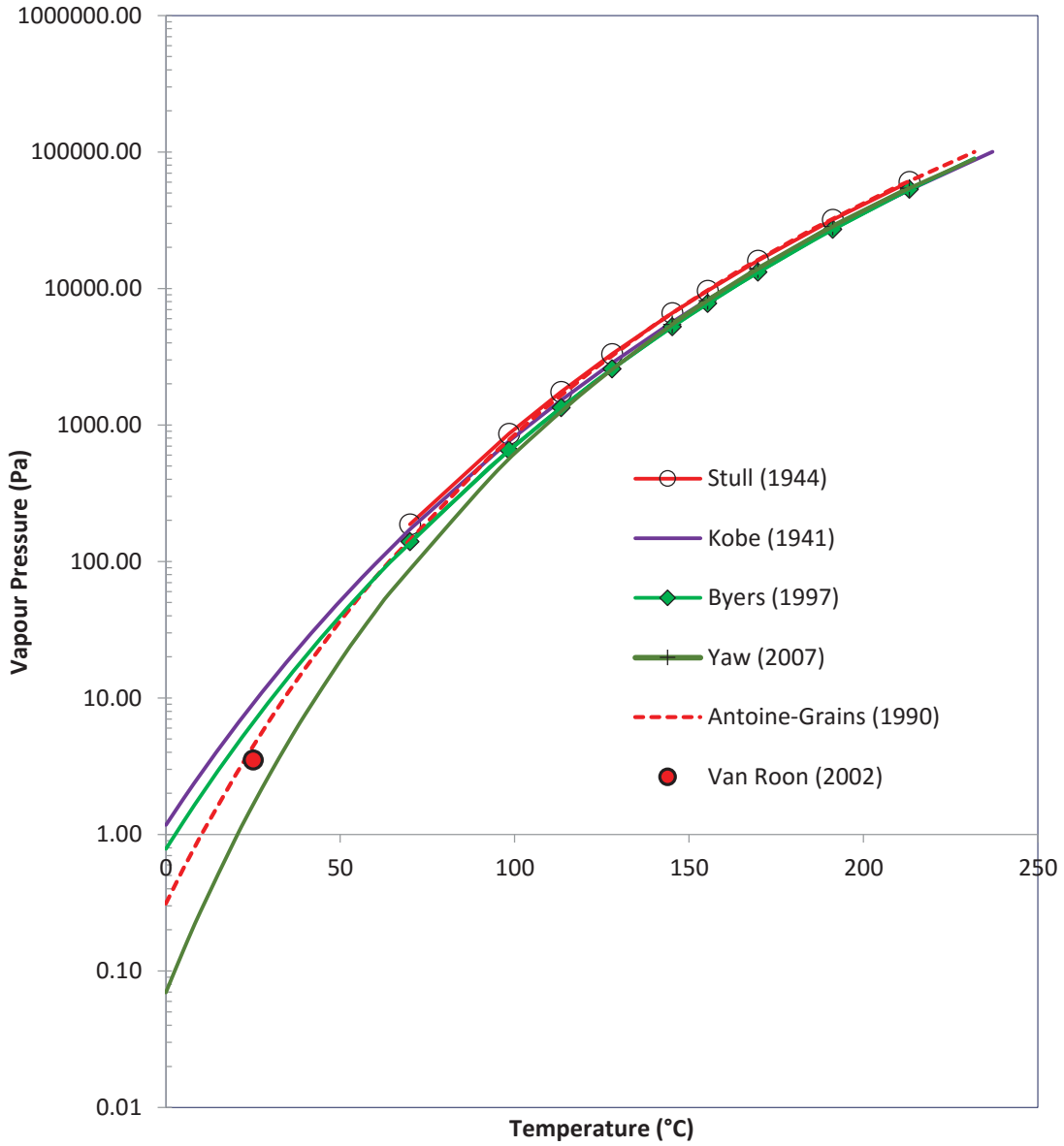


Figure 7-2: **Comparison of carvacrol vapour pressure profiles derived from different sources. The experimental data was obtained from Stull (1947), Van Roon (2002) and Yaw (2007) and predicted results (solid lines) were derived from the Antoine equation while the dotted line (red) was derived from Antoine - Grains equation.**

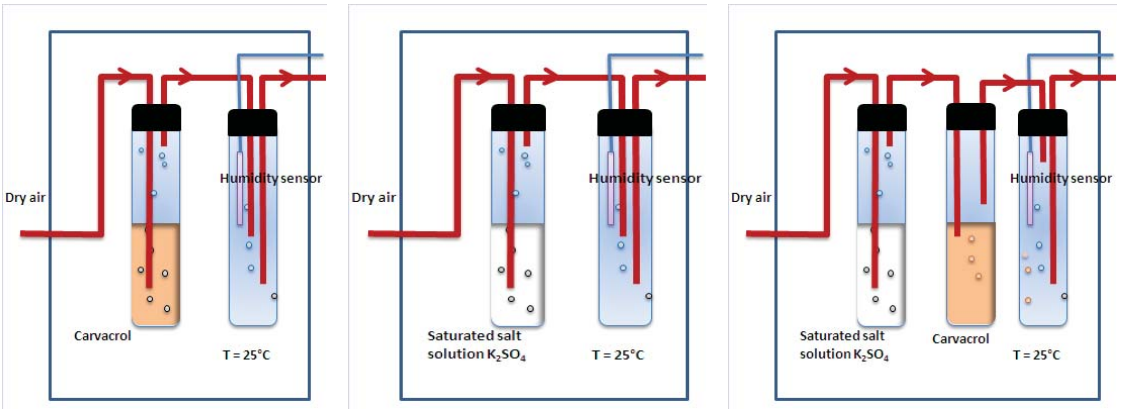
Based on this analysis the Antoine Grain prediction (using the parameters defined in Table 7.3) was used to estimate the saturated vapour pressure of carvacrol in the experimental apparatus design.

7.3 Idea Generation and Concept Screening

Preliminary Studies

As previously stated, a method to deliver stable humidified air flows with well controlled volatile levels was required. The aim of the first preliminary study was to improve the understanding of airstream humidification and its impact on the proposed volatile generating system design. In order to determine the microbial inhibition concentration (MIC) of the microbe, high humidity (97%) should be introduced to the gas delivery system to ensure the inhibition results collected are due to the antimicrobial agent and not due to low water activity. This is because most microbial growth occurs at water activity > 0.9 and fungal growth at water activity > 0.7.

One approach was to humidify the air stream by bubbling it through a saturated salt solution (e.g. potassium sulphate) and then passing this air through a liquid active agent to saturate the airstream with the volatile. To test this concept a series of three experiments were carried out as shown in Figure 7-3 below:



Experiment A Experiment B Experiment C

Figure 7-3: Illustration of experiments conducted to get the required relative humidity in flow through system.

Experiment A was conducted by introducing dry air through carvacrol oil at 45mL/min, in a controlled temperature chamber (25°C). The airstream was then passed through an empty vessel containing a relative humidity sensor, attached to a data acquisition system, (Picolog AC 200). The result (Figure 7-4) shows that constant relative humidity, 0.0% was achieved immediately. Experiment B was performed in the same manner using a saturated salt solution of potassium sulphate (K_2SO_4), (97% RH). The results indicate high humidity level was attained very quickly. It is thought that small temperature differences between the bubbling column and the RH sensor might have caused condensation in the sensor resulting in the unrealistic measurements for the first few hours.

After several hours, the expected equilibrium state 97.0 ± 2.5 % RH was reached. By contrast, for experiment C, where the dry air was first passed through a saturated K_2SO_4 salt solution and then the carvacrol oil in series, the collected data revealed much longer times were required to reach high relative humidity (~ 55% RH) levels. The experiment was conducted for 9 days

continuously (Figure 7-5) and during this time, the relative humidity level increased gradually from approximately 30% to 90% RH.

Based on the calculation, at the high humidity air stream (~97 RH, 45 mL /min), it was estimated that 0.08g water was absorbed per mL carvacrol oil after 9 days. Data from literature reported that the water solubility of carvacrol is 830±10 ppm (Griffin *et al.*, 1999). So this suggests that water droplets must have formed in the oil. The experiments clearly showed that stable high humidity, carvacrol rich air streams could not be achieved using this method.

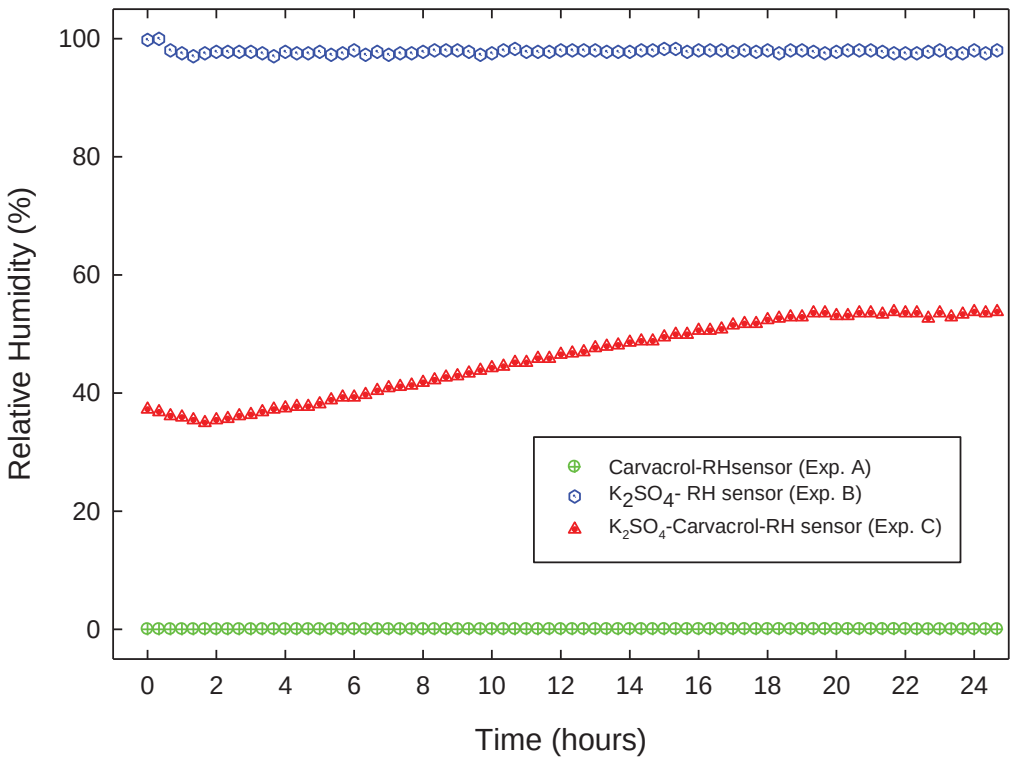


Figure 7-4: **Results of an experiment for the volatile flow through experimental setups A, B and C defined in Figure 7-3 (dry air flow rate 45mL/min; Temperature 25°C).**

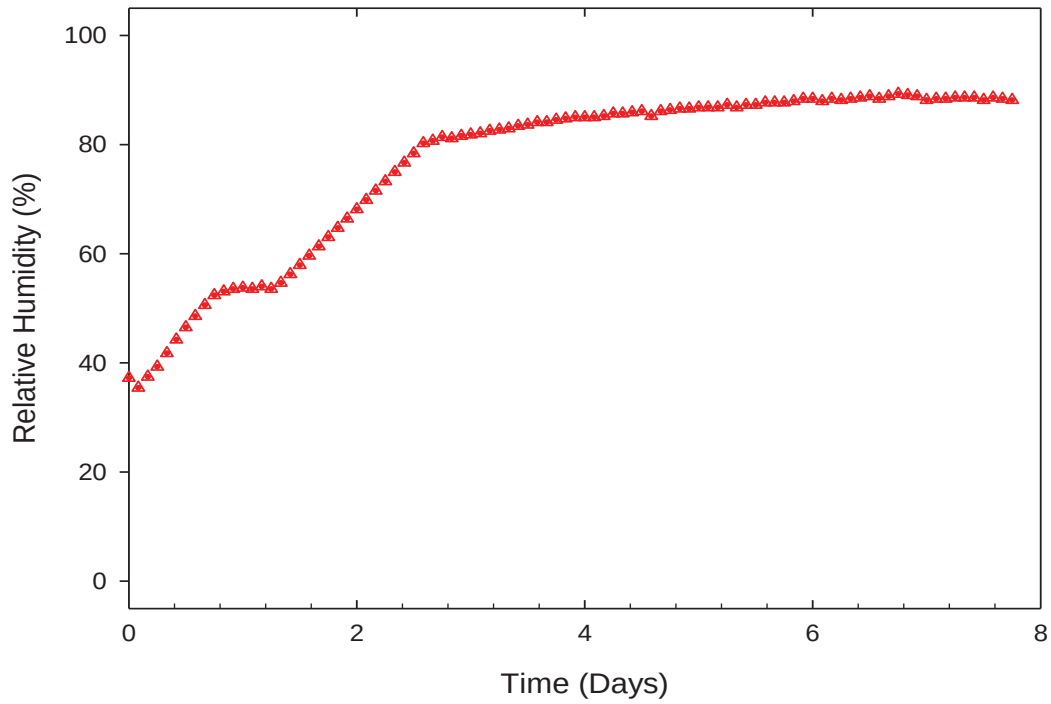


Figure 7-5: **Results of extended experiment C (defined in Figure 7-3) (saturated salt solution – carvacrol oil – RH sensor) for the volatile flow through system (dry air flow rate 45mL/min; temperature 25°C).**

Another experiment was carried where 3% (w/v) of water was added to the carvacrol oil. The 'milky' opaque characteristic of oil in water emulsion was observed when 1% (v/v) emulsifier (Tween 20), was added to the water/oil mixtures. Dry air was introduced into the emulsion and the relative humidity in the outlet air stream was recorded. It was observed that large droplets of water formed in the oil after a few hour of experimentation, as illustrated in Figure 7-6. This was likely to be due to the emulsifiers being insufficient to stabilise the mixtures. The relative humidity of 98.0 ± 1.5 % was attained in a short period of time (less than 30 minutes, data not shown). However, this method was not preferred because it may affect the stability of carvacrol. Hydrolytic oxidation may occur when a high amount of water is present and this may lead to fast

deterioration of carvacrol oil. Additionally, the relative humidity decreased gradually after a few days run, and the stable >95% relative humidity required throughout the experiment was not achieved.

Figure 7-6: Illustration of water/carvacrol oil emulsion in this experiment.

These results suggest water and carvacrol vapour should be generated separately and then mixed as illustrated below (Figure 7-7): -

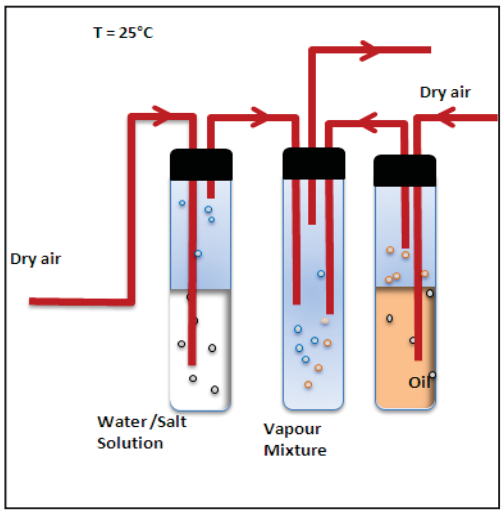


Figure 7-7: Proposed improved volatile generation system

A mass balance was developed to predict the vapour composition after mixing humidified and carvacrol-containing airstreams. Due to the low saturated vapour pressure of carvacrol, 3.45 Pa (derived from Antoine-Grain equation), compared to water's vapour pressure, 3164 Pa at 25°C, a high proportion of carvacrol vapour must be mixed with a small amount of humidified air to get reasonable volatile concentrations. As a result, the relative humidity drops markedly and this will affect the growth of microbial and fungal cultures.

Figure 7.8 presents the theoretical mass balance of water and carvacrol vapour at 25 °C. The graph shows that the maximum relative vapour pressure for carvacrol at T= 25°C at a relative flow state (F_{crv}/F_h) of 3, is 0.75 with 24% relative humidity which could not permit microbial growth. At 97% RH, the carvacrol concentration in the headspace is very low (less than 0.1 relative vapour pressure) which may not be sufficient to inhibit the growth of microbes.

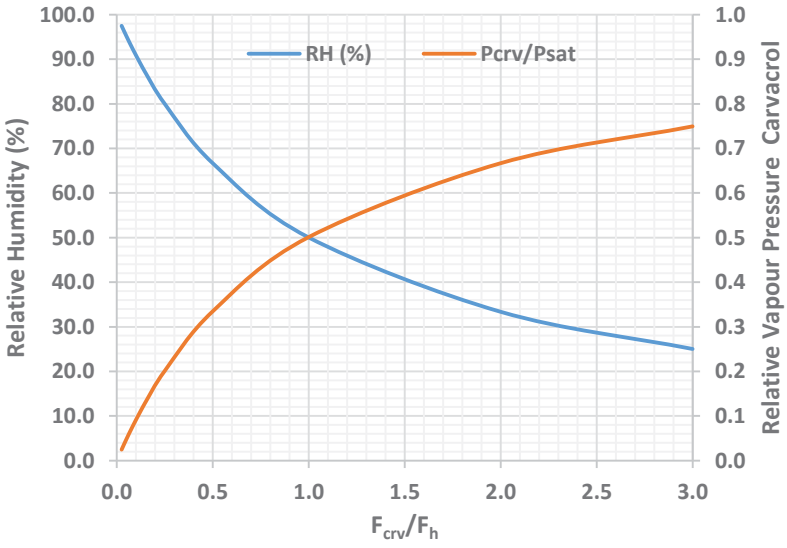


Figure 7-8: Theoretical mass balance of water and carvacrol vapour in the carvacrol delivery system at T_1 and T_2 = 25°C.

This problem can be solved by generating the vapour streams at a higher temperature as demonstrated below (Figure 7-9) and then allowing them to cool to the experiment temperature.

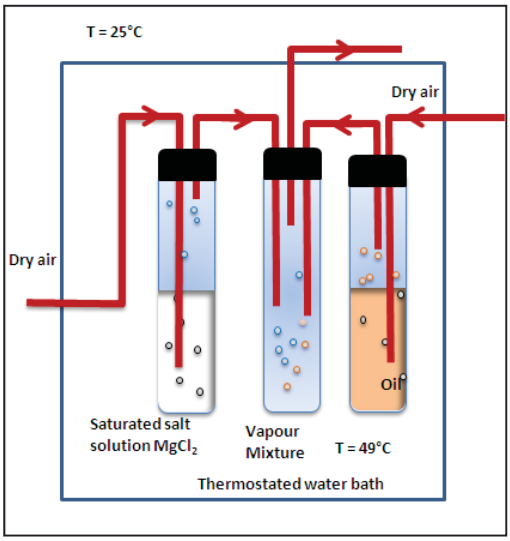


Figure 7-9: The ideal system for the volatile generating system.

In this design, the generation of volatiles is done at high temperature in order to build up a high concentration of carvacrol as well as a high humidity level. However, if conditions are not correct, condensation might occur when the vapour mixture is cooled down to 25°C. The mass balance was used in order to determine the optimum conditions and to avoid condensation. The most favourable condition to avoid condensation from the water and carvacrol vapour after mixing is presented in a schematic diagram (Figure 7-9).

Based on the data collected from the preliminary experiments above, several factors were contemplated in designing this system, such as:

a) Bubble column design to maximise the rate delivery of volatiles in the system.

b) The type of bubble column due to the corrosiveness of the saturated salt solutions (Magnesium Chloride)

c) The volume of oil in the chamber as essential oil is expensive

d) Absorption of volatiles in the tube, no rubber tubes between the connections – the system should be fabricated from stainless steel, glass or Teflon to minimise absorption.

7.4 Detailed Design of the Volatile Generation System

Although the vapour generation system was developed from a single volatile, it was designed such that can be extended to allow the use of multiple volatiles in the future. The schematic design of the system is presented in Figure 7-10.

Each flow-through chamber would contain a number of inoculated agar plates (PDA) into which two vapour streams are mixed and allowed to flow through. The chamber would be designed to ensure constant gas composition over the full incubation period. The gas flow rates through each chamber would be adjustable using a mass flow controller. This will allow different concentrations to be set in each chamber. In this way, the apparatus would be used to record time for observation of growth and assessed over a range of conditions simultaneously.

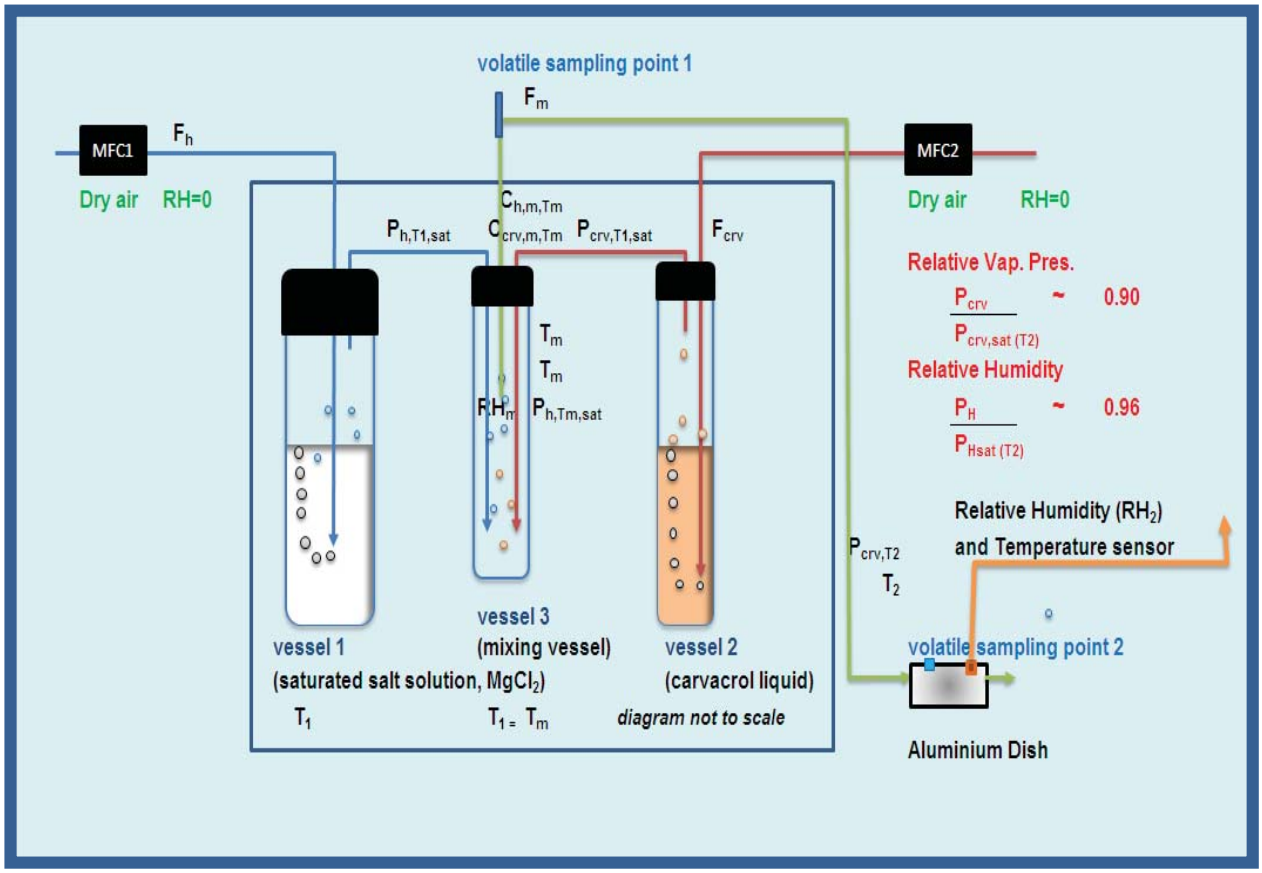


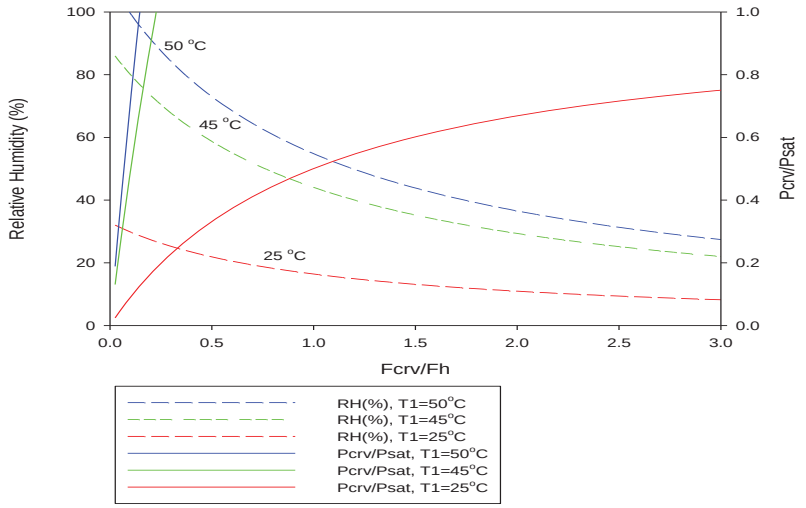
Figure 7-10: Schematic diagram of the volatile generating system.

Nomenclature

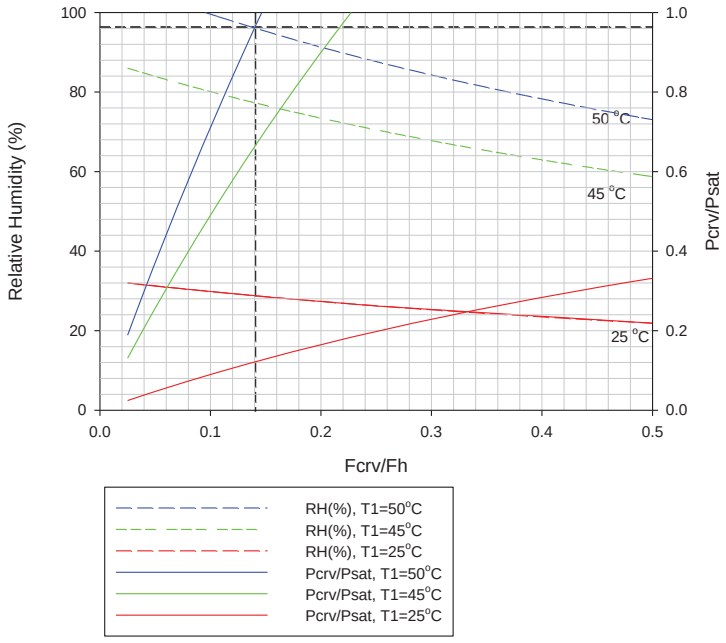
F_h	=	Dry air flow rate for humidity (mL/min)	T_2	=	Room temperature (25.0 ±1.0°C)
F_{crv}	=	Dry air flow for carvacrol (mL/min)	T_m	=	Temperature in mixing vessel (°C)
F_M	=	Mixture of dry air flow of carvacrol and humidity (mL/min)	P_h	=	Humidity Vapour Pressure (Pa)
RH	=	Relative humidity (%)	$P_h/P_{h,sat\ (T2)}$	=	Humidity relative vapour pressure (Pa)
RH_M	=	Relative humidity after mixture of carvacrol (%)	$P_{crc}/P_{crv,sat(T2)}$	=	Carvacrol relative vapour pressure at T_2°C
RH_2	=	Relative humidity at room temperature	MFC1&MFC2	=	Mass Flow Controller (mL/min)
T_1	=	Water bath temperature (°C)	C_{crv}	=	Carvacrol concentration (mol/m^3)

To provide a system capable of delivering stable flows of air with carvacrol volatile at high concentrations in the presence of high relative humidity, an air stream (MFC2) would be saturated with volatile compounds at high temperature (45 - 50°C) by sparging through a bubble column containing pure carvacrol, submersed in a water bath. A second air stream (MFC1) would be equilibrated with water vapour by bubbling through a saturated salt solution (e.g. magnesium chloride; $MgCl_2$ ~ 30% RH at 50°C, T_1), also submerged in the water bath. These two streams would then be mixed. The gas mixture is then allowed to cool to ambient conditions. In this way, a combined air stream could be produced that contained carvacrol up to 90% volatile saturation and 95% relative humidity at ambient temperature.

Mass flow controllers (MFC) would be used to regulate the flows of each gas and to vary the ratio of each component. The equilibrium vapour pressures of antimicrobial compounds generated by the flow through system could be quantified using gas chromatography-mass spectrometry (GC-MS) as described in Chapter 4. This system potentially allows generation of accurate target headspace conditions for controlled volatile release active packaging design. The compositions of air streams predicted to be generated with the volatile generating system proposed are shown in Figure 7-11.



(a)



(b)

Figure 7-11: **Theoretical carvacrol vapour delivery system mass balance of humidity from saturated Magnesium Chloride ($MgCl_2$) at different mass flow fraction (MFC2/MFC1) at T1=25°C, 45°C and 50°C; and T2 = 25°C. (b) is the same as (a) but with smaller x-axis scale.**

The profiles in Figure 7-11 demonstrate that at mass flow fraction (F_{crv}/F_h) of more than 0.2, the relative vapour pressure of carvacrol and relative humidity are more than 1 and 100% respectively. Thus condensation will occur, when the combined vapour is cooled to 25 °C. In order to obtain high volatile carvacrol concentration, the mass flow fraction range is quite narrow (0.10 – 0.14) in the range 0.70 to 0.96 relative vapour pressure with 98 to 96% relative humidity. This indicates that the setup of equipment is very critical to avoid condensation of vapour and production of inconsistent results. The best flow rate fraction was ~ 0.14 (refer to Figure 7-11(b)) that theoretically supplies 0.96 carvacrol relative vapour pressure and humidity above 95% in the system headspace.

7.5 The Experimental Setup and Lab View Program

The experimental set up involved five components which were:

a) Water bath tank to control the temperature. This contained three vessels for carvacrol liquid, mixing vessel and humidity vessel connected together with stainless steel tubing.

b) Aluminium Dish (flow cell) with temperature and relative humidity sensor attached to the aluminium dish lid.

c) Two mass flow controllers to control the dry air flow into the carvacrol vessel (MFC2) and into the humidity vessel (MFC1).

d) Lab View Signal Express 2010 programme display.

The analogue inputs (Figure 7-12 (a)) are converted from the voltage signals into digital values that could be stored and processed in the Lab View software. These consisted of five signals:

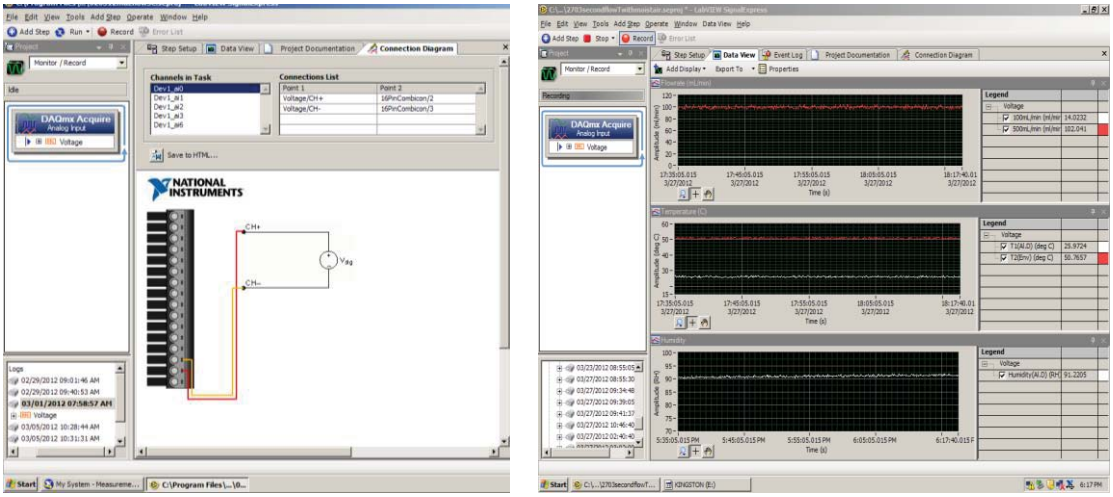
(a) MFC2

(b) MFC1

(c) Temperature, T2 (in Aluminium Disc)

(d) Temperature; T1 (in Water Bath)

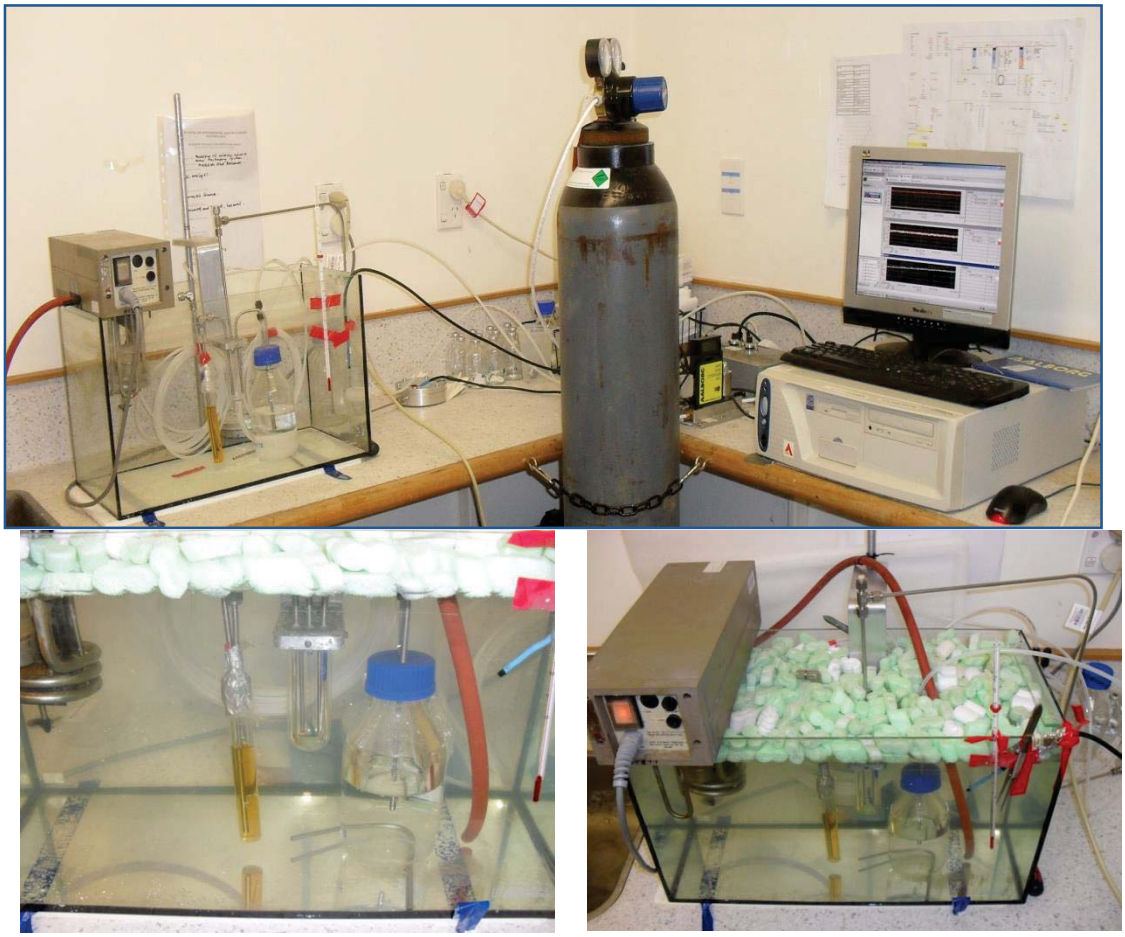
(e) Humidity sensor (in Aluminium Disc)



a) Analogue inputs b) Front Panel of Lab View programme

Figure 7-12: Analogue inputs and front panel of Lab View programme

The experimental setup and the description of the developed Lab View programme are shown in Figure 7-12 and 7-13.



(a) (b) (c) (d)

Figure 7-13: General experimental setup for the experiments

(a) Aluminium Dish (flow cell) with Temperature and Relative Humidity sensor, (b) Volatile and water vapour system and c) Mass Flow controllers d) Lab View 2010 programme display.

Polystyrene cubes were placed in the water bath tank in order to reduce the water evaporation. The experiment was carried out in a temperature-controlled room at 25°C. Before the experiment was carried out, all temperature probes were calibrated. Calibration of the humidity sensor was carried out using saturated salts (Greenspan, 1977). The data collection programme was set up using the default system as presented in Table 7-4.

Table 7-4: Configuration set up of Lab View Lab View Signal Express before Temperature and RH sensor calibration

Code	Configurations	Voltage input settings		Terminal Configuration	Custom scaling	Sampling period (s)	NI-DAQmx Scale		Scaling Parameters		
	Voltage	max	min				Pre-scale	Scale	Slope	Intercepts	Resulting Equation
MCF2	Dev_ai0	101	0	Differential	100 mL Flow	20	Volts	mL/min	20	0	y = 20x + 0
MCF1	Dev_ai1	501	0	Differential	500 mL Flow	20	Volts	mL/min	100	0	y = 100x + 0
T1	Dev_ai2	100	0	RSE	LM35dz	20	Volts	° C	100	0	y = 100x + 0
T2	Dev_ai3	100	0	RSE	LM35dz	20	Volts	°C	100	0	y = 100x + 0
RH	Dev_ai6	100	0	RSE	Humidity	20	Volts	RH	32.75	27.01	y=32.754x-27.01

7.6 Testing the System

The designed apparatus has a number of key functionalities that needed to be tested. These include RH control, characterising the dynamics for when changes to the set points are made, testing the gas mixing and testing volatile generation. Several experiments were carried out to test the design and are documented in this section.

A test was carried out to show that the humidification bubble column worked and to characterise the dynamics of this part of the system. The system was set up at 25 °C and an air stream at 100 mL/min was passed through the bubble column. The column was filled with saturated Magnesium Chloride ($MgCl_{2)}$ solution for the first 2 hours. This was replaced with sodium chloride (NaCl) at 2 hours. This was then replaced with water after 24 hours.

Figure 7.14 demonstrates the results of the relative humidity sensor response with the different salts at 25° C. The results indicate that the equilibrium in the chamber takes about one hour to achieve. Once a steady state was achieved, it remained very stable.

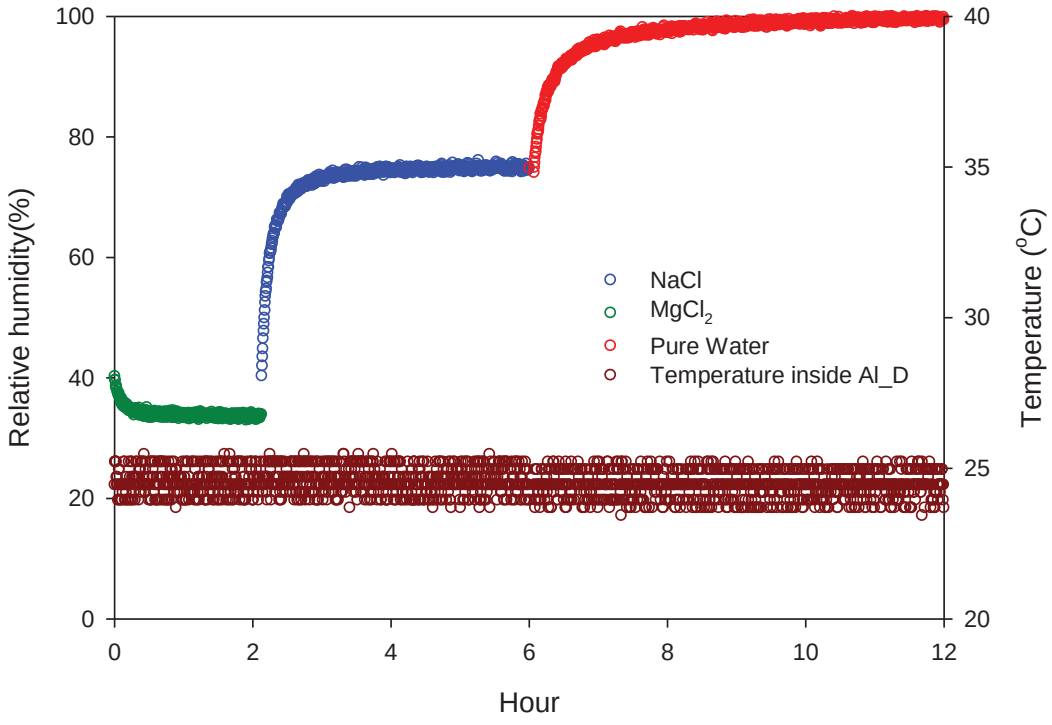


Figure 7-14: **Example experimental data of bubble column operation with saturated salt solutions $MgCl_2$ (0 -2 h), $NaCl_2$ (2- 6 h) and pure water (6 h plus) at 25°C measured in the Aluminium Dish**

Figure 7-15 shows the effect of mass flow fraction against relative humidity. In this experiment, the temperature was controlled at 25°C for both ambient and the temperature in the water bath. An air stream was then bubbled through the humidification bubble column that was full of water. A second dry air stream was then introduced into an empty carvacrol bubble column and mixed with the humid air. By changing the ratio of humid air to dry air flow, tests on the air mixing could be achieved. Figure 7-15 shows that the equilibrium was reached for each flow ratio and that was consistent for at least 3 hours. At the ratio, F_{crv}:F_h = 15:60, 15:120, 5:100 mL/min the humidity profiles were as expected according to the

mass balance (Refer to Figure 7.17, page 192). However, at ratio F_{crv}:F_h = 15:15, the experimental data shows that the humidity was less than the theoretical value.

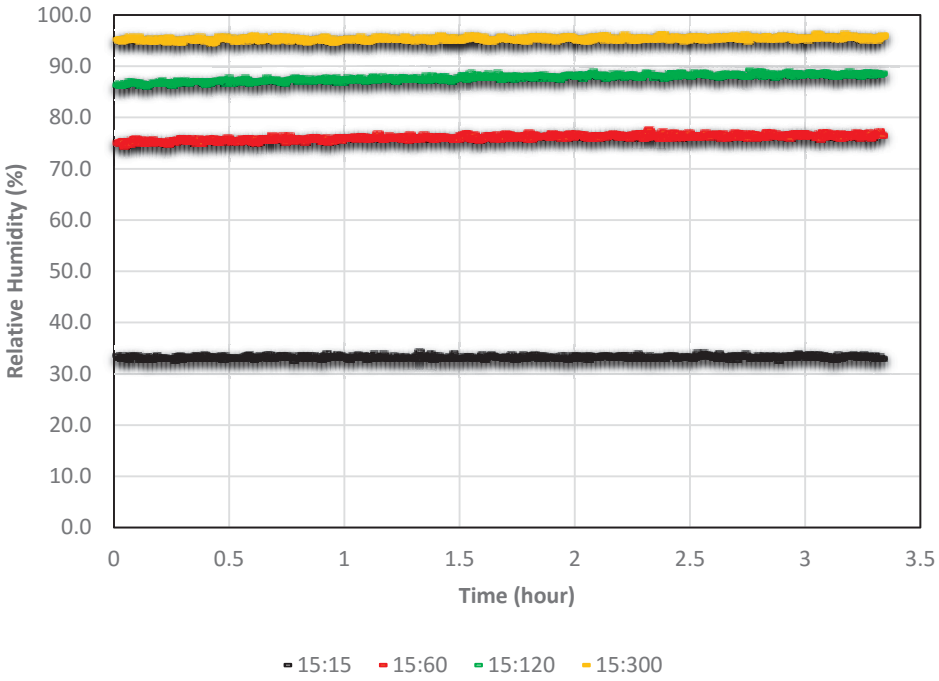


Figure 7-15: **Effect of mass flow fraction against the relative humidity in the system headspace. Legend represent F_{crv}: Fh (T_1 = 25°C, T_2 = 25 °C, F_{crv}: F_h = 15:15, 15:60, 15:120, 5:100 mL / min) (note F_{crv} was effectively dry air as no carvacrol oil was present in the bubble column).**

An experiment was also carried out to determine the effect of changing the mass flow fraction on the relative humidity in the aluminium dish headspace. For this experiment, $MgCl_2$ was used in the humidification bubble column, and the water bath was run at 50°C. The flow rate of humidified air mixed into the dry air stream was then changed from 100 to 500 mL/min after 8 hours. This changed the flow ratio from 15:15 to 60:15. The results of one experiment are presented in Figure 7-16.

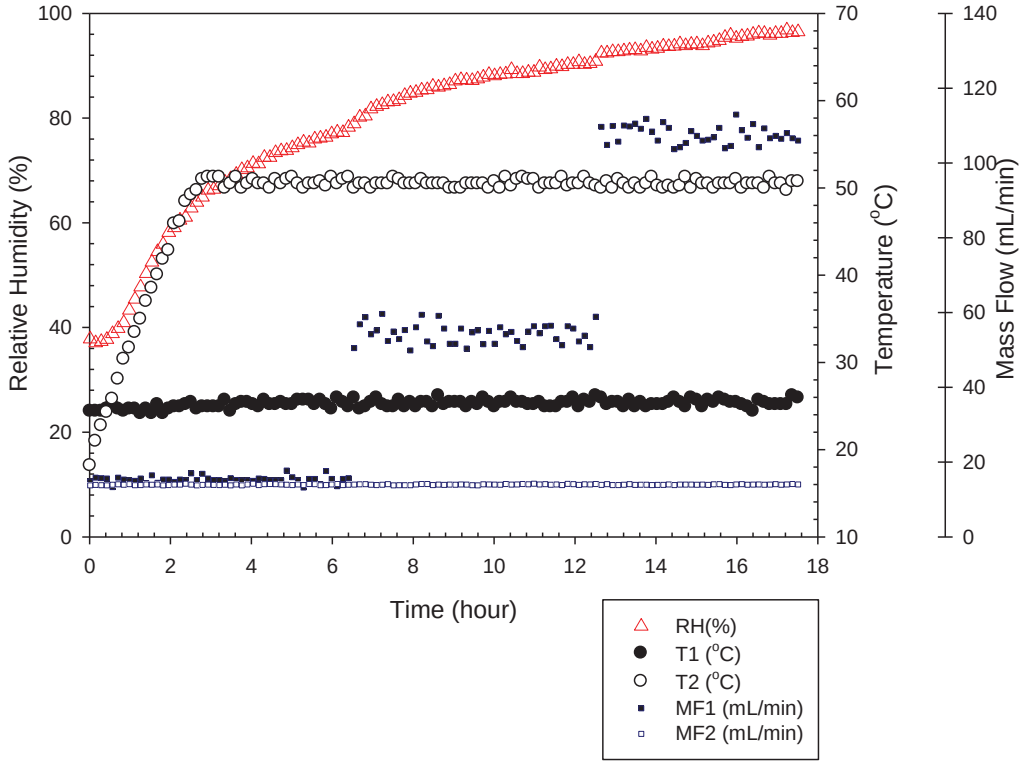


Figure 7-16: **Experimental data to determine the effect of mass flow fraction against the relative humidity in the aluminium dish headspace. Legend represent F_{crv}:F_h (T_1 = 50°C, T_2 = 25 °C, F_{crv}:F_h = 15:15, 15:50 and 15:100mL / min**

In the first hour, some heating dynamics (T2) can be seen, after the system was submerged into the water bath. The outlet air relative humidity changed during this time and then showed an expected 1st order response, although it did not reach equilibrium before the system was changed at 6 hours. Similar 1st order responses can be seen when the flow ratio was changed after 6 hours and 12.5 hours. It can be seen that effectively saturated air at 25 °C was produced from humidifying air in a saturated $MgCl_2$ bubble column at 50 °C.

There were some apparent differences between the equilibrium RH recorded experimentally and the expected result from the mass balance. Figure 7-17 shows how the steady state RH changed with the flow ratio. This experiment was conducted with the water bath at 25 °C using water in the humidification bubble column.

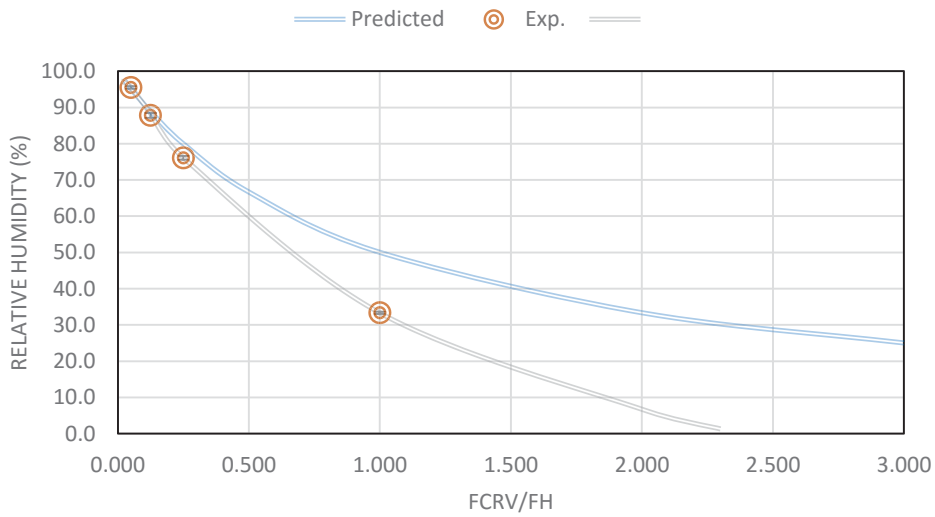


Figure 7-17: **Experimental data of relative humidity (%) in the system (Aluminium Dish) at various ratios of F_{crv} and F_h and the predicted curve obtained by mass balance. Each data point was an average of 3 hours at equilibrium state (at frequency 20 seconds per data, N = 540).**

These results show that the experimental device produced air streams with RHs that were lower than expected using mass balance estimates. This is likely to be due to the bubble column not quite reaching saturation. If a longer bubble residence time in the solution was achieved (by increasing height or using a secondary column in series), then this result could be improved. In spite of this issue, very stable RH streams were achievable with the current design.

Having established that RH control could be achieved, the ability to produce close to saturated carvacrol concentrations were tested. For this trial, the apparatus was run at 50 °C with $MgCl_2$ used in the humidification bubble column. Samples were taken from the aluminium dish headspace through the Teflon septum. Samples were analysed for carvacrol volatile concentration using GC-MS as outlined in Chapter 4. Figure 7-18 shows the steady state RH and carvacrol concentrations at three different flow ratios.

Clearly, the design can produce high RH air streams with high vapour concentrations. This was not possible with the preliminary designs outlined earlier in the chapter. At very low flow ratio's there was general agreement with the values predicted from the mass balance. At equal flows, it was expected that completely saturated/condensed carvacrol would result, however, this was not seen.

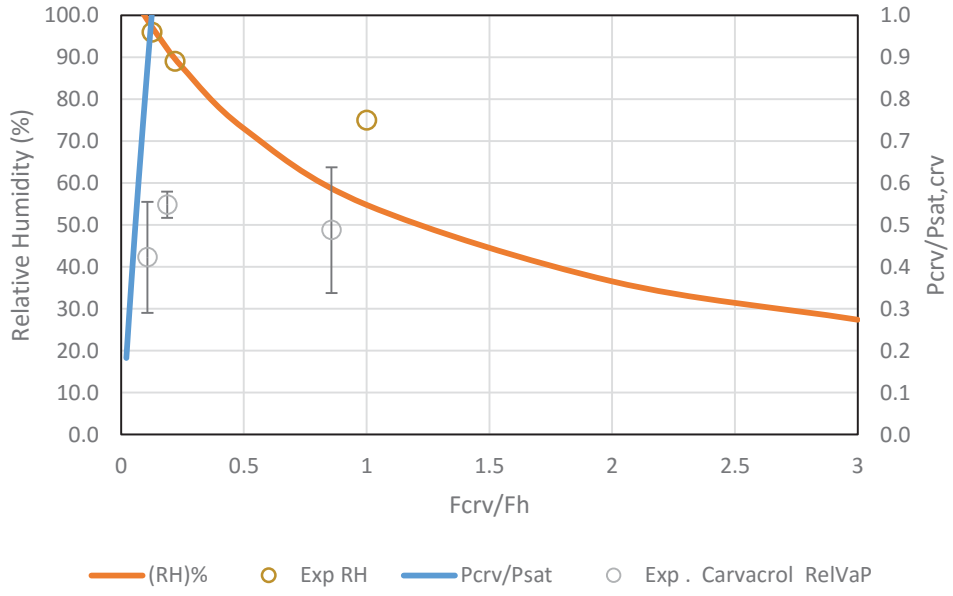


Figure 7-18: **Experimental data of relative humidity (%) and relative carvacrol vapour pressure in the system (Aluminium Dish) at various ratios of F_{crv}/F_h (1(14:14), 0.22 (14:64), 0.13 (14:112) and the descriptive curve obtained by mass balance. Each point of carvacrol concentration was an average of three measurements and humidity experimental data was calculated as an average. T_1 = 50 °C and T_2, 25°C**

It may be that there were some components in the design where carvacrol absorption could still take place, despite efforts to use only inert materials. This may result in some time being required before stable volatile concentrations are achieved. Figure 7-19 shows the changes in volatile concentration over time. This shows approximately 30 hours was required before high vapour concentrations were observed. Once this initial lag was achieved, however, subsequent experiments did not require such large initial delays. This does suggest some absorption occurred somewhere in the system, and once saturated, the system responded more quickly.

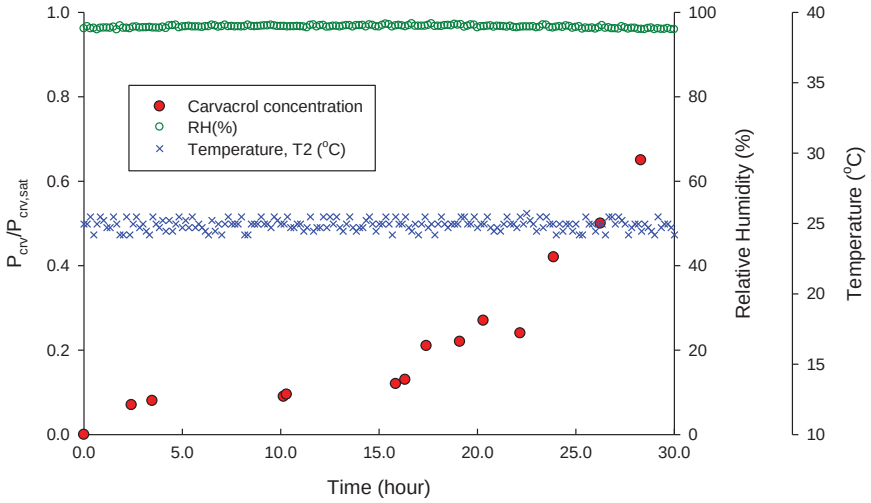


Figure 7-19: **Plot of $P_{crv}/P_{crv,sat}$, Temperature (°C) and Relative Humidity against Time (hour) of the carvacrol volatile delivery system. (T_1, 50 ^{0}C, Water bath; T_2, 25 ^{0}C, Aluminium Dish); MFC1 =100.0 mL/min; MFC2 = 14.0 mL/min).**

Figure 7-20 shows another similar experiment. The data does not exhibit a large delay because the experiment was started soon after the previous trial and so the system was already close to equilibrium. In this experiment, however, carvacrol volatile concentrations,

both at the inlet and outlet of the aluminium samples chamber (empty) were measured. This shows a significant drop in carvacrol concentration due to flow through the chamber. In spite of this and the apparent scatter in the volatile concentration measurement, the system does provide relatively high and constant volatile concentration at high RH. Although further design improvements should be made to provide better control, the device could be used to test the control of the headspace concentration above foods.

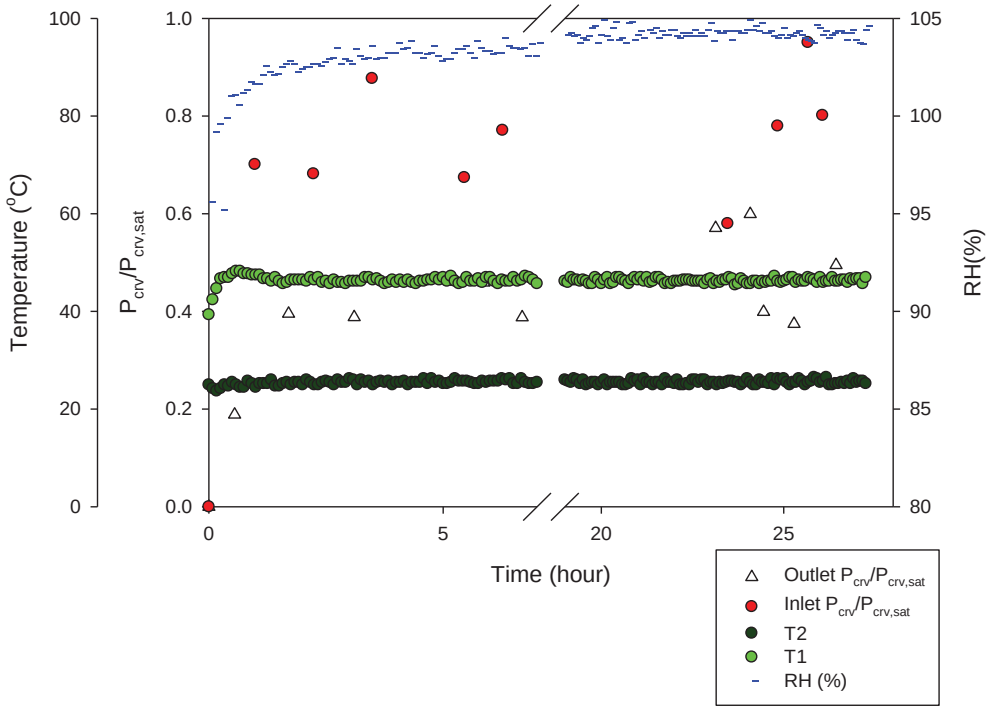


Figure 7-20: **Carvacrol concentration at sampling point 1(SP1, inlet) and in the sampling point (SP2, outlet) of the system delivery**

7.7 Application of Vapour Generator to MIC Measurement

Although the control of the vapour generator was not perfect, it was applied to provide vapour to a food system. This was done in a way to simulate control of the headspace

conditions around food during incubation. Solid potato dextrose agar (PDA) was used as a model food system. PDA was prepared as described in Chapter 4 and poured directly into the aluminium sample chamber and sealed. For the purposes of this experiment, the PDA was not inoculated with *Botrytis cinerea*. Instead, the trial was used to determine how the vapour generator was affected by the presence of the food sample.

The inlet to the sample chamber was fed by the vapour generator. Vapour was generated running the water bath at 50°C using $MgCl_2$ in the humidification bubble column. The total flow rate through the sample chamber was 114 mL/min (humidity, MFC1 = 100mL/min; carvacrol, MFC2 =14mL/min), at a RH of 97%. Control of RH is to reduce water losses or (gains) during an experiment. In this study, the RH 97% target was used as an example but the system can be configured to any desired humidity. Generally, the RH would be set at the water activity of the test product.

The vapour concentration in the sample chamber headspace was measured by gas sampling and analysis using GCMS as described in Chapter 4. Figure 7-21 shows the headspace concentrations changing with time. The headspace concentration reached 60 – 70% saturation within 3 days and the delivery of carvacrol remained relatively fast compared to the rate of absorption into the PDA. This is promising as the release rates from filter paper in the traditional reverse Petri dish method (Chapter 4) could not reach this headspace concentration when PDA was present.

After 8 days, the chamber was sealed off by closing valves on both the inlet and outlet. There was then a gradual drop in headspace carvacrol concentration. This drop is consistent with the continued absorption of the carvacrol into the PDA before a final equilibrium was reached.

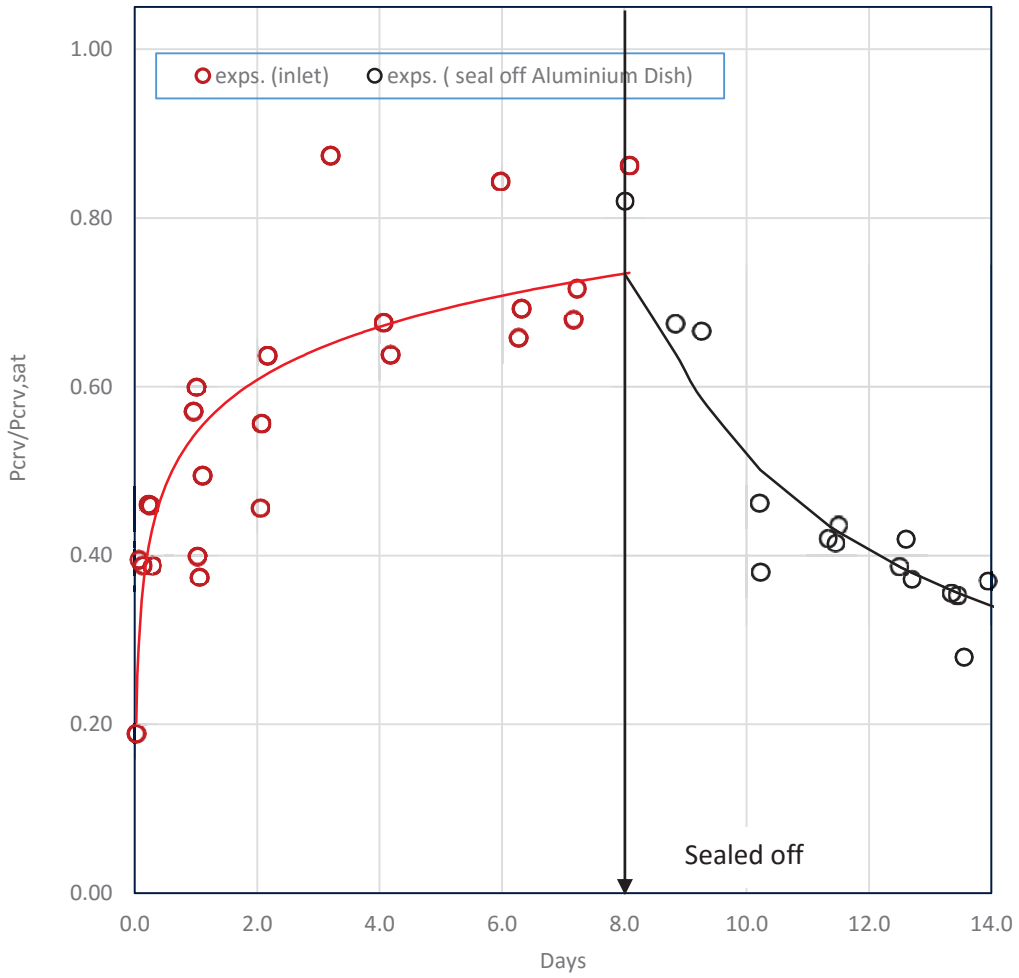


Figure 7-21: Carvacrol concentration in the aluminium dish headspace

This shows that even after 8 days of exposure to high carvacrol concentrations, the PDA did not reach saturation. The method shows promise, in that it can maintain high carvacrol concentration for sustained incubation periods. Further work, however, is required to provide the control and operation of the vapour generator. This is required before accurate exposure of real inoculated food samples to controlled vapour concentrations is possible and meaningful MIC measurements could be made.

7.8 Conclusion

In the previous chapter, the carvacrol and thymol were added directly to the food model system (PDA). This was to reduce the time and to attain equilibrium concentrations in the container headspace. However, in real systems it is impossible to add the antimicrobial component directly to the food, and delivery of volatiles must be achieved through the gas phase in order to measure the MIC. To try to achieve this, a volatile generating system was designed. The generation of airstreams with both high volatile concentration and high relative humidity is a difficult task. In this work a useful start to such a system was designed and implemented. The key mechanism for the device was the generation of volatile and humidified streams at high temperature and mixing. In this way, air flows with the required concentration were achieved. Further work is required to reduce the variations in vapour concentrations and to identify and remove components in the system that absorb carvacrol. When applying the system to real foods, the variations in the rate of absorption into the food could be compensated for by varying the delivery rate.

CHAPTER 8

GENERAL CONCLUSIONS AND RECOMMENDATIONS

The work presented in this thesis was aimed at providing a better understanding of the minimum inhibitory concentration of volatiles against spoilage microorganisms. The design of active packaging systems is dependent on data for the target gas phase concentration required to provide shelf life extension. With this data, an appropriate design of an in-package volatile delivery system can be utilised to control the release of volatiles into a food product at this concentration can be developed. In this way, the concentration of volatiles levels to inhibit the microbial growth can be regulated over the duration of the food's shelf life. Combinations of two or more different antimicrobial volatiles have the potential to achieve shelf life extension with reduced individual and overall concentrations. In this way, active packaging systems can be introduced that minimise sensory impact of the volatile components.

A review of the literature was conducted to provide an overview of antimicrobial active packaging systems with an emphasis on their application in food products. Controlled release antimicrobial active packaging systems are relatively new in food applications. Essential oils, are natural ingredients that can be exploited as active agent compounds in controlled release active packaging systems since their antimicrobial activities are well documented in the literature and because they are generally recognised as safe. The review also outlined principles and approaches to measuring the MICs of targeted microorganism. However, the accuracy of the techniques applied to measure headspace MICs against targeted microorganisms is questionable, particularly when it is evident that many reported MIC values significantly exceed saturated vapour pressures of the essential oils.

Despite the widespread use of the techniques reported in literature, the accuracy to characterised headspace concentration required to inhibit the microbes is questionable. In the traditional techniques, the concentration of active compound vapour in the headspace may not be stable and could be lost during the treatment. The sorption of volatiles by the culture media and other parts of the packaging system must also be considered. The review also identified that combination of the major constituents of some essential oils, such as thymol and carvacrol, may act synergistically to reduce the overall MIC and thus be used at lower concentrations than if used singly which in turn could reduce their undesirable sensory impact on the foods.

Based on the literature, a model food and spoilage system together with two volatiles to use as case studies was selected for the word. Two natural volatiles were selected; thymol and carvacrol, which both showed strong antifungal activity and there is potential to control the growth of spoilage fungi in a closed system thus verifying observations from other authors.

The minimum inhibitory concentration for carvacrol was determined using the widely used reverse Petri dish method. The measured data showed that the minimum inhibitory concentration of carvacrol against *Botrytis cinerea* varies depending on the definition of MIC, however the results obtained were within the range reported in the literature.

In this method, several factors are likely to influence the MIC determination. For example, the reporting of effective concentration in relation to air space, volatile evaporation rate, exposure times were variable, as well as potential method variations in terms of Petri dish sizes, filter paper diameter, and the choice of media. In addition, there was a likelihood for vapour loss through the un-hermetic closure system, the absorption of the volatile into media and the dish walls. The incubation temperature can also affect the partial pressure of the active compound. These factors all contribute to the observed variations in reported MIC's for each essential oil volatile. Because of these factors the MIC data collected using this method could not be used for active packaging design. This study suggests, for the reasons outlined above, that the Petri dish method (particularly using plastic plates) overestimates the MICs and its use should be discontinued.

To further understand the dynamics of the reverse Petri plate system, the headspace concentration changes during the measurements of the minimum inhibitory concentration were characterised. According to the literature, this method is to be considered the premium standard to evaluate the resistance of organisms to antimicrobial agents. Four dish configurations were used, either empty or filled with PDA. The dish configurations were a Polystyrene Petri dish unsealed, Polystyrene Petri dish wrapped with plastic paraffin film at the edge Polystyrene Petri dish wrapped with aluminium foil at the edges and lastly a hermetically sealed aluminium dish. It was concluded from the results that absorption of carvacrol vapour by the PDA strongly influenced the headspace dynamics and was the main

reason for the low volatile concentrations in the headspace in the reverse Petri plate MIC measurement method. The relative partial pressure of the carvacrol was much lower (0.02 to 0.35) than the saturated vapour pressure except in the empty sealed aluminium dish. These results suggest the MICs reported by investigators are much higher than the actual headspace concentrations in the system.

In order to determine reliable minimum inhibitory concentrations, a method that can provide a consistent concentration in the system headspace was required. The study showed that there was a relatively slow vapour release (even in the empty aluminium dish) suggesting that there was an interaction between the volatile and the filter paper and could thus also be an influence on the headspace dynamics. The interaction of the essential oil volatiles and the culture substrate (in this case PDA) is likely to be a key factor influencing the interpretation of MIC measurements.

A Harris isotherm equation was used to describe the sorption equilibrium between the carvacrol concentration in the air phase and on the filter paper. The shape of the desorption isotherm explains some of the release behaviour if the volatile in the reverse petri dish method. At the onset of the experiments, the amount of carvacrol in the filter paper is high and, therefore, the equilibrium vapour pressure is high. This allows the release to match or be greater than absorption into the plastic dish. After the desorption of carvacrol on the filter, paper surface drops to about 1 mol/m^3, the equilibrium vapour pressure dramatically reduces and, therefore, the release rate will decrease. This behaviour would occur earlier if larger filter paper discs were used for the same applied volume of essential oil liquid. If diffusion within the filter paper were also to be partially limiting, then this together with the equilibrium behaviour of the carvacrol and filter paper could explain the relatively low release rate in the empty aluminium dish. The interaction between the volatile and filter

paper suggest that for reliable MIC measurements, the use of the filter paper should be avoided.

The characterisation of the headspace dynamics in the reverse Petri dish method demonstrated that it is difficult to obtain reliable MIC values for volatile antimicrobial agents. This is especially true if the values are needed for active packaging design rather than for testing the comparative activity of different compounds. The dynamics associated with release from the filter paper, losses from the dish and absorption into the dish walls and media result in much lower gas phase concentrations than are normally reported using this method.

These mechanisms also result in changing conditions during incubation and explain why differences in methodology such as filter paper area, dish diameter and the volume of media affect the results. As a result, an alternative method to determine MIC and potential for synergetic behaviour was required.

The strong partitioning of carvacrol and thymol ($K_{A/W}$ values 5.94×10^{-5} and 2.58×10^{-4} respectively) toward the solid phase, provided a basis for the design of a new method to enable better MIC measurement. Chapter 5 outlined the development and application of a new method to measure the MIC in volatile systems. The new method was based on pre-mixing the volatile compound into the liquid media before it was allowed to set in Wheaton bottles. After inoculating and sealing, only a small amount of volatile desorption is required before equilibrium is achieved potentially in a closed system, oxygen limitation could occur. Because control experiments showed growth, it was clear that oxygen availability did not limit the growth.

In comparison to the control, the introduction of carvacrol in the potato dextrose agar at 35ppm (P/P_{sat} = 1.54%) started to inhibit the growth of *Botrytis cinerea*. At 50 ppm (P/P_{sat} = 2.20%), the growth of *Botrytis cinerea* was delayed for four days in comparison to the control. Similarly, the MIC for more than six days was 60 ppm (P/P_{sat} = 2. 7%). In Chapter 3, the MIC was 173mmol/m^3_{air}, corresponding to P/P_{sat} equal to 126, which was not realistic. This observation backs up the investigation that the Petri dish method is inaccurate due to losses and absorption into the dish and media.

The introduction of thymol in the potato dextrose agar at 25 ppm started to inhibit the growth of *Botrytis cinerea*. At 50 ppm, thymol delayed the growth of *Botrytis cinerea* for five days compared to the control. The microbial activity of thymol was found to be slightly stronger compared to carvacrol, which is in agreement with results reported by several authors (Knobloch *et al.*, 1989; Lambert *et al.*, 2001; Liolios *et al.*, 2009). It was concluded that the development of the new method solved the problems identified in Chapter 4. The results of the work provided reliable and repeatable results and showed much lower inhibition concentrations than observed in the Petri dish method and provide a sound basis for active packaging design or addition directly to food to provide protection against spoilage.

The new method was then applied to the characterisation of synergistic effects. In the literature review, researchers identified synergistic effects where the use of multiple antimicrobial agents can provide greater shelf life to lower overall concentrations. This has the potential to reduce the impact of the volatiles on the sensory qualities of foods.

In Chapter 6 an experiment was carried out to investigate the antifungal effect of binary combinations of carvacrol and thymol as antimicrobial aroma compounds. The efficacy of these combinations was compared to that of the compounds used alone. For the traditional

reverse, Petri dish methods applied to multiple volatiles, the release from the filter paper (isotherm), the saturated vapour pressure, the partition coefficient, the diffusivity of the different volatiles will all be different. It is likely that different dynamics for each volatile compound will occur. These phenomena may explain some apparent synergies even if no actual synergy exists. The new method developed in Chapter 5 was used for multi-component systems to achieve constant vapour phase of each volatile for the whole incubation period to investigate more accurately whether synergies occur.

The measurement methodology was successfully used to investigate synergistic effects for multiple volatile systems. It has the advantage that the volatile concentrations in the solid and gas phase are constant throughout the experiment. FIC values were calculated for different combinations of volatiles but it was difficult to apply the FIC data analysis from the literature directly. Problems existed in being able to state the time for the MIC definition such that the MIC values were known for the mixture and individual components alone. Instead, the FIC index was assessed for each volatile mixture by defining the MIC as inhibiting growth by the time observed. From the data for individual volatiles, it was possible to determine an FIC. These values were variable and ranged from 0.8 to 1, indicating a small degree of synergism.

An alternative data analysis approach was adopted by linearising the inhibition time observed for each sample and regressing this against the thymol and carvacrol concentrations. This resulted in a simple model with a significant thymol/carvacrol interaction term. This analysis more clearly demonstrated a synergistic effect, although the FIC values estimated from the model were between 0.89-0.96, suggesting the effect is minor. As a result, the mixture of thymol and carvacrol will be largely additive. Sensory tests would be required to determine if the halving of the concentration of each volatile and using them

together, would avoid changing taste perception less than using them individually. The regression-based analysis method has the advantage that all data can be included in the analysis and it provides a tool that is not dependent on arbitrarily selecting a time frame on which to base the definition of MIC.

Although the antimicrobial activity measurement and analysis methods developed in this work, offer many advantages over traditional methods, they have the disadvantage that they require the essential oil to be mixed directly with the agar broth before pouring. If solid foods are required to be tested as the food system, such mixing is not possible. As such, control of the concentration in the headspace and at the food surface must be carried out through the gas phase. Because the partitioning of the volatiles favours the solids phase, this is difficult.

The last stage of the research involved the preliminary design of a vapour generation system that can produce high vapour concentration at >95% RH. The system was based on two airstreams bubbled through the essential oil and the salt solution respectively before mixing them together. By doing this at high temperature and subsequently cooling the resulting mixture, a high RH, close to the saturated volatile gas stream can be produced. The design was successful, although some components of the system absorbed volatiles and, therefore, the dynamics of the system were initially slow until this had been equilibrated. The system offers promise and after some further development, it could be a useful tool for measurement of MIC for solid food systems.

This work has contributed a new understanding of the dynamics that occur in traditional MIC measurement systems and the development of new measurement methodologies that provide data more useful for active packaging design. However, there are a number of research areas that this work has identified that require further investigation;

1. In this research, only one temperature was used to investigate MIC for the volatile system used. Testing of the developed methods work at other temperature is essential.
2. In this work, the bactericidal effect of carvacrol and thymol was not investigated. Application of the new method to define MIC and explore synergistic effects of these compounds to bacterial (as opposed to fungal) systems is recommended.
3. Similarly, the new methods presented in this work should be applied to other volatile and growth media systems.
4. The single volatile generator developed in this work is promising but must be improved and extended to multi volatiles generator system. One of the key issues to solve is the elimination of materials in the system that absorb volatiles and slow the dynamics of the system. The operation of the system could be optimised to produce a constant and well-controlled headspace environment for MIC measurement in solid food systems.
5. Finally, a fundamental mathematical model based approach for the design of multi-volatile release active packaging systems should be developed. In this work, carvacrol and thymol vapour pressures were low at 25 °C, 3.5 Pa and 2.5 Pa, respectively. A previous study on a single volatile, hexanal (1300 Pa) by Utto (2008) had considerably higher volatility. This contributed to complications in the design of the system. It is recommended to use medium volatility natural volatiles such as 2-nonanone (193 Pa), limonene (192 Pa) or ethyl hexanoate (240 Pa) to mathematically model the controlled release of multiple volatiles. A validated model based design system will allow new packaging systems to be

designed and screened prior to sample testing. This would reduce the use of trial and error design that are time consuming and costly. This capability is important for the rapid design of release systems for volatiles in order to control the growth of microorganisms on food products.

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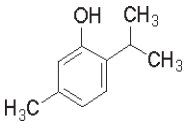
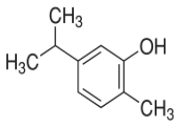
List of Appendices

Appendix 1: Chemical and physicochemical properties of active compounds

	Thymol	Carvacrol
Brand	Sigma - Aldrich	SAFC
CAS No	89-83-8	499-75-2
Assay Purity (%)	> 99	>98%
Melting Point (°C)	49 – 51	3 - 4
Flash Point (°C)	110	106
Boiling Point (°C) at 1 atm	232	236 - 237
Formula Weight	$C_{10}H_{14}0$	$C_{10}H_{14}0$
Molecular Weight	150.22	150.22
Density (g/mL)	0.965 (at 25°C)	0.976 (at 25°C)
Form at 20°C	Crystal powder	Liquid
Molecular Structure		

Appendix 2: Example of Chromatogram analysis of antimicrobial vapour (carvacrol) concentration using GCMS.

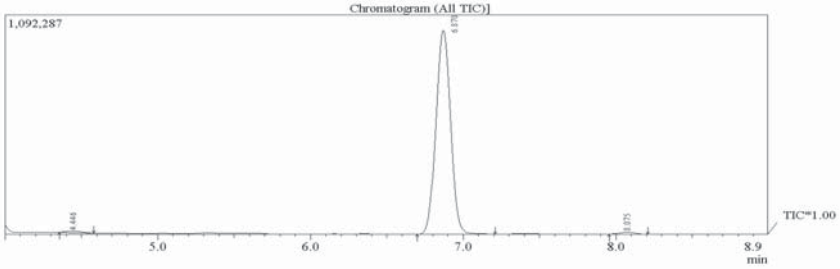
3/11/2009 14:39:49

C:\mazessential oil\031109 carvacrol pure static b.QGD

Sample Information

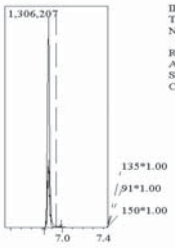
Analyzed by	: Admin
Analyzed	: 3/11/2009 2:28:22 p.m.
Sample Type	: Unknown
Level #	: 1
Sample Name	: 031109 carvacrol pure static b
Sample ID	: 031109 carvacrol pure static b
IS Amount	: [1]=1.000
Sample Amount	: 1.000
Dilution Factor	: 1.000
Vial #	: 1
Injection Volume	: 500.000
Data File	: C:\mazessential oil\031109 carvacrol pure static b.QGD
Org Data File	: C:\mazessential oil\031109 carvacrol pure static b.QGD
Method File	: C:\mazessential oil\211009 carvacrol standard.qgm
Org Method File	: C:\mazessential oil\301009carvacrol standard.qgm
Report File	:
Tuning File	: C:\GCMSsolution\System\Tune1\Zebron-ZB5 02-09-09.qgt
Modified by	: Admin
Modified	: 3/11/2009 2:37:22 p.m.

Chromatogram (All TIC)]



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	4.446	4.358	4.583	45433	0.66	7017	0.69	6.47		
2	6.870	6.700	7.208	6762340	98.32	1003924	98.28	6.73		Carvacrol
3	8.075	7.958	8.208	70464	1.02	10549	1.03	6.67		
				6878237	100.00	1021490	100.00			

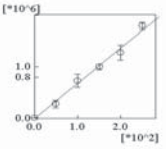


Quantitation
ID#:1 Mass:135.00
Type:Target
Name:Carvacrol

R.T.:6.868
Area:1764146
SI:93
Conc.:258.4ppm

#	m/z	Intensity	Ratio
1	91.00	278560	29.00
2	150.00	304250	31.68

Calibration
ID#:1 Mass:135.00 Name:Carvacrol
f(x)=6827.824000*x+0.000000
rr1=0.995013 rr2=0.990050
MeanRF:5517.94 RFSD:2769.01 RFRSD:50.18
CurveType:Least Square Method
ZeroThrough:Through
WeightedRegression:None
External Standard



#	Conc. (ppm)	Mean Area
1	0.000	10360.00
2	50.000	281452.00
3	100.000	730094.00
4	150.000	997840.00
5	200.000	1273489.00
6	250.000	1789480.00

1 / 2

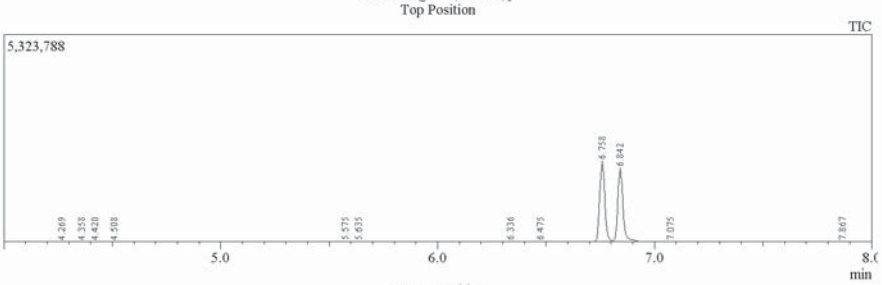
Appendix 3: Example of Chromatogram analysis of antimicrobial vapours (carvacrol and thymol) concentration using GCMS.

2/11/2010 15:55:48

Sample Information

Analyzed by	: Admin
Analyzed	: 2/11/2010 3:46:22 p.m.
Sample Type	: Unknown
Level #	: 1
Sample Name	: 021110 PR0.C0.1c
Sample ID	: 021110 PR0.C0.1c
IS Amount	: [1]=1.000
Sample Amount	: 1.000
Dilution Factor	: 1.000
Vial #	: 1
Injection Volume	: 500.000
Data File	: C:\mazessential oil\021110 PR0.C0.1c.QGD
Org Data File	: C:\mazessential oil\021110 PR0.C0.1c.QGD
Method File	: C:\mazessential oil\021110CTEO.qgm
Org Method File	: C:\mazessential oil\021110CTEO.qgm
Report File	:
Tuning File	: C:\GCMSsolution\System\Tune1\291010maz.qgt
Modified by	: Admin
Modified	: 2/11/2010 3:54:23 p.m.

Chromatogram (All TIC)]
Top Position



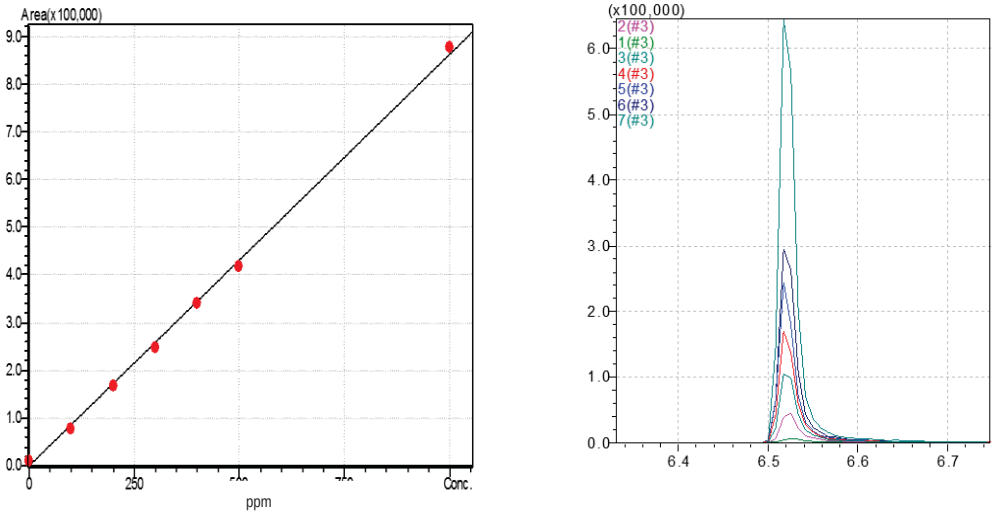
Bottom Position

Picture TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	4.269	4.075	4.333	29203	0.43	3256	0.08	8.96		
2	4.358	4.342	4.383	3553	0.05	1905	0.05	1.86		
3	4.420	4.383	4.492	21919	0.33	4503	0.11	4.86	V	
4	4.508	4.492	4.525	892	0.01	853	0.02	1.04	V	
5	5.575	5.525	5.608	15572	0.23	4391	0.11	3.54		
6	5.635	5.608	5.717	24995	0.37	11955	0.30	2.09	V	
7	6.336	6.200	6.458	23300	0.35	2799	0.07	8.32		
8	6.475	6.458	6.700	10156	0.15	1385	0.03	7.33	V	
9	6.758	6.700	6.808	3323339	49.38	2081390	51.85	1.59	V	Thymol
10	6.842	6.808	6.992	3259427	48.43	1898858	47.30	1.71	V	Carvacrol
11	7.075	6.992	7.142	16028	0.24	2194	0.05	7.30	V	
12	7.867	7.817	7.883	1879	0.03	815	0.02	2.30		
				6730263	100.00	4014304	100.00			

1 / 2

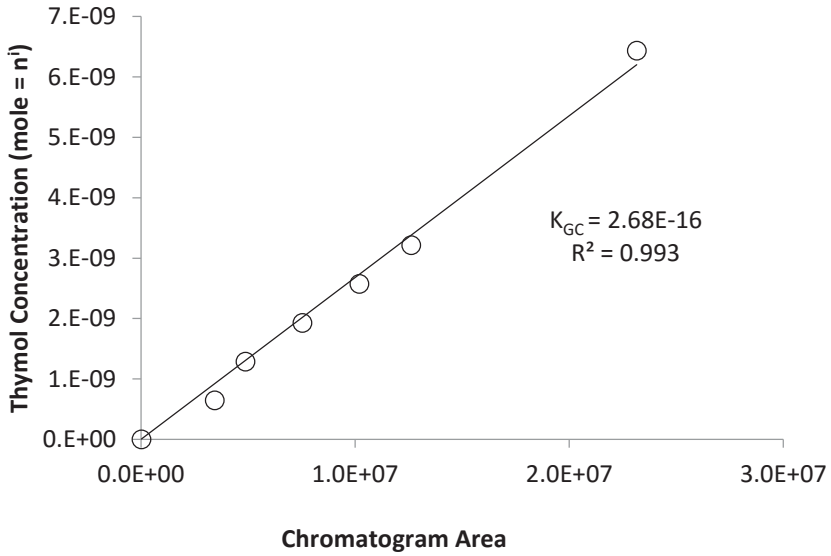
Appendix 4



(a) (b)

Appendix 4: (a) Example of thymol calibration curves plotted by Shimadzu GCMS-QP2010. Correlation coefficient (R) = 0.999
(b) The constant retention time was obtained at different concentration of thymol showing the stability and repeatability of chromatograms.

Appendix 5: An example thymol standard curve and the calculation of thymol vapour concentration.



Volatile Concentration (mol/m^3) was calculated using Eq.

$$C^i = \frac{K_{GC}^i A_{GC}^i}{Vol_{inj}}$$

Where

C^i	=	Concentration of Volatile Organic Compound (VOC) *i*, (mol.m^{-3})
K_{GC}^i	=	Detector response or slope (mol. Area $^{-1}$) of standard curve of Volatile, *i*. (thymol)
A_{GC}^i	=	Area of gas chromatogram peak from the injected volume of Sample (Area).
Vol_{inj}	=	Injection volume of sample (m^3)