Study of Antimicrobial Polymeric Packaging Films Containing Basil Extracts

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Study of Antimicrobial Polymeric Packaging Films Containing Basil Extracts

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Melbourne, Australia

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DEDICATION

To

Boonma Suppakul

Kanya (Khotavivattana) Limtung

and

Ku Heng Sai

and

In loving memory of

Kai Hui Khotavivattana

Veerawat Suppakul

and

Veerapong Suppakul

ABSTRACT

The quality and safety of foods may deteriorate due to the surface growth of microorganisms. The integration of antimicrobial (AM) agents in polymeric packaging films to enhance microbial stability of foods may have a crucial effect on extending the shelf life of packaged food products or on maintaining food safety. Linalool and methylchavicol are the principal constituents of basil and exhibit an AM effect against a wide spectrum of bacteria, yeasts and mould. These compounds are generally recognized as safe (i.e. possess GRAS status) and are stable at the high temperatures that prevail during the extrusion film blowing process. Therefore, films containing these substances might have a potential use as AM packages.

The present study generally aimed at investigating AM polymeric packaging films containing the principal constituents of basil. This thesis first reports the results of a study in which linear low-density polyethylene (LLDPE) films containing either linalool or methylchavicol as AM additives, were prepared by absorption, coating or extrusion film blowing. All films showed a significantly positive AM activity against *Escherichia coli* in solid media. The microencapsulation of AM additive in β-cyclodextrin (β-CD) was investigated because of a considerable loss of active agent *via* evaporation during the extrusion film blowing process. However, due to the high cost of β-CD and the low inclusion efficiency of β-CD for linalool and methylchavicol encapsulation,

it was concluded that it may not be economical to prepare concentrates of linalool and methylchavicol in β-CD to be used in AM films.

The method of bacterial enumeration by optical density (OD) measurement was initially applied in the determination of the AM efficacy in liquid media and was critically assessed. It was found that this method is insufficiently sensitive due to the required minimum inoculum size of *E. coli* (around 5×10^5 cfu mL^{-1}).

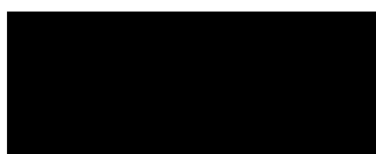
In the second stage of the research, films containing linalool or methylchavicol were prepared by the extrusion of a blend made of low-density polyethylene (LDPE) and ethylene vinyl acetate (EVA) and the AM effectiveness of these films was tested. All films showed a significantly positive AM activity against *E. coli* in both solid and liquid media. These films were characterised in regards to barrier, optical, and thermal properties. A significant decrease in the water vapour and oxygen transmission rates of the AM-LDPE-EVA films compared to the control film was observed. There was a non-significant difference in the degree of crystallinity between the AM-LDPE-EVA films and the control. The study of the release of AM additives from the films into iso-octane as a fatty food simulant showed that the kinetics of linalool and methylchavicol release fitted better a time-response function using Hill coefficient than a Fickian diffusion model. In film storage tests, the AM-LLDPE films showed AM activity against *E. coli* even after 1 year of storage at ambient conditions.

LDPE-EVA films containing linalool or methylchavicol were applied to wrap samples of Cheddar cheese. Linalool-LDPE-EVA and methylchavicol-LDPE-EVA films decreased *E. coli* populations by a factor of 28 and 47, respectively and *Listeria innocua* populations by a factor of 2.7 and 3.4 after 35 days of storage at 4 °C. In addition, sensory panelists did not perceive a difference in flavour between Cheddar cheese wrapped in linalool-LDPE-EVA film and in the control film throughout the storage period of 6 weeks at 4 °C. These studies highlight the encouraging potential use of polymeric films containing the principal constituents of basil as AM films for enhancing quality and safety of packaged foods.

DECLARATION OF ORIGINALITY

I, Panuwat Suppakul, hereby declare that the work presented in this thesis is my own and has been undertaken through the Packaging and Polymer Research Unit (formerly the Centre for Packaging, Transportation and Storage) at the School of Molecular Sciences, Victoria University. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of diploma at a university or another institution of higher learning, except where due acknowledgement is made in the text.

Signed

.....

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Date

22/09/04.................................

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"The search for truth is in one way hard and in another easy. For it is evident that no one can master it fully nor miss it wholly. But, each adds a little to our knowledge of nature and from all the facts assembled, there arises a certain grandeur."

~Aristotle

PREFACE

This thesis presents the study of antimicrobial polymeric packaging films containing basil extracts. The work was conducted at the Packaging and Polymer Research Unit (formerly the Centre for Packaging, Transportation and Storage) at the School of Molecular Sciences, Victoria University, Melbourne, Victoria, Australia, during the period July 2000 to February 2004.

Part of the work presented has been disseminated by various publications and conference presentations. A complete list is presented in **Appendix F**. Additionally, a number of further publications is in preparation. They will deal with the subject of "Characterisation of antimicrobial polyethylene films containing basil extracts"; "Release of linalool and methylchavicol from antimicrobial polyethylene films into a food simulant"; "Efficacy of antimicrobial polyethylene films containing basil extracts" and "Loss of antimicrobial additives from polyethylene films containing basil extracts".

This work has also been attracted media interest resulting in various references in magazines, trade publications, newspapers, internet sites, a selection of which is listed below:

Biever, C. Herb extracts wrap up lethal food bugs. *New Scientist* **June 14, 2003**, *178*, 26.

Molnar, M. Wrap mine in basil, please. [On-line]. Available:

http://www.ats.org/news.php?id=67. 2003, Accessed June, 2003.

Tucker, L. Common herb used to overpower bugs. *Werribee Banner* **July 16, 2003**, 14.

Svenson, N. Herbal remedy on way for food poisoning. *The Sun-Herald* **July 20, 2003**, 29.

Angebrandt, A. M. Humble herb a new food essential. *The Star* **July 22, 2003**, 2.

Anonymous. Basil packaging film improves product shelf life. *Microbial Update International* **August, 2003**, *9*, 2.

Cole, B. Basil packaging improves shelf life. *SAAFoST SNIPPETS* **September, 2003**, *8*, 5.

TABLE OF CONTENTS

Dedication....................i

Abstract....................ii

Declaration of originality....................v

Acknowledgements....................vi

Preface....................xi

Table of contents....................xiii

List of figures....................xx

List of tables....................xxiv

Abbreviations....................xxvii

Nomenclature....................xxxii

1 Introduction....................1

1.1 Background to the research....................1

1.2 Aims of the research....................5

1.3 Scope of work....................6

1.4 Outline of the thesis....................7

2 Literature review....................10

2.1 AP technologies with an emphasis on AM and its applications....................10

2.1.1 Oxygen scavenging systems....................11

2.1.2 Moisture-absorbing and controlling systems....................17

2.1.2.1 Water absorption....................17

2.1.2.2 Humidity buffering....................18

2.1.3 Carbon dioxide generating systems....................19

2.1.4 Ethanol generating systems....................21

2.1.5 AM migrating and non-migrating systems....................23

2.1.5.1 Gas emission or flushing....32

2.1.5.2 Coating of films with AM additives....34

2.1.5.3 Incorporation of AM additives....37

2.1.5.4 Immobilisation....45

2.1.5.5 Surface modification....46

2.1.6 The future for AM packaging....48

2.2 Factors to consider in the manufacturing of AM films....49

2.2.1 Process conditions and residual AM activity....50

2.2.2 Characteristics of AM additives and foods....50

2.2.3 Chemical interaction of AM additives with film matrix....51

2.2.4 Storage temperature....52

2.2.5 Mass transfer coefficients and modeling....52

2.2.6 Physical properties of packaging materials....52

2.2.7 Cost....53

2.2.8 Food contact approval....54

2.3 AM activity of basil and its application in food packaging....57

2.3.1 Composition of basil extracts....60

2.3.1.1 Cultivar and chemotaxonomic classification....60

2.3.1.2 Compositional variation....62

2.3.1.3 Enantiomeric considerations....70

2.3.1.4 Other factors affecting composition....72

2.3.2 Testing methods of the AM activity of essential oils....74

2.3.2.1 Principal methods....74

2.3.2.2 Limitation of the test methods....75

2.3.3 Results for the AM activity of basil extracts....76

2.3.3.1 Studies involving diffusion methods....77

2.3.3.2 Studies involving agar and broth dilution methods............87
2.3.3.3 Studies involving the micro-atmosphere method..............94
2.3.4 Food preservation...95
2.3.5 The future developments in food preservation..........................98
2.4 Microencapsulation of AM additives..98
2.5 Estimation of population size of microorganism.............................103
2.6 Release of AM additives...104
2.7 Microorganisms in foods...105
2.8 Cheddar cheese...108
2.9 Food contamination from packaging material.................................114
3 Materials and methods..116
3.1 Materials..116
3.1.1 Polymers..116
3.1.2 AM additives...116
3.1.3 Chemicals...116
3.1.4 Media..117
3.1.5 Count plates..117
3.1.6 Microorganisms..117
3.2 Methods...118
3.2.1 Preliminary study of AM films..118
3.2.1.1 Preparation of AM LLDPE films.......................................118
3.2.1.2 Additive quantification in LLDPE film..............................119
3.2.1.3 AM activity of LLDPE films in solid media........................120
3.2.1.4 Data analysis...121
3.2.2 Microencapsulation of AM additives.......................................122
3.2.2.1 Precipitation method...122

3.2.2.2 Moisture determination..123

3.2.2.3 Additive quantification in β-CD complex.....................123

3.2.3 Sensitivity enhancement of OD measurement.........................123

3.2.3.1 Design of the spectrophotometer..............................123

3.2.3.2 Microorganism and medium.....................................124

3.2.3.3 Growth experiments...124

3.2.4 Study of AM films...125

3.2.4.1 Preparation of AM LDPE-EVA films...........................125

3.2.4.2 Additive quantification in LDPE-EVA film.................125

3.2.4.3 AM activity of LDPE-EVA films in solid media............126

3.2.4.4 AM activity of LDPE-EVA films and additives in liquid media..127

3.2.4.5 Data analysis...128

3.2.5 Characterisation of AM films..129

3.2.5.1 Water vapour transmission rate measurement................129

3.2.5.2 Oxygen transmission rate measurement......................129

3.2.5.3 Ultraviolet-visible spectroscopy.............................129

3.2.5.4 Infrared spectroscopy...129

3.2.5.5 Differential scanning calorimetry............................130

3.2.5.6 Thermogravimetric analysis...................................130

3.2.5.7 Scanning electron microscopy.................................131

3.2.5.8 Data analysis...131

3.2.6 Release of AM additive from AM films into a food simulant........132

3.2.6.1 Film thickness measurement...................................132

3.2.6.2 Release experiments...132

3.2.6.3 Additive quantification in aliquot...........................132

3.2.6.4 Data analysis..133

3.2.7 Storage of AM films..134

3.2.7.1 Long-term storage of AM LLDPE films......................134

3.2.7.2 AM activity of LLDPE films after long-term storage.......135

3.2.7.3 Accelerated storage of AM LDPE-EVA films...............136

3.2.7.4 Additive quantification in AM film............................136

3.2.7.5 Data analysis..136

3.2.8 Application of AM films on Cheddar cheese.........................137

3.2.8.1 Inhibition of microbial growth on cheese using AM films..137

3.2.8.2 Inhibition of bacterial growth on cheese using AM films...138

3.2.8.3 Sensory analysis of cheese wrapping with AM films........140

4 Results and discussion..142

4.1 Preliminary study of AM films..142

4.1.1 Concentration of AM additive in LLDPE film........................142

4.1.2 AM activity of LLDPE films in solid media..........................144

4.2 Microencapsulation of AM additive..150

4.2.1 Complexation..150

4.2.2 Concentration and additive retention in β-CD complex..............152

4.3 Sensitivity enhancement of OD measurement...............................153

4.3.1 Bacterial growth..153

4.3.2 Correlation curve between viable cell count and OD.................155

4.4 Study of AM films..155

4.4.1 Concentration of AM additive in LDPE-EVA film...................155

4.4.2 AM activity of LDPE-EVA films in solid media......................157

4.4.3 AM activity of LDPE-EVA films and additives in liquid media....161

4.5 Characterisation of AM films..167

4.5.1 Water vapour and oxygen transmission rates....167
4.5.2 Transparency....168
4.5.3 IR spectra as polymer 'fingerprint'....169
4.5.4 Carbonyl group detection....170
4.5.5 Degree of crystallinity and melting temperature....174
4.5.6 TGA mass-loss and peak temperature....177
4.5.7 Electron micrograph....181
4.6 Release of AM additive from films into a food simulant....181
4.6.1 Kinetics of additive released from LDPE-EVA films....181
4.6.2 Effect of temperature on migration....184
4.7 Storage of AM films....191
4.7.1 Residual concentration and AM activity after long-term storage....191
4.7.2 Effect of temperature on additive retention after accelerated storage....191
4.8 Application of AM films on Cheddar cheese....197
4.8.1 Microbial growth....197
4.8.2 Bacterial growth....200
4.8.3 Sensory evaluation....206
5 Conclusions and recommendations....209
5.1 Conclusions....209
5.2 Significance of the findings....212
5.3 Recommendations for further research....212
Bibliography....215
Appendix A-Typical properties of polymers....258
Appendix B-Description and typical properties of linalool and methylchavicol....259

Appendix C-Standard curves of linalool and methylchavicol.....................260

Appendix D-IR spectra of linalool and methylchavicol.............................261

Appendix E-Scorecard for the triangle test..262

Appendix F-List of publications..263

Biography...265

LIST OF FIGURES

Figure 2.1 Number of articles related to AM packaging in journal publications....24

Figure 2.2 Chemical structures of major constituents found in *O. basilicum* L. 65

Figure 2.3 Computerised 3-D structure of cyclodextrins....99

Figure 2.4 Molecular structure of β-cyclodextrin....101

Figure 4.1 Distribution of AM additive in various film matrices....143

Figure 4.2 Growth inhibition of *E. coli* by LLDPE film containing 0.05% w/w linalool or methylchavicol....146

Figure 4.3 Growth curves of *E. coli* using turbidimetry at different wavelengths....154

Figure 4.4 Growth curve of *E. coli* using viable cell count....154

Figure 4.5 Relationship between viable cell count and optical density at 470 nm....156

Figure 4.6 Growth inhibition of *E. coli* by LDPE-EVA film containing 0.34% w/w linalool or methylchavicol....158

Figure 4.7 Growth inhibition of *E. coli* by LDPE-EVA film containing linalool or methylchavicol with different film cutting....158

Figure 4.8 Normalised inhibitory of AM films against *E. coli*....160

Figure 4.9 Growth curve of *E. coli* after addition of pieces of film containing 0.34% w/w linalool or methylchavicol at different ratios of film area/medium volume......163

Figure 4.10 Growth curve of *E. coli* after addition of linalool or methylchavicol at particular concentration (mg mL^{-1})......165

Figure 4.11 IR spectra of AM LDPE-EVA films......170

Figure 4.12 IR spectra of AM LLDPE films......171

Figure 4.13 Changes in carbonyl index corresponding to ester, aldehyde, ketone and acid with CH_2 scissoring peak for AM LLDPE films......172

Figure 4.14 DSC thermograms of AM LDPE-EVA films......176

Figure 4.15 TGA mass-loss curves of AM LDPE-EVA films......178

Figure 4.16 First derivative TGA mass-loss curves of AM LDPE-EVA films..179

Figure 4.17 SEM micrographs of the edge surfaces for AM LDPE-EVA films......180

Figure 4.18 Migration curves of linalool and methylchavicol from LDPE-EVA films into iso-octane at different temperatures......182

Figure 4.19 Migration curves of linalool and methylchavicol from LDPE-EVA films into iso-octane at 4 °C......183

Figure 4.20 Ward clustering of the rate constant at different temperatures......187

Figure 4.21 Arrhenius plots of linalool and methylchavicol incorporated in LDPE-EVA films....188

Figure 4.22 Growth inhibition of *E. coli* by LLDPE film containing linalool or methylchavicol after 1 year of storage....192

Figure 4.23 Decrease in additive retention of AM LDPE-EVA films during storage at 25 and 35 °C....194

Figure 4.24 Plot of ln(C) versus time as first order decay in additive retention of AM LDPE-EVA films during storage at 25 and 35 °C....194

Figure 4.25 Plot of $\ln(C-C_{\infty})$ versus time as first order decay with offset in additive retention of AM LDPE-EVA films during storage at 25 and 35 °C....195

Figure 4.26 Inhibition of total aerobic mesophilic bacteria on Cheddar cheese with AM LPDE-EVA films refrigerated at 4 °C....198

Figure 4.27 Inhibition of *E. coli* and *L. innocua* on Cheddar cheese with AM LDPE-EVA films refrigerated at 4 °C....201

Figure 4.28 Inhibition of *E. coli* and *L. innocua* on Cheddar cheese with AM LDPE-EVA films subjected to temperature abuse condition of 12 °C....202

Figure A.1 Standard curve of linalool quantification by gas chromatography...260

Figure A.2 Standard curve of methylchavicol quantification by

gas chromatography..260

Figure A.3 IR spectrum of linalool..261

Figure A.4 IR spectrum of methylchavicol..261

LIST OF TABLES

Table 2.1 Commercial oxygen scavengers..14

Table 2.2 Commercial carbon dioxide generators..20

Table 2.3 Commercial ethanol generators..22

Table 2.4 Examples of AM additives for potential use in food packaging materials..26

Table 2.5 Trade names and manufacturers of commercial AM materials.........31

Table 2.6 List of permitted food additives that could be used as AM additives in packaging materials..55

Table 2.7 Current and future applications of AM packaging........................58

Table 2.8 Chemotaxonomic classification of *O. basilicum* L. based on geographical origins..61

Table 2.9 Chemotaxonomic classification of *O. basilicum* L. based on the USDA germplasm collection..63

Table 2.10 Major constituents of *O. basilicum* L. 64

Table 2.11 Compositional variation in *O. basilicum* L. 66

Table 2.12 Comparative evaluation of North American commercially available *O. basilicum* L. cultivars..71

Table 2.13 Range of concentrations of basil extracts reported to inhibit the growth of microorganisms..78

Table 2.14 Range of concentrations of the principal constituents of basil extracts reported to inhibit the growth of microorganisms........................79

Table 2.15 Physical properties of β-cyclodextrin....................................99

Table 3.1 Experimental-conditions used for the agar disc diffusion assay on AM films in the preliminary study..121

Table 3.2 Experimental-conditions used for the agar disc diffusion assay on AM films in the study..126

Table 4.1 AM activity of LLDPE films against *E. coli* as observed by agar disc diffusion assay...145

Table 4.2 AM activity of PE films incorporated with basil extract in comparison with some other AM films...148

Table 4.3 Recovery of linalool and methylchavicol complexes..................151

Table 4.4 Complexation on co-crystallisation of β-cyclodextrin and the recovery of inclusion compound..151

Table 4.5 Actual concentration and retention of volatile content in complexes..153

Table 4.6 AM activity of LDPE-EVA films against *E. coli* as observed by agar disc diffusion assay..157

Table 4.7 AM activity of LDPE-EVA films on the maximum specific growth rate of *E. coli* in liquid medium...163

Table 4.8 AM activity of linalool and methylchavicol on the maximum specific growth rate of *E. coli* in liquid medium....................................165

Table 4.9 Barrier and optical properties of AM LDPE-EVA films...............167

Table 4.10 Thermal properties and polymer morphology of AM LDPE-EVA films..174

Table 4.11 Effect of temperature on the migration of linalool and methylchavicol from LDPE-EVA films into iso-octane.................................185

Table 4.12 Activation energies of linalool and methylchavicol incorporated in LDPE-EVA films...189

Table 4.13 AM activity of LLDPE films against *E. coli* as observed by agar disc diffusion assay after long-term storage.................................192

Table 4.14 Effect of temperature on the loss of AM additive in LDPE-EVA films...196

Table 4.15 Triangle test results of Cheddar cheese wrapped with linalool-LDPE-EVA film refrigerated at 4°C..207

Table 4.16 Triangle test results of Cheddar cheese wrapped with methylchavicol-LDPE-EVA film refrigerated at 4°C..................................207

Table A.1 Typical properties of polymers used in this research.................258

Table A.2 Description and typical properties of linalool and methylchavicol..259

ABBREVIATIONS

AIT	Allyl isothiocyanate
AM	Antimicrobial
ANOVA	Analysis of variance
ANZFA	Australian and New Zealand Food Authority
AP	Active Packaging
β-CD	beta-Cyclodextrin
β-CD-IC	beta-Cyclodextrin inclusion compound
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BMC	Basil methylchavicol
BSL	Basil sweet linalool
CDs	Cyclodextrins
CDT	*Escherichia coli* producing cytolethal-distending toxin
CFU	Colony forming unit
COS	Chito-oligosaccharide
CTA	Cellulose triacetate
DAEC	Diffuse-adhering *Escherichia coli*
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimetry

EAEC	Eenteroaggregative *Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
ERH	Equilibrium relative humidity
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Entertoxigenic *Escherichia coli*
ETP	Ethyl paraben
EVA	Ethylene vinyl acetate
EVOH	Ethylene vinyl alcohol
FDA	Food and Drug Administration
FSA	Food Science Australia
FTIR	Fourier transform infrared
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GFSE	Grapefruit seed extract
GRAS	Generally recognised as safe
HACCP	Hazard Analysis Critical Control Points
HDPE	High density polyethylene
HMT	Hexamethylenetetramine
HPMC	Hydroxypropyl methylcellulose

HUS	Haemorrhagic uremic syndrome
IC	Inclusion compound
IMF	Intermediate moisture foods
IR	Infrared
LAB	Lactic acid bacteria
LDPE	Low density polyethylene
LLDPE	Linear low density polyethylene
MAP	Modified atmosphere packaging
MC	Methylcellulose
MIC	Minimum inhibitory concentration
MIC	Minimum inhibitory concentration
MLC	Minimum lethal concentration
MRS	Man Rogosa Sharpe
NMA	*N*-methylol acrylamide
NSLAB	Nonstarter lactic acid bacteria
NTEC	Necrotoxigennic *Escherichia coli*
OD	Optical density
OSP	Oxygen scavenging packet
OTR	Oxygen transmission rate
PAA	Poly(acrylic acid)
PABA	*p*-aminobenzoic acid

PCL	Poly(ε-caprolactone)
PDA	Potato dextrose agar
PE	Polyethylene
PEG	Polyethylene glycol
PEMA	Polyethylene-co-methacrylic acid
PLLA	Poly(*L*-lactic acid)
PPIX-ED	Ethylene diamine derivative of protoporphyrin
PRP	Propyl paraben
PVA	Polyvinylacetate
PVC	Polyvinylchloride
PVDC	Polyvinylidenechloride
RGR	Reduction in growth rate
SEM	Scanning electron microscopy
SM	Salt-in-moisture
SPPS	Solid-phase peptide synthesis
STEC	Shiga toxigenic *Escherichia coli*
TBHQ	Tertiary-butyl-hydroquinone
TG	Thermogravimetric
TGA	Thermal gravimetric analysis
TIP	Triclosan-incorporated plastic
UOD	Unit of optical density

USDA	United States Department of Agriculture
UV	Ultra-violet
VA	Vinylacetate
VC	Viable cell
WPI	Whey protein isolate
WVTR	Water vapour transmission rate
XPS	X-ray photoelectron spectroscopy
YM	Yeast-malt extract
YMPG	Yeast-malt extract peptone glucose
Zn-PPIX-ED	Ethylene diamine derivative of zince protoporphyrin

NOMENCLATURE

T	Absolute temperature
A	Absorbance
E_a	Activation energy
C	Additive retention
M_∞	Amount of additive released from film after an infinite time
M_t	Amount of additive released from film at time t
r^2	Coefficient of determination
c	Concentration
χ	Crystallinity
ΔA	Difference between absorbance
D	Diffusion coefficient
t_d	Diffusion time
L	Film thickness
$\theta_{1/2}$	Half-life
ΔH_f	Heat of fusion
n	Hill coefficient
C_∞	Infinite additive concentration
X_0	Initial bacterial concentration

I_0	Intensity of incident light
I	Intensity of transmitted light
λ	Lag time
X_{max}	Maximum bacterial concentration
μ_{max}	Maximum specific growth rate
T_m	Melting point
ε	Molar absorptivity or molar extinction coefficient
l	Path length
k	Rate constant
Q_{10}	Sensitivity of film deterioration
t	Time
T	Transmittance
T_{600}	Transmittance of light at 600 nm
R	Universal gas constant
a_w	Water activity

1 INTRODUCTION

1.1 Background to the research

Articles made from polymers are ubiquitous in modern life. Microorganisms can grow on many of their surfaces causing displeasing or harmful results. Different methods have been developed to incorporate antimicrobial (AM) substances in polymeric materials. These additives are slowly released onto the product and interfere or suppress the growth of a broad spectrum of microbes such as bacteria, moulds and yeasts. For instance, Mildewcide was added to an extruder during the film fabrication stage in manufacturing of household products such as shower curtains to prevent mildew growth (Paik et al. 1998). The range of AM products in the form of films, fibres and moulded items has developed significantly in recent years. These products comprise, among others, chopping boards, kitchen wear, hosiery, carpets, hospital waste bags and refuse containers (Simmons 2000).

In most foods, surface growth of microorganisms is the major cause of food spoilage (Gill 1979; Maxcy 1981). Antimicrobial agents are often mixed directly into the foods to control microbial growth and to extend shelf life (Weng and Hotchkiss 1993). Nevertheless, direct application of AM additives in foods was found to be of limited benefit because the substances were neutralized or diffused out rapidly from the bulk of the food (Torres et al. 1985a; Siragusa and Dickson 1992). Food may also be treated with AM additives by washing (Dickson 1988), dipping (Fallik and Grinberg 1992), or spraying (Xiong et al.

1988). Antimicrobials have also been added to wax-based food coatings in cheeses, edible coatings for fruits and vegetables (Chen et al. 1999) and intermediate moisture foods (IMF) (Torres et al. 1985a, b; Torres and Karel 1985).

Most food spoilage reactions occur on the surface of the food. Antimicrobial substances added directly to food might lead to an overdose. Treatments like washing, dipping or spraying may be required for achieving an AM effect. For that purpose, AM compounds have to be dissolved in suitable solvents prior to food treatment.

In recent years, consumers have become more concerned about the many food additives (i.e., chemical preservatives) associated with the food chain. For this reason, naturally derived AM additives and biopreservatives are becoming more important since they are perceived by consumers as posing a lower risk (Nicholson 1998). Efforts are being made to discover new natural AM, and/or to reduce the amount of direct additives incorporated into the food by the development of AM films from which the preservative is slowly and continuously released onto the food surface. In this way, smaller quantities of AM agent are needed than when incorporated directly in the food (Yalpani et al. 1992). This is viewed to be a good approach for food preservation. Antimicrobial packages can also simplify the food manufacturing process by integrating packaging and AM activity in one step. Begin and Calsteren (1999) claimed that direct incorporation of preservatives into the film is an economical method for

the manufacturing of AM packaging. Following the packaging process, no additional treatment is required other than refrigeration. Refrigerated storage and incorporation of AM into packaging films, possibly in combination with gas or vacuum-packaging, could potentially provide enhanced microbial stability and safety of food products. The greatest potential for AM packaging in food applications include meat, fish, poultry, bread, cheese, fruits and vegetables (Labuza and Breene 1989). Although AM food packaging has been studied in the last two decades, no known commercial product has emerged as yet (Shearer et al. 2000).

As a result of the increasing demand for natural food additives, more extensive attempts are presently being made in the search for alternative AM compounds based on plant extracts. For example, the AM effect of essential oils and their active constituents against many food-borne pathogenic bacteria including *Campylobacter jejuni* (Friedman et al. 2002), *Escherichia coli* (Eloff 1999; Friedman et al. 2002), *Listeria monocytogenes* (Aureli et al. 1992; Friedman et al. 2002), *Salmonella enterica* (Friedman et al. 2002), *Salmonella typhimurium* (Juven et al. 1994; Mahrour et al. 1998), *Staphylococcus aureus* (Juven et al. 1994; Mahrour et al. 1998; Opalchenova and Obreshkova 2003), and *Vibrio parahaemolyticus* (Koga et al. 1999)have been studied. The AM effect of essential oils against various food spoilage bacteria have been reported in the literature. Examples are: meat spoilage bacteria like *Brochothrix thermosphacta*, *Carnobacterium piscicola*, *Pseudomonas fluorescens*, *Serratia liquefaciens* (Ouattara et al. 1997), seafood spoilage bacteria like *Photobacterium*

phosphoreum and *Pseudomonas putida* (Ouattara et al. 2001; Mejlholm and Dalgaard 2002), and vegetable spoilage bacteria like *Aeromonas hydrophila* and *P. fluorescens* (Wan et al. 1998). However, there are few published data in the scientific literature related to AM food packaging films incorporated with natural plant extracts. As no single AM agent can cover all the needs for food preservation, it is essential to investigate a range of such additives for their potential use in this technology.

Sweet basil (*Ocimum basilicum* L.) is a popular culinary herb that has been widely used as a food ingredient (Dziezak 1989). Additionally, basil essential oils have been reported to possess AM activity against a spectrum of Gram-positive, Gram-negative bacteria (Deans and Ritchie 1987), important food-borne pathogens (Fyfe et al. 1998) and moulds (Arora and Pandey 1977) as well as against yeasts (Conner and Beuchat 1984). It was also found that the AM activities of essential oils of different species of Ocimum were predominately related to the major components of linalool and methylchavicol (Sinha and Gulati 1990, Wan et al.1998).

The following questions and considerations should be taken into account when studying AM films:

1. What are the target microorganisms against which the film should be effective? Antimicrobial films should diminish food spoilage and/or pathogenic microflora.

2. What is the AM effectiveness in terms of the retained AM activity? The AM activity of an incorporated active substance may deteriorate during film fabrication, and/or storage of the packaging material.

3. What is the effect of the AM additives on the properties of the film? The AM compound may affect certain properties of the films such as mechanical and optical properties.

4. What is the diffusion behaviour of the AM additives in the polymeric material?

5. To what extent can the AM film maintain the microbiological quality and/or safety of food wrapped in that material?

1.2 Aims of the research

The general aim of this research was to investigate polymeric packaging films incorporated with naturally derived AM agents for their potential use in food applications.

The specific aims were:

1. To study the feasibility of producing AM films based on an incorporation of the - commercially produced - compounds linalool and methylchavicol, which are the principal constituents of basil extract.

2. To determine the inhibitory effect of such films against tested common microorganisms that are known to be associated with food spoilage and safety.

3. To investigate the release of the AM agent into a food product.

4. To evaluate the suitability of such films as a food packaging material in terms of its functional properties other then providing an AM effect.

5. To investigate the retention of the AM additives during film storage in regards to time and temperature.

6. To investigate the feasibility for using such films to inhibit surface growth of microorganisms in a packaged food.

1.3 Scope of Work

Antimicrobial packaging materials are of increasing interest as a potential means of safely preserving food products. Many compositions have been studied and the results reported in the literature. However, most of these systems concentrate on synthetic additives. The present work concentrates on the principal constituents of basil (incorporated in a polymeric packaging film). These naturally derived compounds have been shown to possess AM activities as is reviewed in Chapter 2. The aim of the present work was to study whether the principal constituents of basil could be incorporated into packaging films and to evaluate their inhibitory effects against selected microorganisms. Basil contains numerous components and it was decided to concentrate on the two main components, namely linalool and methylchavicol.

A standard commercial grade of low-density polyethylene (LDPE) was selected as the major component of the AM films to be manufactured. The effectiveness of the prepared AM films was evaluated by means of an agar

diffusion method and a growth experiment, performed in solid and liquid matter. One part of the work was devoted to studying the release of the active substance and to characterizing the prepared material, including the retention of the active substance during film storage. The other part concentrated on the evaluation of the effectiveness of the films as AM packaging for cheese. The selected microorganisms in the present research were *Staphylococcus aureus*, *Listeria innocua*, *Escherichia coli*, and *Saccharomyces cerevisiae*. Yellow Cheddar cheese was chosen as it is one of many products whose commercial market would benefit by this type of technology. Iso-octane was chosen as a non-polar solvent recommended as an alternative fatty food simulant in migration studies.

1.4 Outline of the thesis

This thesis contains five chapters, a bibliographical list and five appendices. The aim of Chapter 2 is to first review and summarise the area of "Active Packaging Technologies" and their characteristics. The focus of this chapter then turns specifically to Antimicrobial Packaging. Factors to be considered in the manufacturing of AM packaging materials and the future prospects of this technology are also discussed. Furthermore an extensive literature review on the topic of basil essential oils with regards to their chemical composition, their effect on microorganisms, the test methods for AM activity determination, and their possible use in food preservation, is presented. As an adjunct to this review, the subjects of the microencapsulation of AM additives and the estimation of population size of microorganism is also presented. Finally, topics involving Cheddar cheese, microorganisms in foods, contamination in food

from packaging materials and the release of AM additives onto food surfaces are reviewed in this chapter.

The experimental approach adopted for this research is presented in Chapter 3, which is composed of 6 sections describing the major track, and two sections on side-tracks of the study. It begins with a preliminary study including the preparation of AM LDPE films, produced by methods ranging from simple absorption to industrially extrusion film blowing. It also includes a description of the testing of these films for their AM activity in a solid medium.

The chapter continues with the description of a side-track study involving the microencapsulation of AM additives. This study was undertaken because of a manufacturing problem that had become apparent during the preliminary study.

A second side-track study relates to the methods of AM effect evaluation of LDPE-based AM films with solid and, especially, liquid media. Since these methods are labour-intensive, an indirect method for enumeration of populations of microorganisms was tried by using optical density (OD) measurements with sensitivity enhancement.

Returning to the major track of the study, the chapter then deals with the characterisation of the prepared AM LDPE-based films, the release of the AM additives from the films, and the retention of the AM additives during film

storage. In the final part the experimental program with regards to the application of the prepared AM films as a cheese-wrapper is outlined. These experiments aimed at testing the inhibition effect on microbial and bacterial growth on the surface of the cheese, as well as evaluating the sensory characteristics, primarily in regards to tainted flavour in the cheeese.

In Chapter 4 the findings of the particular experiments described in Chapter 3 are reported. The chapter presents discussions on the findings of each of the experiments including their interrelation and how they correspond to the findings of other studies reported in the literature.

In Chapter 5 the overall findings in regards to the research questions are summarised. Recommendations for future research are given. The chapter concludes with a discussion of the significance of the findings of the presented work.

2 LITERATURE REVIEW

2.1 AP technologies with an emphasis on AM and its applications

In recent years, the major driving forces for innovation in food packaging have been the increase in consumer demand for minimally processed foods, the change in retail and distribution practices associated with globalisation, new consumer product logistics, new distribution trends (e.g. internet shopping), automatic handling systems at distribution centers, and stricter requirements regarding consumer health and safety (Vermeiren et al. 1999; Sonneveld 2000). Active packaging (AP) technologies are being developed as a result of these driving forces. Active packaging is an innovative concept that can be defined as a mode of packaging in which the package, the product and the environment interact to prolong shelf life, and/or enhance safety and/or sensory properties, while maintaining the quality of the product. This is particularly important in the area of fresh and extended shelf life foods as originally described by Labuza and Breene (1989); and followed by Guilbert et al. (1996) and Rooney (1998).

Floros et al. (1997) reviewed the products and patents in the area of AP and identified antimicrobial (AM) packaging as one of the most promising versions of an AP system. Han (2000), Brody et al. (2001), Cooksey et al. (2001), Appendini and Hotchkiss (2002), Quintavalla and Vicini (2002), Vermeiren et al. (2002) and Suppakul et al. (2003a) recently published articles focused on AM systems with a detailed discussion of some of the principal AP concepts. The present literature review outlines the general principles of AP and AM packaging concepts including oxygen scavenging, moisture absorption and control, carbon

dioxide and ethanol generation, and it reviews in detail AM migrating and non-migrating systems as was summarised by Suppakul et al. (2003a).

2.1.1 Oxygen scavenging systems

The presence of O_2 in a packaged food is often a key factor in limiting the shelf life of food products. Oxidation can cause changes in flavour, colour, and odour, as well as destroy nutrients and facilitate the growth of aerobic bacteria, moulds and insects. Therefore, the removal of O_2 from the package headspace and from the solution, in liquid foods and beverages, has long been a target of food-packaging scientists. The deterioration in quality of O_2-sensitive products can be minimised by recourse to O_2 scavengers that remove the residual O_2 after packing. Existing O_2 scavenging technologies are based on oxidation of one or more of the following substances: iron powder, ascorbic acid, photo-sensitive dyes, enzymes (e.g. glucose oxidase and ethanol oxidase), unsaturated fatty acids (e.g. oleic, linoleic and linolenic acids), rice extract, or immobilised yeast on a solid substrate (Floros et al. 1997). These materials are normally contained in a sachet. Details on O_2 scavenging can be obtained from other reviews (Labuza and Breen 1989; Miltz et al. 1995; Miltz and Perry 2000; Floros et al. 1997; Vermeiren et al. 1999).

Oxygen scavenging is an effective way to prevent growth of aerobic bacteria and moulds in dairy and bakery products. Oxygen concentrations of 0.1% v/v or less in the headspace may be required for this purpose (Rooney 1995). Packaging of crusty rolls in a combination of CO_2 and N_2 (60% CO_2) has

shown to be an effective measure against mould growth for 16-18 days at ambient temperature. However, the study also revealed that such an "anaerobic environment" is not totally effective without the incorporation of an oxygen scavenger into the package to ensure that the headspace O_2 concentration never exceeds 0.05%. Under such conditions the rolls remain mould-free even after 60 days (Smith et al. 1986).

Many researchers have expressed concern about the safety of modified atmosphere packaged (MAP) foods, especially with respect to the growth of psychrotrophic pathogens such as *Listeria monocytogenes* and anaerobic pathogens such as *Clostridium botulinum* (Farber 1991). Lyver et al. (1998) monitored the physical, chemical, microbiological, textural and sensory changes in surimi nuggets inoculated with *L. monocytogenes*, packaged in either air or 100% CO_2 with and without an oxygen scavenger, and stored at 4 °C and 12 °C. They found that MAP was not effective in controlling the growth of the pathogen in either raw or cooked nuggets, and also that the pathogen overcame competitive inhibition and pH reduction caused by lactic acid bacteria. They concluded that nuggets packaged under these conditions and contaminated with this pathogen could pose a risk to the consumer. More importantly, it was found that the product retained acceptable odour and appearance scores at the above storage temperatures, even though the level of the pathogen increased over the storage period. The latter is indeed a cause for concern, since the contaminated product may appear safe from the sensory point of view (Lyver et al. 1998).

Oxygen scavenging is advantageous for products that are sensitive to O_2 and light. One important advantage of AP over MAP is that the capital investment involved is substantially lower; in some instances, only sealing of the system that contains the oxygen absorbing sachet is required. This is of extreme importance to small and medium-sized food companies for which the packaging equipment is often the most expensive item (Ahvenainen and Hurme 1997).

An alternative to sachets involves the incorporation of the O_2 scavenger into the packaging structure itself. This minimises negative consumer responses and offers a potential economic advantage through increased outputs. It also eliminates the risk of accidental rupture of the sachets and inadvertent consumption of their contents. A summary of available O_2 scavengers is given in **Table 2.1**.

Since the share of polymers in primary packages for foods and beverages increases constantly, they have become the medium for incorporation of active substances such as antioxidants, O_2 scavengers, flavour compounds, pigments, enzymes and AM agents (Hotchkiss 1997). BP Amoco Chemical (USA) is marketing Amosorb® 2000 and 3000, which are polymer-concentrates containing iron-based O_2 scavengers. These can be used in polyolefins and in certain polyester packaging applications for wines, beers, sauces, juices and other beverages. Other recent developments include OS2000™ developed by Cryovac Division, Sealed Air Corporation, USA (Butler 2002) and $ZERO_2$™ developed by Food Science Australia, a Division of CSIRO, Australia, in collaboration with Southcorp Packaging (now VisyPak), Australia (Brody et al. 2001). Both of the

Table 2.1 Commercial oxygen scavengers

Format	Trade Name	Manufacturer	References
Card	Ageless®	Mitsubishi Gas Chemical Co (Japan)	
Closure Liner	Darex®	Grace Performance Chemicals (USA)	Teumac (1995), Brody et al. (2001)
	PureSeal®	Advanced Oxygen Technologies Inc (USA)	Teumac (1995)
	Smartcap®	Advanced Oxygen Technologies Inc (USA)	Teumac (1995)
Concentrate	Amosorb®, 2000, 3000	BP Amoco Chemical (USA)	
	Oxbar™	Crown Cork and Seal (USA)	Brody et al. (2001)
	Oxyguard™	Toyo Seikan Kaisha (Japan)	
	Oxysorb®	Pillsbery Co (USA)	
Film	Bioka®	Bioka Ltd (Finland)	
	OS2000™	Sealed Air Corporation (USA)	Butler (2002)
	ZERO2™	CSIRO and VisyPak (Australia)	Brody et al. (2001)
Label	Ageless®	Mitsubishi Gas Chemical Co (Japan)	
	ATCO®	Standa Industrie (France)	
	FreshMax®	Multisorb Technologies Inc (USA)	

Table 2.1 (Continued)

Format	Trade Name	Manufacturer	References
Sachet	Ageless®	Mitsubishi Gas Chemical Co (Japan)	Nakamura and Hoshino (1983), Smith et al. (1995), Lyver et al. (1998)
	ATCO®	Standa Industrie (France)	Hurme and Ahvenainen (1996)
	Bioka®	Bioka Ltd (Finland)	Ahvenainen and Hurme (1997)
	Freshilizer®	Toppan Printing Co (Japan)	Smith et al. (1995)
	FreshPax®	Multisorb Technologies Inc (USA)	Smith et al. (1995)
	Keplon™	Keplon Co (Japan)	Brody et al. (2001)
	Modulan™	Nippon Kayaku Co (Japan)	Brody et al. (2001)
	Negamold®[1]	Freund Industrial Co (Japan)	Smith et al. (1995)
	Oxyeater™	Ueno Seiyaku Co (Japan)	Brody et al. (2001)
	Oxysorb®	Pillsbury Co (USA)	
	Sanso-cut®	Finetech Co (Japan)	Hurme and Ahvenainen (1996)
	Sansoless™	Hakuyo Co (Japan)	Brody et al. (2001)
	Secule®	Nippon Soda Co (Japan)	Brody et al. (2001)
	Sequl®	Dai Nippon Co (Japan)	Brody et al. (2001)
	Tamotsu™	Oji Kako Co (Japan)	Brody et al. (2001)
	Vitalon®[2]	Toagosei Chemical Co (Japan)	Hurme and Ahvenainen (1996)
Thermoformed Tray	Oxycap®	Standa Industrie (France)	

[1] Combined actions between O_2 scavenging and ethanol generation
[2] Combined actions between O_2 scavenging and CO_2 generation

latter are organic-based, UV light-activated O_2 scavengers that can be tailored to allow them to be bound into various layers of a wide range of packaging structures. Oxbar™ is a system developed by Carnaud-Metal Box (now Crown Cork and Seal) that involves cobalt-catalysed oxidation of a MXD6 nylon that is blended into another polymer. This system is used especially in the manufacturing of rigid PET bottles for packaging of wine, beer, flavoured alcoholic beverages and malt-based drinks (Brody et al. 2001).

Another O_2 scavenging technology involves utilising directly the closure lining. Darex® Container Products (now a unit of Grace Performance Chemicals) has announced using an ethylene vinyl alcohol (EVOH) with a proprietary oxygen scavenger developed in conjunction with Kararay Co. Ltd. In a dry form, pellets containing unsaturated hydrocarbon polymers with a cobalt catalyst are used as oxygen scavengers in mechanical closures, plastic and metal caps, and steel crowns (both PVC and non-PVC lined). These reportedly can prolong the shelf life of beer by 25% (Brody et al. 2001).

Oxygen scavengers have opened new horizons and opportunities in preserving the quality and extending the shelf life of foodstuffs. However, much more information is needed on the action of O_2 scavengers in different environments before optimal, safe and cost-effective packages can be designed. The need for such information is especially acute on O_2 scavenging films, labels, sheets and trays that have begun to appear in recent years (Miltz et al. 1995; Miltz and Perry 2000).

2.1.2 Moisture-absorbing and controlling systems

In solid foods, a certain amount of moisture may be trapped during packaging or may develop inside the package due to generation or permeation. Unless it is eliminated, it may form a condensate with the attendant spoilage and/or low consumer appeal. Moisture problems may arise in a variety of circumstances, including respiration in horticultural produce, melting of ice, temperature fluctuations in food packs with a high equilibrium relative humidity (ERH), or drip of tissue fluid from cut meats and produce (Rooney 1995). Their minimisation *via* packaging can be achieved either by liquid water absorption or humidity buffering.

2.1.2.1 Water absorption

The main purpose of water control is to lower the water activity, a_w, of the product, thereby suppressing the growth of microorganisms on the foodstuff (Vermeiren et al. 1999). Temperature cycling of high a_w foods has led to the use of plastics with an anti-fog additive that lowers the interfacial tension between the condensate and the film. This contributes to the transparency of the films and enables the customer to see clearly the packaged food (Rooney 1995) although it does not affect the amount of water present inside the package.

Several companies manufacture drip-absorbent sheets such as Thermarite® or Peaksorb® (Australia), or Toppan™ (Japan) for liquid water control in high a_w foods such as meat, fish, poultry and fresh produce. Basically, these systems consist of a super-absorbent polymer located between two layers of

a micro-porous or non-woven polymer. Such sheets are used as drip-absorbing pads placed under, for example, whole chickens or chicken cuts. Large sheets are also utilised for absorption of melted ice during air transportation of packaged seafood. The preferred polymers used for this purpose are polyacrylate salts and graft copolymers of starch (Rooney 1995).

2.1.2.2 Humidity buffering

This approach involves interception of moisture in the vapour phase by reducing the in-pack relative humidity and thereby the surface-water content of the food. It can be achieved by means of one or more humectants between two layers of a plastic film that is highly permeable to water vapour or by a moisture-absorbing sachet. An example of this approach is the Pichit™ film manufactured by Showa Denko (Japan). It is marketed in Japan for wrapping fish and chicken and reduces the ERH in the vicinity of the product, but has not been evaluated experimentally. Pouches containing NaCl have also been used in the US tomato market (Rooney 1995).

Desiccants have been successfully used for moisture control in a wide range of foods, such as cheeses, meats, chips, nuts, popcorn, candies, gums and spices. Silica gel, molecular sieves, calcium oxide (CaO) and natural clays (e.g. montmorillonite) are often provided in Tyvek™ sachets (Brody et al. 2001). Other desiccant systems include the MiniPax® and StripPax® packets, the DesiMax® (United Desiccants, USA) and the DesiPak®, Sorb-it®, Tri-sorb® and 2-in-1™ sachets (Multisorb Technologies Inc., USA).

2.1.3 Carbon dioxide generating systems

Carbon dioxide is known to suppress microbial activity. Relatively high CO_2 levels (60-80%) inhibit microbial growth on surfaces and, in turn, prolong shelf life. Therefore, a complementary approach to O_2 scavenging is the impregnation of a packaging structure with a CO_2 generating system or the addition of the latter in the form of a sachet. **Table 2.2** lists the main commercial CO_2 generators. Since the permeability of CO_2 is 3-5 times higher than that of O_2 in most plastic films, it must be continuously produced to maintain the desired concentration within the package. High CO_2 levels may, however, cause changes in taste of products and the development of undesirable anaerobic glycosis in fruits. Consequently, a CO_2 generator is only useful in certain applications such as fresh meat, poultry, fish and cheese packaging (Floros et al. 1997). In food products for which the volume of the package and its appearance are critical, an O_2 scavenger and CO_2 generator could be used together (Smith et al. 1995) in order to prevent package collapse as a result of O_2 absorption.

Nakamura and Hoshino (1983) reported that an oxygen-free environment alone is insufficient to retard the growth of *Staphylococcus aureus*, *Vibrio* species, *Escherichia coli*, *Bacillus cereus* and *Enterococcus faecalis* at ambient temperatures. For complete inhibition of these microorganisms in foods, the authors recommended a combined treatment involving O_2 scavenging with thermal processing, or storage under refrigeration, or using a CO_2 enriched atmosphere. They found that an O_2 and CO_2 absorber inhibited the growth of *Clostridium sporogenes* while an O_2 absorber and a CO_2 generator enhanced the growth of this microorganism, which is quite a surprising result. This result

Table 2.2 Commercial carbon dioxide generators

Trade Name	Manufacturer	References
Ageless® G[1]	Mitsubishi Gas Chemical Co (Japan)	Nakamura and Hoshino (1983), Smith et al. (1995)
Freshilizer® C[1] and CW[1]	Toppan Printing Co (Japan)	Smith et al. (1995)
FreshPax® M[1]	Multisorb Technologies Inc (USA)	
Vitalon® G[1]	Toagosei Chemical Co (Japan)	Vermeiren et al. (1999)
Verifrais®	SARL Codimer (France)	Vermeiren et al. (1999)

[1] Combined actions between CO_2 generating and O_2 scavenging

indicates the importance of selecting the correct scavenger to control the growth of *Clostridium* species in MAP foods.

2.1.4 Ethanol generating systems

Ethanol is used routinely in medical and pharmaceutical packaging applications, indicating its potential as a vapour phase inhibitor (Smith et al. 1987). It prevents microbial spoilage of intermediate moisture foods (IMF), cheeses, and bakery products. It also reduces the rate of staling and oxidative changes (Seiler 1989). Ethanol has been shown to extend the shelf life of bread, cake and pizza when sprayed onto product surfaces prior to packaging. Sachets containing encapsulated ethanol release its vapour into the packaging headspace thus maintaining the preservative effect (Labuza and Breene 1989).

Many applications of ethanol-generating films or sachets have been patented (Floros et al. 1997) and marketed (Smith et al. 1995), including an adhesive-backed film that can be taped on the inside of a package to provide AM activity (Labuza and Breene 1989). Mitsubishi Gas Chemical Company patented a sachet containing encapsulated ethanol, glucose, ascorbic acid, a phenolic compound and an iron salt (Floros et al. 1997), thereby achieving the combined effect of O_2 scavenging and ethanol generation. **Table 2.3** lists examples of commercial ethanol generators.

An ethanol-generating technology was originally developed in Japan whereby foodgrade ethanol is encapsulated in a fine inert powder inside a sachet.

Table 2.3 Commercial ethanol generators

Trade Name	Manufacturer	Reference
Ethicap®	Freund Industrial Co (Japan)	Smith et al. (1995)
Negamold®[1]	Freund Industrial Co (Japan)	Smith et al. (1995)
Oitech™	Nippon Kayaku Co (Japan)	Smith et al. (1995)
ET Pack	Ueno Seiyaku Co (Japan)	Smith et al. (1995)
Ageless® SE[1]	Mitsubishi Gas Chemical Co (Japan)	Floros et al. (1997)
Fretek®	Techno International Inc (USA)[2]	Brody et al. (2001)

[1] Combined actions between ethanol-generating and O_2 scavenging
[2] Under license from Freund Industrial Co (Japan)

The rate of ethanol vapour release can be tailored by controlling the permeability of the sachet. Several Japanese companies manufacture this type of ethanol generator, the most widely used being Ethicap® or Antimold Mild® produced by the Freund Industrial Company (Smith et al. 1995). These systems, approved for use in Japan, extend the mould-free shelf life of various bakery products.

Smith et al. (1987) demonstrated the usefulness of ethanol vapour in extending the shelf life of apple turnovers. The shelf life was found to be 14 days for the product packaged in air or in a CO_2/N_2 gas mixture (60% CO_2) and stored at ambient temperature. Afterwards, visible swelling occurred as a result of *Saccharomyces cerevisiae* growth and additional CO_2 production. When encapsulated ethanol was incorporated in the package, yeast growth was totally suppressed and the shelf life was extended to 21 days. On the other hand, this solution caused the packages to contain 1.5% ethanol at the end of the storage period as compared to only 0.2% when packed without ethanol. Consequently, the final products may be unacceptable to the consumer due to elevated ethanol contents. This problem can be partially resolved by heating the contents of the package prior to consumption, thereby evaporating the ethanol.

2.1.5 AM migrating and non-migrating systems

Quintavalla and Vicini (2002) have claimed that AM packaging is an extremely challenging technology and Appendini and Hotchkiss (2002) have reported that AM packaging is expected to grow in the next decade. Additionally, recent publications demonstrate AM packaging to be a rapidly emerging

technology (Suppakul et al. 2003a). As shown in **Figure 2.1**, the number of publications, available in the public domain and relevant to AM packaging, has significantly increased during the past decade.

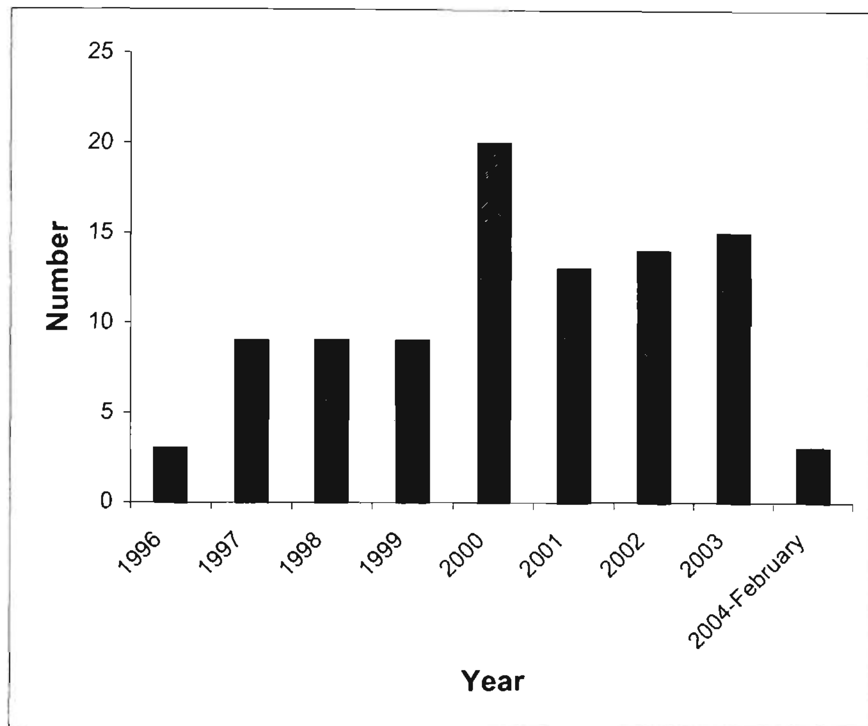


Figure 2.1 Number of articles related to AM packaging in journal publications

Antimicrobial food packaging materials have to extend the lag phase and/or reduce the growth rate of microorganisms in order to extend shelf life and to maintain product quality and safety (Han 2000). Alternatives to direct additives for minimising the microbial load are canning, aseptic processing and MAP. However, canned foods cannot be marketed as "fresh". Aseptic processing may be expensive and hydrogen peroxide, which is restricted in level of use by regulatory authorities, is often used as a sterilising agent. In certain cases, MAP can promote the growth of pathogenic anaerobes and the germination of spores, or prevent the growth of spoilage organisms which indicate the

presence of pathogens (Farber 1991). If packaging materials have self-sterilising abilities due to their own AM effectiveness, the need for chemical sterilisation of the packages may be obviated and the aseptic packaging process simplified (Hotchkiss 1997).

Food packages can be made AM active by incorporation and immobilisation of AM agents or by surface modification and surface coating. Present plans envisage the possible use of naturally derived AM agents in packaging systems for a variety of processed meats, cheeses, and other foods, especially those with relatively smooth product surfaces that come in contact with the inner surface of the package. This solution is becoming increasingly important, as it represents a perceived lower risk to the consumer (Nicholson 1998). Nonetheless, the "natural" origins of the chemicals are likely to be a selling point, but this does not necessarily make them safer than artificial additives (Biever 2003). **Table 2.4** lists a number of substances, which can be bound to polymers to impart AM properties. Such substances can be used in AM films, containers and utensils (Ishitani 1995). Antimicrobial materials have been known for many years. However, AM packages have had relatively few commercial successes, except in Japan. **Table 2.5** (Brody et al. 2001) summarises some of the AM systems. Antimicrobial films can be classified in two types: (i) those that contain an AM agent that migrates to the surface of the food, and (ii) those that are effective against surface growth of microorganisms without migration.

Table 2.4 Examples of AM additives for potential use in food packaging materials

Class	Examples	References
Acid Anhydride	Benzoic anhydride	Weng and Hotchkiss (1993), Huang et al. (1997), Dobias et al. (2000)
	Sorbic anhydride	Weng and Chen (1997)
Alcohol	Ethanol	Luck and Jager (1997)
Amine	Hexamethylenetetramine (HMT)	Luck and Jager (1997), Devlieghere et al. (2000b)
Ammonium Compound	Silicon quaternary ammonium salt	
Antibiotic	Natamycin	Luck and Jager (1997)
	Neomycin sulfate	Huang et al. (1999)
Antimicrobial Peptide	Attacin	Dillon (1994)
	Cecropin	Dillon (1994)
	Defensin	Dillon (1994)
	Magainin	Abler et al. (1995), Cleveland et al. (2001)
	14-residue synthetic peptide (6K8L)	Appendini and Hotchkiss (2000)
Antioxidant Phenolic[1]	Butylated hydroxyanisole (BHA)	Hotchkiss (1997)
	Butylated hydroxytoluene (BHT)	Hotchkiss (1997)
	Tertiary butylhydroquinone (TBHQ)	Hotchkiss (1997)
Bacteriocin	Bavaricin	Nettles and Barefoot (1993)
	Brevicin	Nettles and Barefoot (1993)
	Carnocin	Nettles and Barefoot (1993)
	Lacticin	Nettles and Barefoot (1993), An et al. (2000), Scannell et al. (2000), Kim et al. (2002b)
	Mesenterocin	Nettles and Barefoot (1993)

Table 2.4 (Continued)

Class	Examples	References
Bacteriocin	Nisin	Delves-Broughton et al. (1996), Luck and Jager (1997), An et al. (2000), Kim et al. (2000) Natrajan and Sheldon (2000a, b), Padgett et al. (2000), Scannell et al. (2000), Cleveland et al. (2001), Cutter et al. (2001), Hoffman et al. (2001), Bower et al. (2002), Cha et al. (2002; 2003a, b), Dawson et al. (2002), Kim et al. (2002a, b), Leung et al. (2002; 2003), Ross et al. (2002), Sebti et al. (2002), Buonocore et al. (2003b), Sebti et al. (2003), Chi-Zhang et al. (2004)
	Pediocin	Barnby-Smith (1992), Nettles and Barefoot (1993), Cleveland et al. (2001)
	Sakacin	Nettles and Barefoot (1993)
	Subtilin	Barnby-Smith (1992)
Chelator	Citrate	Hotchkiss (1997)
	Conalbumin	Conner (1993)
	EDTA	Luck and Jager (1997), Rodrigues and Han (2000), Hoffman et al. (2001), Cutter et al. (2001), Cha et al. (2002)
	Lactoferrin	Conner (1993)
	Polyphosphate	Shelef and Seiter (1993)
	Protoporphyrin 1X	Sherrill et al. (2003)
	Zinc protoporphyrin 1X	Sherrill et al. (2003)

Table 2.4 (Continued)

Class	Examples	References
Enzyme	Chitinase	Fuglsang et al. (1995)
	Ethanol oxidase	Fuglsang et al. (1995)
	b-Glucanase	Fuglsang et al. (1995)
	Glucose oxidase	Fuglsang et al. (1995)
	Lactoperoxidase	Conner (1993), Fuglsang et al. (1995)
	Lysozyme	Conner (1993), Fuglsang et al. (1995), Appendini and Hotchkiss (1997), Luck and Jager (1997), Rodrigues and Han (2000), Cha et al. (2002), Buonocore et al. (2003a, b)
	Myeloperoxidase	Fuglsang et al. (1995)
Fatty Acid	Lauric acid	Ouattara et al. (1997; 2000b), Padgett et al. (2000), Hoffman et al. (2001), Dawson et al. (2002), Walters et al. (2003)
	Palmitoleic acid	Ouattara et al. (1997)
Fatty Acid Ester	Monolaurin (lauricidin®)	Luck and Jager (1997), Blaszky and Holley (1998)
Fungicide	Benomyl	Halek and Garg (1989)
	Imazalil	Hale et al. (1986), Weng and Hotchkiss (1992)
	Sulfur dioxide	Thomas et al. (1995), Christie et al. (1997), Luck and Jager (1997), Opperman et al. (1999)
Germicide	Triclosan	Cutter et al. (1999), Lu et al. (2001), Chung et al. (2003)
Inorganic Acid	Phosphoric acid	Hotchkiss (1997)
Metal	Copper	Ishitani (1995)
	Silver	Ishitani (1995), Luck and Jager (1997), An et al. (1998), Chung et al. (1998)
Miscellaneous	Reuterin	Helander et al. (1997)
	Propionic acid	Doores (1993), Ouattara et al. (2000a, b), Luck and Jager (1997)

Table 2.4 (Continued)

Class	Examples	References
Natural Phenol	Catechin	Walker (1994)
	p-Cresol	Hotchkiss (1997)
	Hydroquinones	Hotchkiss (1997)
Oligosaccharide	Chitooligosaccharide	Cho et al. (2000), Hong et al. (2000)
Organic Acid	Acetic acid	Doores (1993), Ouattara et al. (2000a, b), Luck and Jager (1997)
	p-Aminobenzoic acid	Cagri et al. (2002; 2003)
	Benzoic acid	Luck and Jager (1997), Weng et al. (1997), Chen et al. (1999), Weng et al. (1999)
	Citric acid	
	Lactic acid	Doores (1993), Luck and Jager (1997)
	Malic acid	Doores (1993)
	Propionic acid	Doores (1993), Ouattara et al. (2000a, b), Luck and Jager (1997)
	Sorbic acid	Luck and Jager (1997), Weng et al. (1999), Cagri et al. (2002)
	Succinic acid	Doores (1993)
	Tartaric acid	Doores (1993)
Organic Acid Salt	Potassium sorbate	Chen et al. (1996), Han and Floros (1997, 1999), Devlieghere et al. (2000a), Ozdemir and Floros (2001)
	Sodium benzoate	Chen et al. (1996), Buonocore et al. (2003b)
	Sodium citrate	Blaszky and Holley (1998)
	Sodium propionate	Soares et al. (2002)
Paraben	Ethyl paraben	Davidson (1993), Luck and Jager (1997), Dobias et al. (2000)
	Methyl paraben	Davidson (1993), Luck and Jager (1997)
	Propyl paraben	Davidson (1993), Luck and Jager (1997), Dobias et al. (2000), Chung et al. (2001a, b)

Table 2.4 (Continued)

Class	Examples	References
Plant-Volatile Component	Allyl isothiocyanate (AIT)	Isshiki et al. (1992), Delaquis and Mazza (1995), Kyung and Fleming (1996), Delaquis and Sholberg (1997), Luck and Jager (1997), Shofran et al. (1998), Nielsen and Rios (2000), Brody et al. (2001)
	Carvacrol	Ouattara et al. (1997), Scora and Scora (1998)
	Cineole	Lis-Balchin et al. (1998), Scora and Scora (1998)
	Cinnamaldehyde	Ouattara et al. (1997; 2000b; 2001)
	Citral	Lis-Balchin et al. (1998), Scora and Scora (1998)
	p-Cymene	Scora and Scora (1998)
	Estragole (methylchavicol)	Scora and Scora (1998), Suppakul et al. (2002; 2003b, c, d)
	Eugenol	Ouattara (1997), Blaszky and Holley (19998), Scora and Scora (1998)
	Geraniol	Scora and Scora (1998)
	Hinokitiol (b-thujaplicin)	Fallik and Grinberg (1992), Brody et al. (2001)
	Linalool	Lis-Balchin et al. (1998), Scora and Scora (1998), Suppakul et al. (2002; 2003b, c, d)
	Terpineol	Scora and Scora (1998)
	Thymol	Ouattara et al. (1997; 2002), Scora and Scora (1998)
Polysaccharide	Chitosan	Sudarshan et al. (1992), Begin and Calsteren (1999), Shahidi et al. (1999), Hong et al. (2000), Coma et al. (2002)
	Konjac glucomannan	Xiao et al. (2000)

[1] Although generally used as Antioxidants, they have shown also antimicrobial activity (Hotchkiss, 1997)

Table 2.5 Trade names and manufacturers of commercial AM materials

Format	Trade Name	Manufacturer	References
Concentrate	AgION™	AgIon Technologies LLC(USA)	www.agion-tech.com
	Apacider-A®	Sangi Co (Japan)	Brody et al. (2001)
	MicroFree™	DuPont (USA)	Brody et al. (2001), Vermeiren et al. (2002)
	Microban®	Microban Products (USA)	Brody et al. (2001), Vermeiren et al. (2002)
	Novaron®	Milliken Co (USA)	Vermeiren et al. (2002)
	Sanitized®	Sanitized AG / Clariant (Switzerland)	Vermeiren et al. (2002)
	Surfacine®	Surfacine Development Co (USA)	Vermeiren et al. (2002)
	Ultra-Fresh®	Thomson Research Associates (Canada)	Vermeiren et al. (2002)
	Zeomic®	Shinanen New Ceramics Co (Japan)	Brody et al. (2001)
Extract	Citrex™ (Grapefruit seed)	Quimica Natural Brasileira Ltda (Brazil)	Lee et al. (1998)
	Nisaplin® (Nisin)	Integrated Ingredients (USA)	Scannell et al. (2000), Brody et al. (2001)
	Take Guard (Bamboo)	Takex Co (Japan)	Brody et al. (2001)
	WasaOuro® (Mustard)	Green Cross Co (Japan)	Brody et al. (2001)
Film	MicroGard™	Rhone-Poulenc (USA)	Brody et al. (2001)
	Piatech	Daikoku Kasei Co (Japan)	Brody et al. (2001)

2.1.5.1 Gas emission or flushing

Gas emission or flushing controls the growth of mould. Typical spoilage moulds include *Botrytis cinerea*, *Penicillium*, *Aspergillus* and *Rhizopus* species commonly found in citrus and berry fruits. To extend the storage period of these fruits, fungicides or antimycotic agents can be applied.

Sulfur dioxide (SO_2) is known to be the most effective material in controlling the decay of grapes and is superior to the gamma irradiation and heat-radiation combination methods (Smilanick 1990). However, a SO_2-releasing material entails a number of problems, including bleaching and SO_2 residues.

Thomas et al. (1995) studied the effect of SO_2 generating pads on the decay and quality of table grapes. In Australia, two different SO_2 release sheets were tested for packaging of the white "Thompson Seedless" and the purple "Red Globe" grapes (Christie et al. 1997). Sulfur dioxide in the surrounding atmosphere is absorbed into the grapes and initially converted to sulfite and then metabolised into the sulfate form. At the end of the experiment (after 4 days at 21°C), the sulfite levels in the "Red Globe" were found to be lower than those in the "Thompson Seedless", even though the former was subjected to a higher SO_2 level. This reflects the different metabolic rates of the two varieties (Christie et al. 1997). The authors suggested development of a controlled release polymer that would apply the fungicide at a sufficient level to retain satisfactory fungistatic action, while minimising undesirable effects.

Opperman et al. (1999) considered controlling the decay of table grapes with monolithic-type polymer structures that release SO_2 at a constant rate over an extended period. Two different systems containing either two or four SO_2-containing polymer discs were tested. In the four disc system, a disc was placed in the corner of each carton whereas in the two-disc system they were placed in a central location, approximately 10 cm from the edges of the carton. In 50% of the monolithic device treatments, the discs were placed directly on top of the grapes, while in the other treatments, they were placed on top of a corrugated paper liner. It was found that the liner acted as a physical barrier between the grapes and the SO_2 generator and that the carton absorbed much of the free SO_2. The controlling effect was vastly improved by raising the level of sodium metabisulfite ($Na_2S_2O_5$) impregnated in the polymer structure, but the product suffered from SO_2 damage. The optimum range for the $Na_2S_2O_5$ concentration was found to be 10-20% w/w.

Another volatile compound exhibiting AM effects is allyl isothiocyanate (AIT), the major pungent component of black mustard (*Brassica nigra*), brown mustard (*Brassica juncea*) and wasabi (*Eutrema wasabi* Maxim.). Isshiki et al. (1992) compared the minimum inhibitory concentration (MIC) of AIT vapour against microorganisms on agar. In the experiments, a mixture (500 mg) of AIT and beef fat (2:98, w/w) was placed on top of a perforated cellophane film, and packed in the bag with the sample food. It was claimed that at such low concentrations, only slight odours were perceived, which suggests that AIT can be employed in MAP. The shelf life of various foods (e.g. fresh beef, cured pork, sliced raw tuna, cheese, egg sandwich, noodles, and pasta) packaged in barrier plastic bags was enhanced when the package was flushed with AIT.

The AM effectiveness of AIT inside a package depends on its interaction with the particular packaging materials. Lim and Tung (1997) determined the vapour pressure of pure AIT and that of AIT above AIT-canola oil mixtures. Canola oil is effective in depressing the vapour pressure of AIT, and may be used as a controlling diluent for this purpose in MAP applications. It was found that the diffusion, solubility and permeability coefficients of AIT in polyvinylidenechloride (PVDC)/polyvinylchloride (PVC) copolymer films are concentration and temperature dependent. At a fixed vapour activity, the diffusion and permeability coefficients increased whereas the solubility coefficient decreased with an increase in temperature.

2.1.5.2 Coating of films with AM additives

Appropriate coatings can sometimes impart AM effectiveness. An et al. (2000) claimed that a polymer-based solution coating would be the most desirable method in terms of stability and adhesiveness of attaching a bacteriocin to a plastic film. It was found that low-density polyethylene (LDPE) films coated with a mixture of polyamide resin in isopropanol/propanol and a bacteriocin solution provided AM activity against *Micrococcus flavus*. The migration of bacteriocins reached equilibrium within 3 days, but the level attained was too low to affect several bacterial strains spread on an agar plate medium. When the films were in contact with a phosphate buffer solution containing strains of *M. flavus* and *L. monocytogenes*, a marked inhibition of microbial growth of both strains was observed.

LDPE film was successfully coated with Nisin using methylcellulose (MC)/hydroxypropyl methylcellulose (HPMC) as a carrier. Nisin was found to be effective in suppressing *S. aureus* and *L. monocytogenes* respectively (Cooksey 2000). Natrajan and Sheldon (2000a) studied the efficacy of Nisin-coated polymeric films such as PVC, linear low-density polyethylene (LLDPE), and nylon, in inhibiting *Salmonella typhimurium* on fresh broiler drumstick skin. As anticipated, the more hydrophobic LLDPE film repelled the aqueous Nisin formulations to a greater extent than the other films and caused coalescence of the treatment solution droplets. The repulsion between the LLDPE film and the treatment solution may have affected the overall inhibitory activity of the formulations by causing more localised inactivation of the target. An agar-based film containing nisin was also studied. It was found that in this film, the degree of cross-linking depends on the agar concentration, which may affect the migration of Nisin to the surface of a broiler drumstick skin (Natrajan and Sheldon 2000b). Thus, 0.75% w/w compared with 1.25% w/w gels formed a more open and elastic network, allowing greater migration of the treatment components over time. The respective levels of bacterial inhibition exhibited by the films, especially after 96 h, appeared to support this postulation.

Chung et al. (2001a) coated a styrene-acrylic copolymer with propyl paraben and observed an inhibition of *S. cerevisiae* by the slow release of propyl paraben as distinct from the inhibition by direct addition of propyl paraben. It was found that *S. cerevisiae* cells isolated from the culture under slow release conditions were less tolerant of propyl paraben than cells isolated from the

culture under direct addition. A key merit of slow release over direct addition is the continuous microbial inhibition by slow delivery of active AM agent to the food during an extended period. Therefore, the continuing slow release from a packaging material may be valuable in reducing contamination on multiple opening of food stored in reclosable packaging. This may have a clear advantage over aseptic packaging systems where the effect of sterilisation is lost upon the first opening of the packaging. However, to achieve this advantage and prevent initial microbial outgrowth, the rate and amount of slow release should be carefully controlled in each application (Chung et al. 2001b).

Kim et al. (2002b) coated LDPE film with a bacteriocin (Nisin or Lacticin NK24) to control naturally-contaminated bacteria on packaged fresh oysters and ground beef, stored at 3 and 10 °C. Compared to plain LDPE film, bacteriocin-coated LDPE film slowed down microbial growth at both temperatures. The effect of the AM coated films on the suppression of coliform bacterial growth were more pronounced at 10 °C than 3 °C, whereas the effect on total aerobic bacteria were consistantly evident at both temperatures.

Recently, a polyethylene (PE) film was also coated with Nisin and then tested for packaging of tofu topically inoculated with *L. monocytogenes* (Cha et al. 2003a). Results from this study show that the application of Nisin-coated PE film in the packaging of tofu has the potential to overcome the problems associated with the growth and survival of *L. monocytogenes* Brie-1 and the secondary contamination of opened packages in household refrigerators.

2.1.5.3 Incorporation of AM additives

The direct incorporation of AM additives in packaging films is a convenient means by which AM activity can be achieved. Several compounds have been proposed and/or tested for AM packaging using this method. Han and Flores (1997) studied the incorporation of 1.0% w/w potassium sorbate in LDPE films. A 0.1 mm thick film was used for physical measurements, while a 0.4 mm thick film was used for AM effectiveness tests. It was found that potassium sorbate lowered the growth rate and maximum growth of yeast, and lengthened the lag period before mould growth became apparent. The results of this study, however, contradict those obtained by Weng and Hotchkiss (1993) with LDPE films (0.05 mm thick) containing 1.0% w/w sorbic acid. In the latter case, the films failed to suppress mould growth when brought into contact with inoculated media. Devlieghere et al. (2000a) investigated these contradictory results. Their results confirm that ethylene vinyl alcohol/linear low density polyethylene (EVA/LLDPE) film (70 μm thick) impregnated with 5.0% w/w potassium sorbate is unable to inhibit the growth of microorganisms on cheese and to extend its shelf life. As suggested by Weng and Hotchkiss (1993), very limited migration of potassium sorbate into water as well as into cheese cubes occurs, probably because of the incompatibility of the polar salt with the non-polar LDPE. The choice of an AM agent is often restricted by the incompatibility of that agent with the packaging material or by its heat instability during extrusion (Weng and Hotchkiss 1993; Han and Floros 1997).

While polyethylene (PE) has been widely employed as the heat-sealing layer in packages, in some cases the copolymer polyethylene-co-methacrylic acid

(PEMA) was found to be preferable for this purpose. Weng et al. (1999) reported a simple method for fabricating PEMA films (0.008-0.010 mm thick) with AM properties by the incorporation of benzoic or sorbic acids. The experimental results suggest that 'sodium hydroxide and preservative-treated' films exhibit dominantly AM properties for fungal growth, presumably due to the higher amount of preservatives released from the films (75 mg benzoic acid or 55 mg sorbic acid per g of film) than 'hydrochloric acid and preservative-treated' films. Chen et al. (1996) found that chitosan films made from dilute acetic acid solutions block the growth of *Rhodotorula rubra* and *Penicillium notatum* if the film is applied directly to the colony-forming organism. Since chitosan is soluble only in slightly acidic solutions, production of such films containing the salt of an organic acid (e.g. benzoic acid, sorbic acid) that is an AM agent is straight forward. However, the interaction between the AM agent and the film-forming material may affect the casting process, the release of the AM agent and the mechanical properties of the film.

Begin and Calsteren (1999) showed that films containing AM agents with a molecular weight larger than that of acetic acid are soft and can be used in multi-layer systems or as a coating. Acetic acid diffusion was, however, not as complete as that of propionic acid when chitosan-containing films were used in contact with processed meats (Ouattara et al. 2000a) in spite of the fact that in an aqueous medium, acetic acid diffused out of chitosan more rapidly than propionic acid (Ouattara et al. 2000b). These results suggest that the release of organic acids from chitosan is a complex phenomenon that involves many factors such as

electrostatic interactions, ionic osmosis, and structural changes in the polymer induced by the presence of the acids.

According to Weng and Hotchkiss (1993), anhydrides are more compatible with PE than their corresponding free acids or salts, due to the lower polarity and higher molecular weight of the former compared to the latter. Hence, anhydrides may serve as appropriate additives to plastic materials for food packaging. LDPE films impregnated with benzoic anhydride completely suppressed the growth of *Rhizopus stolonifer*, *Penicillium* species and *Aspergillus toxicarius* on potato dextrose agar (PDA). Similarly, LDPE films that contained benzoic anhydride delayed mould growth on cheese (Weng and Hotchkiss 1993). PE films (0.010-0.015 mm thick) containing benzoic anhydride (20 mg benzoic anhydride per g of PE in the initial preparation) alone or in combination with minimal microwave heating, were effective in controlling microbial growth of tilapia fillets during a 14-day storage at 4 °C (Huang et al. 1997). Shelf-life studies of packaged cheese and toasted bread demonstrated the efficiency of LDPE film containing benzoic anhydride against mould growth on the food surface during storage at 6 °C (Dobias et al. 2000). Dobias et al. (2000) also studied the migration of benzoic anhydride, ethyl paraben (ETP) and propyl paraben (PRP) in LDPE films. It was found that the incorporation of these parabens in the polymer was more difficult than that of benzoic anhydride due to their higher volatilities.

No single AM agent can cover all the requirements for food preservation. Weng and Chen (1997) investigated a range of anhydrides for use in food

packaging. It is known that for mould growth inhibition, the effectiveness of sorbic anhydride (initial concentration of 10 mg sorbic anhydride per g of PE) incorporated in PE films (0.10-0.12 mm thick) is much better with slow-growing (*Penicillium* species) than with fast-growing mould (*Aspergillus niger*). This is due to the time required for the PE to release sorbic acid to an inhibitory concentration.

Apart from organic acids and anhydrides, Imazalil has also been used with LDPE film. Weng and Hotchkiss (1992) showed that an Imazalil concentration of 2000 mg kg^{-1} LDPE film (5.1 μm thick) delayed *A. toxicarius* growth on potato dextrose agar, while LDPE film containing 1000 mg kg^{-1} Imazalil substantially inhibited *Penicillium* sp. growth and the growth of both of these moulds on cheddar cheese.

Little published data exist on the incorporation of bacteriocins into packaging films. Siragusa et al. (1999) highlighted the potential of incorporating Nisin directly into LDPE film for controlling food spoilage and enhancing product safety. Devlieghere et al. (2000b) were probably the first to use hexamethylene-tetramine (HMT) as an AM packaging agent. The AM activity of the latter is believed to be due to the formation of formaldehyde when the film comes into contact with an acidic medium (Luck and Jager 1997). It was found that a LDPE film containing 0.5% w/w HMT exhibited AM activity in packaged cooked ham and therefore this agent is a promising material for food packaging applications.

In Japan, the ions of silver and copper, quaternary ammonium salts, and natural compounds such as Hinokitiol are generally considered safe AM agents. Silver-substituted zeolite (Ag-zeolite) is the most common agent with which plastics are impregnated. It retards a range of metabolic enzymes and has a uniquely broad microbial spectrum. As an excessive amount of the agent may affect the heat-seal strength and other physical properties such as transparency, the normal incorporation level used is 1-3% w/w. Application to the film surface (i.e. increasing the surface area in contact with the food) is another approach that could be investigated in the future (Ishitani 1995).

Another interesting commercial development is Triclosan-based antimicrobial agents such as Microban®, Sanitized® and Ultra-Fresh®. Vermeiren et al. (2002) reported that LDPE films containing 0.5 and 1.0% w/w triclosan exhibited AM activity against *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7, *Salmonella enteritidis* and *Brocothrix thermosphacta* in agar diffusion assay. The 1.0% w/w Triclosan film had a strong AM effect in *in vitro* simulated vacuum-packaged conditions against the psychrotrophic food pathogen *L. monocytogenes*. However, it did not effectively reduce spoilage bacteria and growth of *L. monocytogenes* on refrigerated vacuum-packaged chicken breasts stored at 7 °C. This is because of its ineffectiveness towards microbial growth.

Other compounds with AM effects are natural plant extracts. Recently, Korean researchers developed certain AM films impregnated with naturally-derived AM agents (An et al. 1998; Chung et al. 1998; Lee et al. 1998; Hong et al. 2000; Ha et al. 2001; Cha et al. 2002; Suppakul et al. 2002, 2003c). These

compounds are perceived to be safer and are claimed to alleviate safety concerns (Lee et al. 1998). It was reported that the incorporation of 1% w/w grapefruit seed extract (GFSE) in LDPE film (30 μm thick) used for packaging of curled lettuce reduced the growth rate of aerobic bacteria and yeast. In contrast, a level of 0.1% GFSE yielded no significant effect on the rate of microbial growth in packaged vegetables, except for lactic acid bacteria on soybean sprouts (Lee et al. 1998). Ha et al. (2001) studied GFSE incorporated (by co-extrusion or a solution-coating process) in multi-layered PE films and assessed the feasibility of their use for ground beef. They found that coating with the aid of a polyamide binder resulted in a higher level of AM activity than when incorporated by co-extrusion. A co-extruded film (15 μm thick) with 1.0% w/w GFSE showed AM activity against *M. flavus* only, whereas a coated film (43 μm of LDPE with 3 μm of coating) with 1.0% w/w GFSE showed activity also against *E. coli*, *S. aureus*, and *Bacillus subtilis*. Both types reduced the growth rates of bacteria on ground beef stored at 3 °C, as compared to plain PE film. The two investigated GFSE levels (0.5 and 1.0% w/w) did not differ significantly in the efficacy of the film in terms of its ability to preserve the quality of beef.

Chung et al. (1998) found that LDPE films (48-55 μm thick) impregnated with either 1.0% w/w *Rheum palmatum* and *Coptis chinensis* extracts or silver-substituted inorganic zirconium retarded the growth of total aerobic bacteria, lactic acid bacteria and yeast on fresh strawberries. However, the study of An et al. (1998) showed that LDPE films (48-55 μm thick) containing 1.0% w/w *R. palmatum* and *C. chinensis* extracts or Ag-substituted inorganic zirconium did not exhibit any AM activity in a disc test (Davidson and Parish 1989) against *E. coli*,

S. aureus, *Leuconostoc mesenteroides*, *S. cerevisiae*, *A. niger*, *Aspergillus oryzae*, *Penicillium chrysogenum*. A film containing sorbic acid showed activity against *E. coli*, *S. aureus*, and *L. mesenteroides*. The reasons for this unusual result are not clear. During diffusion assays, the AM agent is contained in a well or applied to a paper disc placed in the center of an agar plate seeded with the test microorganism. This arrangement may not be appropriate for essential oils, as their components are partitioned through the agar due to their affinity for water (Davidson and Parish 1989). Accordingly, broth and agar dilution methods are widely used to determine the AM effectiveness of essential oils (Davidson and Parish 1989).

According to Hong et al. (2000), the AM activity of 5.0% w/w propolis extract, chitosan polymer and oligomer, or clove extract in LDPE films (0.030-0.040 mm thick) against *Lactobacillus plantarum*, *E. coli*, *S. cerevisiae*, and *Fusarium oxysporum* is best determined through viable cell counts. Overall, LDPE films with incorporated natural compounds show a positive AM effect against *L. plantarum* and *F. oxysporum*. Preliminarily studies by Suppakul et al. (2002) with LLDPE films (45-50 μm thick) containing 0.05% w/w linalool or methylchavicol showed a positive activity against *E. coli*. In a later investigation, Suppakul et al. (2003c) incorporated linalool or methylchavicol into low-density polyethylene (LDPE)-ethylene vinyl acetate (EVA) films of 45-50 μm thickness to minimise the loss of active agent. A net loss of the active agent was observed during the extrusion process but this was significantly lower than the loss observed in a previous study (Suppakul et al. 2002).

Edible films and various AM compounds incorporated in edible food packages have also been investigated recently (Rodrigues and Han 2000; Coma et al. 2001, 2002; Hoffman et al. 2001; Ozdemir and Floros 2001; Cagri et al. 2002, 2003; Cha et al. 2002; Sebti et al. 2002). Rodrigues and Han (2000) investigated edible AM materials produced by incorporating Lysozyme, Nisin and ethylenediamine tetracetic acid (EDTA) in whey protein isolate (WPI) films. Such Lysozyme or Nisin-containing films are effective in inhibiting *B. thermosphacta* but fail to suppress *L. monocytogenes*. The incorporation of EDTA in WPI films improved the inhibitory effect on *L. monocytogenes* but had a marginal effect only on *E. coli* O157:H7. Cagri et al. (2002) investigated WPI films containing 0.5 to 1.0% w/w *p*-aminobenzoic acid (PABA) and/or sorbic acid (SA) and assessed the AM properties of these films when brought in contact with sliced Bologna and summer sausage that were inoculated with three pathogens, *L.* monocytogenes, *E. coli* O157: H7, and *Salmonella enterica* subsp. *Enterica* serovar Typhimurium DT104. Whey protein isolate films containing SA or PABA reduced *L. monocytogenes*, *E. coli*, and *S. enterica* populations by 3.4 to 4.1, 3.1 to 3.6, and 3.1 to 4.1 logs, respectively, on both products after 21 days at 4 °C. Cagri et al. (2003) also incorporated PABA into WPI edible casings and then assessed these for the packaging of hot dogs that were topically surface-inoculated with *L. monocytogenes* and stored for 42 days at 4 °C. It was found that *Listeria* populations on hot dogs prepared with 1.0% w/w PABA WPI casings remained relatively unchanged, but in control treatments, there was an increase in *Listeria* populations of about 2.5 logs during 42 days of refrigerated storage.

Coma et al. (2001) studied the moisture barrier and the AM properties of HPMC-fatty acid films (30-50 μm thick) containing Nisin (10^5 IU mL^{-1}) as the AM agent and its efficacy against *Listeria innocua* and *S. aureus* growth in food products. Stearic acid was chosen as the fatty acid because of its ability to reduce the rate of water vapour transmission. However, it impaired the effectiveness of the film against both strains. This may be explained by electrostatic interaction between the cationic Nisin and the anionic stearic acid.

2.1.5.4 Immobilisation

Besides diffusion and sorption, some AM packaging systems utilise covalently immobilised AM substances that suppress microbial growth. Appendini and Hotchkiss (1997) investigated the efficiency of Lysozyme immobilised on different polymers. It is known that cellulose triacetate (CTA) containing Lysozyme yields the highest AM activity. The viability of *Micrococcus lysodeikticus* was reduced in the presence of immobilised Lysozyme on CTA film (Appendini and Hotchkiss 1997). Scannell et al. (2000) showed that PE/polyamide (70:30) film formed a stable bond with Nisin in contrast to Lacticin 3147. Nisin-adsorbed bioactive inserts reduced the level of *L. innocua* and *S. aureus* in sliced cheese and in ham.

Several peptides isolated from animals, plants, microorganisms, and insects, as well as chemically-synthesised analogs, have shown AM activity against microorganisms (Appendini and Hotchkiss 2002). Since peptides can be covalently immobilised through amino and carboxylic groups, they might be

suitable for attachment to functionalised polymer surfaces. Appendini and Hotchkiss (2001) have studied the potential uses of covalently immobilised peptides for packaging applications. A 14-amino-acid residue peptide was immobilised on polystyrene by solid-phase peptide synthesis (SPPS) and tested against several food-borne microorganisms. The merit of SPPS is that the peptide is constructed directly on the resin by protecting the amino acid functional groups. The resulting polymer was microcidal in a concentration and time-dependent behaviour against several microorganisms including *E. coli* O157: H7, *L. monocytogenes*, *S. aureus*, *Pseudomonas fluorescens* and *Kluyveromyces marxianus* when suspended in a phosphate buffer.

2.1.5.5 Surface modification

Ozdemir et al. (1999) introduced (by chemical methods) functional groups possessing AM activity into polymer films with the purpose of preventing the transfer of the AM agents from the polymer to the food. Cho et al. (2000) synthesised a new biopolymer containing a chito-oligosaccharide (COS) side chain. The COS was introduced on polyvinylacetate (PVA) by cross-linking with the bifunctional compound, *N*-methylolacrylamide (NMA). It was found that the growth of *S. aureus* was almost completely suppressed by this means.

Surface amine groups formed in polymers by electron irradiation were also shown to impart AM effectiveness (Cohen et al. 1995; Ozdemir et al. 1999). Another AM film has recently been developed using a UV excimer laser (Endert et al. 1999). Nylon 6,6 films irradiated in air by a laser at 193 nm (Pireaux et al.

1995) exhibited AM activity, apparently due to a 10% conversion of the amide groups on the nylon surface to amines bound to the polymer chain (Cohen et al. 1995). By contrast, irradiation at 248 nm did not change the surface chemistry or initiate conversion of the amide (Ozdemir and Sadikoglu 1998).

Paik et al. (1998) and Shearer et al. (2000) observed a decrease in all bacterial cells, including *S. aureus*, *P. fluorescens*, and *E. faecalis* in bulk fluid when using an AM nylon film. The results indicate that this decrease is more likely to be due to the bactericidal action than to surface adsorption (Paik et al. 1998). Although the mechanism of the reduction in the bacteria population remained uncertain, electrostatic attractive forces between the positively charged film surface and the negatively charged *E. coli* and *S. aureus* were presumed to be the reason for this effect (Shearer et al. 2000). Further research is needed to characterise the AM active groups on the irradiated film surface and the mechanism of AM action.

Recently, a technique has been described for the grafting of an ethylene diamine derivative of protoporphyrin IX (PPIX-ED) or an ethylene diamine derivative of zinc protoporphyrin IX (Zn-PPIX-ED) onto a nylon surface with pregrafted poly(acrylic acid) (PAA) polymer chains (Sherrill et al. 2003). This technique has been shown to be a consistent and reliable method for permanently attaching light-activated AM molecules to nylon surfaces. An analysis of the samples using X-ray photoelectron spectroscopy (XPS) confirmed the presence of the grafting of PAA, PPIX-ED, and Zn-PPIX-ED to the nylon films. The

biological testing of these materials is currently underway at the Georgia Institute of Technology.

Ionomers, with their unique properties such as a high degree of transparency, strength, flexibility, stiffness and toughness, as well as inertness to organic solvents and oils, have also drawn much attention as food packaging materials. Halek and Garg (1989) successfully incorporated the Benomyl fungicide into ionomer films *via* its carboxyl groups. Unfortunately, Benomyl is not an approved food preservative.

Weng et al. (1997) investigated application of AM ionomers combined with approved food preservatives. Anhydride linkages in the modified films were formed by reaction of acid/or base-treated films with benzoyl chloride. The AM activity was characterised in terms of the release of benzoic acid, which was higher in the base-treated version indicating the superiority of the latter. The AM effect of modified ionomer films was further demonstrated by their ability to inhibit the growth of *Penicillium* species and *A. niger*.

2.1.6 The future for AM packaging

Antimicrobial packaging is a rapidly emerging technology. The need to package foods in a versatile manner for transportation and storage, along with the increasing consumer demand for fresh, convenient, and safe food products presages a bright future for AM Packaging (Floros et al. 1997). However, more information is required on the chemical, microbiological and physiological

effects of these systems on the packaged food especially on the issues of nutritional quality and human safety (Floros et al. 1997). So far, research on AM packaging has focused primarily on the development of various methods and model systems, whereas little attention has been paid to its preservation efficacy in actual foods (Han 2000). Research is essential to identify the types of food that can benefit most from AM packaging materials. It is likely that future research into a combination of naturally-derived AM agents, biopreservatives and biodegradable packaging materials will highlight the merits of AM packaging in terms of food safety, shelf life and environmental friendliness (Nicholson 1998; Rodrigues and Han 2000; Coma et al. 2001).

The reported effectiveness of natural plant extracts suggests that further research is needed in order to evaluate their AM activity and potential side effects in packaged foods (Devlieghere et al. 2003). An additional challenge is in the area of odour/flavour transfer by natural plant extracts to packaged food products. Thus, research is needed to determine whether natural plant extracts could act as both an AM agent and as an odour/flavour enhancer. Moreover, in order to secure safe food, amendments to regulations might require toxicological and other testing of compounds prior to their approval for use (Vermeiren et al. 2002).

2.2 Factors to consider in the manufacturing of AM films

It is clear that the selection of both the substrate and the AM substance is important in developing an AM packaging system. Furthermore, when an AM

agent is added to a packaging material, it may affect the inherent physico-mechanical properties of the latter.

2.2.1 Process conditions and residual AM activity

The effectiveness of an AM agent applied by impregnation may deteriorate during film fabrication, distribution and storage (Han 2000). The chemical stability of an incorporated AM substance is likely to be affected by the extrusion conditions, namely, the high temperatures, shearing forces and pressures (Han and Floros 1999). To minimise this problem, Han (2000) recommended using master batches of the AM agent in the resin for preparation of AM packages. Also, all operations such as lamination, printing and drying as well as the chemicals (adhesives and solvents) used in the process may affect the AM activity of the package. In addition, some of the volatile AM compounds may be lost during storage. All these parameters should be evaluated.

2.2.2 Characteristics of AM additives and foods

The mechanism and kinetics of growth inhibition are generally studied in order to permit mathematical modeling of microbial growth (Han 2000). Foods with different biological and chemical characteristics are stored under different environmental conditions, which, in turn, may cause different patterns of microflora growth. Aerobic microorganisms can exploit headspace O_2 for their growth. The pH of a product affects the growth rate of target microorganisms and changes the degree of ionisation of the most active chemicals, as well as the activity of the AM agents (Han 2000). Weng and Hotchkiss (1993) reported that

LDPE film containing benzoic anhydride was effective in inhibiting moulds at low pH values. Rico-Pena and Torres (1991) found that the diffusion of sorbic acid decreased with an increase in pH. The food a_w may alter the microflora, AM activity, and chemical stability of active ingredients applied by impregnation. Vojdani and Torres (1989a) showed that the diffusion of potassium sorbate through polysaccharide films increases with a_w; this has a negative impact on the amount available for protection. Rico-Pena and Torres (1991) found that potassium sorbate diffusion rates in MC/HPMC film containing palmitic acid were much higher at higher values of a_w.

2.2.3 Chemical interaction of AM additives with film matrix

During incorporation of additives into a polymer, the polarity and molecular weight of the additive have to be taken into consideration. Since LDPE is non-polar, additives with a high molecular weight and low polarity are more compatible with this polymer (Weng and Hotchkiss 1993). Furthermore, the molecular weight, ionic charge and solubility of different additives affect their rates of diffusion in the polymer (Cooksey 2000). Wong et al. (1996) compared the diffusion of ascorbic acid, potassium sorbate and sodium ascorbate in calcium-alginate films at 8, 15 and 23 °C. They found that ascorbic acid had the highest and sodium ascorbate the lowest diffusion rate at all studied temperatures. These findings were attributed to the different ionic states of the additives.

2.2.4 Storage temperature

The storage temperature may also affect the activity of AM packages. Several researchers found that the protective action of AM films deteriorated at higher temperatures, due to high diffusion rates in the polymer (Vojdani and Torres 1989a; 1989b; Wong et al. 1996). The diffusion rate of the AM agent and its concentration in the film must be sufficient to remain effective throughout the shelf life of the product (Cooksey 2000). Weng and Hotchkiss (1993) stated that low levels of benzoic anhydrides in LDPE might be as effective at refrigeration temperatures as high levels at room temperature.

2.2.5 Mass transfer coefficients and modeling

Mathematical modeling of the diffusion process could permit prediction of the AM agent release profile and the time during which the agent remains above the critical inhibiting concentration. With a higher diffusivity and much larger volume of the food component compared to the packaging material, a semi-infinite model in which the packaging component has a finite thickness and the food component has infinite volume could be practical. The initial and boundary conditions that could be used in mass transfer modeling have been identified (Han 2000).

2.2.6 Physical properties of packaging materials

Antimicrobial agents may affect the physical properties, processability or machinability of the packaging material. Han and Flores (1997) reported no significant differences in the tensile properties before and after the incorporation

of potassium sorbate in LDPE films, but the transparency of the films deteriorated as the sorbate concentration increased. Weng and Hotchkiss (1993) reported no noticeable differences in clarity and strength of LDPE film containing 0.5 and 1.0% benzoic anhydride. Similar results were reported for naturally-derived plant extracts such as propolis at 5.0% (Hong et al. 2000), clove at 5.0% (Hong et al. 2000), *R. palmatum* at 1.0% (An et al. 1998; Chung et al. 1998), and *C. chinensis* at 1.0% (An et al. 1998; Chung et al. 1998). On the other hand, LDPE film coated with MC/HPMC containing Nisin was difficult to heat-seal (Cooksey 2000).

Dobias et al. (2000) found statistically significant differences between the physical properties of films without AM agents and with different agents at concentrations of 5 g kg^{-1} and 10 g kg^{-1}. It was found that the tensile and sealing strengths were lower in all samples containing AM agents including benzoic anhydride, ethyl paraben (ETP) or propyl paraben (PRP). In all studied cases, the coefficient of friction increased with the addition of AM substances, water vapour permeability declined by the incorporation of PRP, and oxygen permeability decreased by the impregnation of benzoic anhydride or PRP.

2.2.7 Cost

There are no published data on the cost of films impregnated with AM agents, but they can be expected to be more expensive than their basic counterparts. Commercialisation of such films could therefore become viable for high-value food products only (Cooksey 2000).

2.2.8 Food contact approval

Some organic acids, bacteriocins and volatile compounds derived from plants have FDA approval as additives for certain foods (**Table 2.6**). Allyl isothiocyanate is currently not approved by the FDA for use in the USA (Brody et al. 2001) due to a safety concern that this synthetic compound may be contaminated with traces of the toxic allyl chloride used in the manufacturing process (Clark 1992). In Japan, the use of AIT is allowed only when this compound is extracted from a natural source (Isshiki et al. 1992). Weng and Hotchkiss (1993) pointed out that the rapid hydrolysis of benzoic anhydride to benzoic acid should not pose a safety concern, although at the time of their study benzoic anhydride did not have FDA approval. The use of Ag-zeolite as an acceptable food additive in Europe has not been clarified (Brody et al. 2001). However, recently, Ag-zeolites such as AgION™ and Zeomic® received the approval of the FDA for use in food-contact materials. Triclosan is also not accepted by US regulatory authorities for food contact materials (Brody et al. 2001). In Europe, the legislative status of Triclosan is unclear. Triclosan does not appear on the EU directive list of approved food additives that may be used in the manufacturing of plastics intended for food contact materials (Vermeiren et al. 2002).

No European regulations currently exist on the use of active and intelligent packaging. Packages intended for food contact applications are required to belong to a positive list of approved compounds, and an overall migration limit from the material into the food or food simulant was set at 60 mg kg^{-1}. This is incompatible with the aim of active packaging, especially when the

Table 2.6 List of permitted food additives that could be used as AM additives in packaging materials

Additive	Code Assigned by Legislative Authority		
	Australia/New Zealand[1]	Europe[2]	USA[3]
Acetic acid	260	E260	GRAS
Benzoic acid	210	E210	GRAS
Butylated hydroxyanisole (BHA)	320	E320	GRAS
Butylated hydroxytoluene (BHT)	321	E321	GRAS
Carvarcol			FA
Citral			GRAS
Citric acid	330	E330	GRAS
p-Cresol			FA
EDTA			FA
Estragole (methyl chavicol)			GRAS
Ethanol		E1510	GRAS
Ethyl paraben		E214	GRAS
Eugenol			GRAS
Geraniol			GRAS
Glucose oxidase	1102		GRAS
Hexamethylenetetramine (HMT)		E239	
Konjac glucomannan		E425	GRAS
Lactic acid	270	E270	GRAS
Lauric acid			FA
Linalool			GRAS
Lysozyme	1105	E1105	GRAS

Table 2.6 (Continued)

Additive	Code Assigned by Legislative Authority		
	Australia/New Zealand[1]	Europe[2]	USA[3]
Malic acid	296	E296	GRAS
Methyl paraben	218	E218	
Natamycin	235	E235	FA
Nisin	234	E234	GRAS
Phosphoric acid	338	E338	GRAS
Polyphosphate		E452	GRAS
Potassium sorbate	202	E202	GRAS
Propionic acid	280	E280	GRAS
Propyl paraben	216	E216	GRAS
Sodium benzoate	211	E211	GRAS
Sorbic acid	200	E200	GRAS
Succinic acid		E363	GRAS
Sulfur dioxide	220	E220	GRAS
Tartaric acid	334	E334	GRAS
Tertiary butylhydroquinone (TBHQ)	319		FA
a-Terpineol			FA
Thymol			FA

Source: CFR (1988); Davidson and Branen (1993); Maga and Tu (1995); Luck and Jager (1997); Saltmarsh (2000); Taubert (2000)

[1] Assignment of a number signifies that additive is approved by the Australian and New Zealand Food Authority (ANZFA) and The Australian New Zealand Food Standards Council (ANZFSC) as being safe for food use

[2] Assignment of an "E" number signifies that additive has been approved by the European Communities (EC) Scientific Committee on Food (SCF)

[3] Classification in accordance with Food and Drug Administration (FDA) Title 21 of the Code of Federal Regulations (21 CFR) wherein substances intended for use in the manufacture of foodstuffs for human consumption are classified into three categories: food additives (FA), prior-sanctioned food ingredients and substances generally recognised as safe (GRAS)

system is designed to release active ingredients into the foods. Consequently, as was also stated by van Beest (2001), a new approach in food packaging regulations is needed. The current applications of AM food packaging are rather limited, although promising. This is because of the legal status of the tested additives (Vermeiren et al. 2002). In 2003, a draft amendment of the EU Directive 89/109/EEC (food contact of packaging materials) has been approved by the EU commission. The draft which, under certain conditions, will allow the use of some active packaging, still need approval by the EU Council and the EU Parliament. It is expected that a specific directive for active and intelligent packaging is still some four years away (Anonymous 2004). The major potential food applications of AM films include meat, fish, poultry, bakery goods, cheese, fruits and vegetables (Labuza and Breene 1989). **Table 2.7** lists the current and potential future applications of AM packaging technologies.

2.3 AM activity of basil and its application in food packaging

Basil is one of the oldest spices belonging to the *Ocimum* genus and to the Lamiaceae (Labiatae) family. The botanical nomenclature of the *Ocimum basilicum* L. varieties from which the different types of basil oil are distilled is complicated. The reason for this complexity stems from the fact that botanists have assigned several designations to the same varieties and, in some instances, have confused some varieties with forms of other species (Guenther 1975). The *Ocimum* genus contains approximately 30 species of herbs and shrubs from the tropical and subtropical regions of Asia, Africa and Central and South America. However, the major place of diversity appears to be in Africa (Paton 1992). This

Table 2.7 Current and future applications of AM packaging

SYSTEM	FOOD GROUP						
	Beverage	Minimally Processed	Meat/Poultry	Seafood	Dairy	Bakery	Produce/ Vegetarian
O_2 Scavenging	Wine Beer Fruit juice		Fresh meat Processed meat Sausages		Cheese	Bread	Vegetables
H_2O absorb/control			Meat Chicken	Fish			Vegetables Fruit
CO_2 generating			Fresh meat Poultry	Fish	Cheese		
Ethanol generating				Fish	Cheese	Bread Cake	
Antimicrobial[1]	Fruit juice[2] Tea	Noodles[2] Pasta[2] Sandwiches[2] Sushi[2]	Meat Ham Pastrami Bologna Chicken	Fish Pre-cooked shrimp Oyster	Cheese	Bread Cake[2]	Vegetables Fruit Tofu

[1] Including both migrating and non-migrating systems
[2] Possible future application

genus is characterised by a great variability in its morphology and chemotypes (Lawrence 1988). The ease of its cross-pollination contributes to a myriad of subspecies, varieties and forms (Guenther 1975).

Basil is a popular culinary herb and its essential oils have been used extensively for many years in the flavouring of confectionary and baked goods, condiments (e.g. ketchups, tomato pastes, chili sauces, pickles, vinegars), sausages and meats, salad dressings, non-alcoholic beverages, ice cream and ices. Basil oil has also found wide application in perfumery, as well as in dental and oral products (Guenther 1975). In addition, since the public nowadays prefers natural over synthetic direct or indirect food additives (Nicholson 1998), naturally derived AM agents like basil, are becoming increasingly more important in AM packaging as they present a perceived lower risk to consumers. Antimicrobial packaging is part of the broader area of "Active Packaging" which has become, in the last decade, one of the major areas of research in food packaging (Miltz et al. 1995). In view of their possible future use in food preservation, this section reviews the basil essential oils in regards to their chemical composition, their effect on microorganisms and their potential use in AM packaging for food preservation.

There are several types of basil oil traded commercially. These oils are conventionally extracted by steam distillation from leaves and flowering tops. An alternative to the conventional steam distillation method is carbon dioxide (CO_2) extraction, under liquid or supercritical conditions.

Numerous investigations on basil essential oils have been reported in the scientific literature. These include studies of: (i) taxonomy (Paton 1992; Grayer et al. 1996; Paton and Putievsky 1996), (ii) chemistry (Lawrence et al. 1972; Amvam Zollo et al. 1998; Keita et al. 2000; Vieira and Simon 2000; Yayi et al. 2001), and (iii) AM activity (Prasad et al. 1986; Sinha and Gulati 1990; Lis-Balchin et al. 1998; Hammer et al. 1999; Friedman et al. 2002; Opalchenova and Obreshkova 2003).

2.3.1 Composition of basil extracts

2.3.1.1 Cultivar and chemotaxonomic classification

Most commercial basil cultivars available on the market belong to the *O. basilicum* L species. Darrah (1980) classified the *O. basilicum* cultivars into seven types: (i) Tall, slender types, including the sweet basil group, (ii) Large leafed, robust types, which include 'Lettuce Leaf' also called 'Italian' basil, (iii) Dwarf types, being short and small leafed, such as 'Bush' basil, (iv) Compact types such as *O. basilicum* var. *thyrsiflora*, commonly termed 'Thai' basil, (v) *Purpurascens*, the purple coloured basil types, with conventional sweet basil flavour, (vi) Purple types such as 'Dark Opal', a possible hybrid between *O. basilicum* and *O. forskolei* having lobed leaves, with a sweet basil plus clove-like aroma, and (vii) *Citriodorum* types including lemon-flavoured basils. The essential oils derived from *O. basilicum* L. have been traditionally classified into four distinct chemotypes with many subtypes (**Table 2.8**), based on the biosynthetic pathways that produce the principal components in the oil (Lawrence 1988). There is an enormous variation in composition of basil

Table 2.8 Chemotaxonomic classification of *O. basilicum* L. based on geographical origins

Chemotype	Major constituent	Country of origin
European	linalool, methylchavicol	France, Italy, Egypt, Hungary, South Africa, USA
Reunion	methylchavicol	Comoro Islands, Malagasy Republic, Thailand, Vietnam, Seychelles
Tropical	methylcinnamate	Bulgaria, India, Guatemala, Pakistan
Java	eugenol	Indonesia, North Africa, Russia

Adapted from: Simon et al. (1990), Grayer et al. (1996), Marotti et al. (1996)

essential oils. The different chemotypes contain various proportions of allyl-phenol derivatives, like methylchavicol (estragole), eugenol and methyleugenol as well as linalool, a monoterpene alcohol.

Simon et al. (1990) have classified chemotaxonomically the selected *Ocimum* sp. including *basilicum* (**Table 2.9**). Other basil essential oils have been reported to contain two or more major constituents in almost equal proportions or in various quantities as shown in **Table 2.10**. Clearly, these oils cannot be readily classified using the conventional system of chemotypes. Therefore it has been suggested that the profiles of the essential oils of basil be classified on the basis of all the main components, even if there are only three or four major volatile compounds present (Nicholson 1998). The chemical structures of the major constituents of basil are shown in **Figure 2.2**. The taxonomy of basil is also complicated due to the existence of numerous botanical varieties and cultivar names within the species that may not differ significantly in morphology (Simon et al. 1990). Paton and Putievsky (1996) proposed a system of standardised descriptions that includes volatile oils. This should allow easier communication and identification of the different forms of *O. basilicum*. Investigations to revise the genus are underway at the Royal Botanical Garden, Kew, London (Paton 1992) and at Delaware State University (Simon et al. 1999).

2.3.1.2 Compositional variation

The chemical analysis of essential oils derived from *O. basilicum* L. has been the subject of many studies with varying results from country to country

Table 2.9 Chemotaxonomic classification of *Ocimum basilicum* L. based on the USDA germplasm collection

PI number or cultivar name	Major constituent	Country of origin
175793	linalool	Turkey
368699	linalool, 1,8-cineole	Yugoslavia
358465	linalool, geraniol	Yugoslavia
174285	linalool, methylchavicol	Turkey
190100	methylchavicol, linalool	Iran
253157	methylchavicol, citral	Iran
170579	methylcinnamate, methylchavicol, linalool	Iran
Purdue selection	methyleugenol	Thailand

Adapted from: Simon et al. (1990)

Table 2.10 Major constituents of *O. basilicum* L.

Proportion	Major constituent	Reference
Almost equal	linalool, eugenol	Grayer et al. (1996)
	methylchavicol, methyleugenol	Grayer et al. (1996)
	linalool, methylcinnamate	Lachowicz et al. (1997)
Various quantities	linalool, methylchavicol	Opalchenova and Obreshkova (2003)
	linalool, eugenol	Hasegawa et al. (1997), Yayi et al. (2001)
	linalool, *trans*-α-bergamotene	Yayi et al. (2001)
	methylcinnamate, linalool, 1,8-cineole	Vieira and Simon (2000)
	methylchavicol, 1,8-cineole	Vieira and Simon (2000)
	methylchavicol, linalool, geraniol	Grayer et al. (1996)
	methylchavicol, citral	Simon et al. (1990)

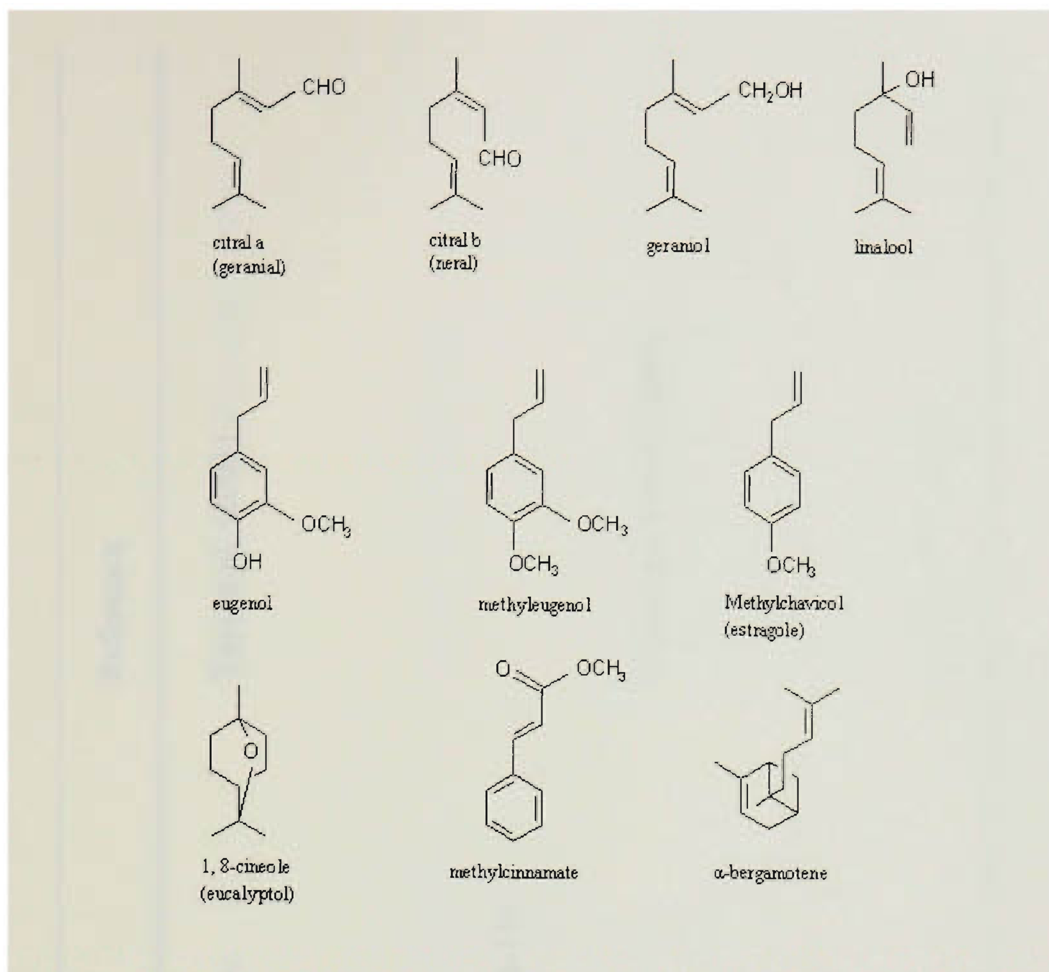


Figure 2.2 Chemical structures of major constituents found in *O. basilicum* L.

(Table 2.11). The variation in the chemical composition of basil essential oils is thought to be due to polymorphism in *O. basilicum* L., which in turn is caused by interspecific hybridisation (Paton and Putievsky 1996). In an early paper, Lawrence et al. (1972) listed a total of 75 chemical constituents in *O. basilicum* L. obtained from Thailand. They identified, for the first time, *trans*-ocimene oxide, germacrene D, caryophyllene oxide and τ-cadinol. Later, over 100 constituents were separated in various basil essential oils and 10 new compounds were identified (Vernin et al. 1984). Hasegawa et al. (1997), characterised the components of the essential oils produced from nine different cultivars of *O. basilicum* L. and oils from a local cultivar found in the Philippines. More than

Table 2.11 Compositional variation in *Ocimum basilicum* L.

Country of origin	Chemical composition/ % w/w	Reference
Benin		Yayi et al. (2001)
"methylchavicol"	methylchavicol (> 65)	
"methylchavicol-linalool"	methylchavicol (55), linalool (20-30)	
"linalool-eugenol"	linalool (42-45), eugenol (15), with or without *trans*-α-bergamotene (6-15)	
Brazil		Vieira and Simon (2000)
"linalool"	linalool (49.73)	
"1,8-cineole"	1,8-cineole (22)	
"methylchavicol"	methylchavicol (47)	
"methylcinnamate"	methyl-(*E*)-cinnamate (65.5)	

Table 2.11 (Continued)

Country of origin	Chemical composition/ % w/w	Reference
Bulgaria	linalool (54.95), methylchavicol (11.98), methylcinnamate (7.24)	Opalchenova and Obreshkova (2003)
Cameroon	linalool (50.8), eugenol (13.5), limonene (10.4), 1,8-cineole (3.1)	Amvam Zollo et al.(1998)
Cuba	methylchavicol (66.75), 1,8-cineole (5.44), linalool (4.95), α-bisabolene (3.60), (*E*)-α-bergamotene (2.96)	Pino et al. (1993)
Germany	methylchavicol (86.1)	Baratta et al. (1998)

Table 2.11 (Continued)

Country of origin	Chemical composition/ % w/w	Reference
Italy		Marotti et al. (1996)
"linalool"	linalool (70), 1,8-cineole (13)	
"linalool-methylchavicol"	linalool (41-60), methylchavicol (18-41), 1,8-cineole (2-6)	
"linalool-eugenol"	linalool (61-76), eugenol (4), 1,8-cineole (1-11)	
"eugenol", low linalool	eugenol (2.22-3.89), linalool (60.76-64.14)	
"eugenol", high linalool	eugenol (trace-1.99), linalool (69.06-76.20)	
Mongolia	methylchavicol (52), linalool (23.8), τ-cadinol (4.4)	Shatar and Altantsetseg (2000)
Republic of Guinea	linalool (69), eugenol (10), *trans*-α-bergamotene (3), thymol (2)	Keita et al.(2000)

Table 2.11 (Continued)

Country of origin	Chemical composition/ % w/w	Reference
Somalia	dihydrotagetone (> 80)	Ruberto et al. (1991)
Thailand	methylchavicol and α-humulene (88.2)	Lawrence et al. (1972)
Togo		Sanda et al. (1998)
"Reunion"	methylchavicol (84-89)	
"European"	linalool (41), methylchavicol (22)	
Turkey	linalool (17-24), methyl-(*E*)-cinnamate (12-16), 1,8-cineole (7-13), τ-cadinol (5-7)	Ozek et al.(1995)

130 compounds were identified in the latter cultivar, 32 of which were found in basil oil for the first time. Simon et al. (1999), reported a comparative evaluation of North American commercially available *Ocimum* sp. cultivars, including *basilicum*, as shown in **Table 2.12**.

2.3.1.3 Enantiomeric considerations

Ravid et al. (1997) determined the amount and the enantiomeric composition of linalool in the essential oils of seven chemotypes of *O. basilicum* L., in the oils of *Ocimum sanctum* L., *Ocimum gratissimum* L. and *Ocimum canum* Sims., originating from Thailand, and in commercial basil oils. The linalool isolated from cultivars of *O. basilicum* L., from various origins and from commercial basil oils consisted of (*R*)(-)-linalool and was optically pure in most cases. On the other hand, (*S*)(+)-linalool was the main enantiomer in essential oils of *O. sanctum* L. and *O. canum* Sims. Consequently, the enantiomeric differentiation of linalool may be useful in interspecific taxonomy in the genus *Ocimum*.

Tateo et al. (1999) studied the quantity and the enantiomeric composition of camphor in the essential oils of two types of *O. basilicum* L. from Italy. In regards to *O. basilicum* L. essential oils having a camphor content below 1%, a chiral analysis showed that the camphor (isolated from the so-called "Genuese type" containing mainly linalool (47-52% w/w) and a trace of methylchavicol) consisted of (*R*)(+)-camphor and was optically pure, in this type. For the so-called "Neapolitan type" (containing primarily linalool (47-63% w/w) and 9-12% w/w methylchavicol), (*R*)(+)-camphor is the main enantiomer with a content of

Table 2.12 Comparative evaluation of North American commercially available *O. basilicum* L. cultivars[1]

Cultivar	Colour Leaf	Colour Flower	Oil yield[2]/ % v/dw	Major Compounds[3]
Anise	green-purple	light pink	0.62	L-56%, MC-12%
Cinnamon	green	pink	0.94	L-47%, MCM-30%
Dark Opal	purple	pink	1.08	L-80%, 1,8-C-12%
Fino Verde	green	white	0.50	L-48%, MC-7%
Genovese	green	white	0.90	L-77%, 1,8-C-12%
Green Ruffles	light-green	white	0.55	L-33%, 1,8-C-18%
Holy Sacred Red	purple	pink	0.83	L-77%, 1,8-C-14%
Italian Large Leaf	green	white	0.83	L-65%, MC-18%
Lettuce Leaf	green	white	0.78	L-60%, MC-29%
Licorice	green-purple	pink	0.43	L-58%, MC-13%
Mammoth	green	white	0.77	L-60%, MC-32%
Napoletano	green	white	0.89	L-66%, MC-10%
Opal	purple	pink	0.91	L-80%, 1,8-C-13%
Osmin Purple	purple	pink	0.66	L-77%, 1,8-C-15%
Purple Ruffles	purple	bright purple	0.49	L-55%, 1,8-C-20%, MC-6%, ME-9%
Red Rubin Purple Leaf	purple	pink	0.74	L-70%, 1,8-C-9%, MC-10%
Sweet	green	white	0.84	L-69%, 1,8-C-11%, MC-13%
Sweet Fine	green	white	0.98	L-86%, 1,8-C-6%
Sweet Thai	green	pink	0.40	L-6%, MC-60%
Thai (Companion Plants)	green	pink	0.75	L-12%, MC-65%
Thai (Richters)	green	pink	0.52	MC-90%
Thai (Rupp Seeds)	green	white	0.25	L-15%, MC-13%

Adapted from: Simon et al. (1999)
[1] Plant density of 12,000 plants ha^{-1}
[2] oil yield % volume per dry weight
[3] L = linalool; MC = methylchavicol; MCM = methylcinnamate; 1,8-C = 1,8-cineole; ME = methyleugenol

above 94 %. It appears that the enantiomeric composition of camphor is a useful parameter in assessing the genuineness and is used as a characterising element of various botanical genuses.

2.3.1.4 Other factors affecting composition

A number of factors were found to affect the composition of basil extracts: (i) The harvest season and plant phenological stages including vegetative, early blooming, full blooming and seeding (Fleisher 1981). For instance, *O. basilicum* L. Cv Genovese Gigante, the basil cultivar used most in the production of typical Italian pesto sauce, contains linalool as its main component at the beginning of the flowering stage but methyleugenol and eugenol become significant between 4 and 6 weeks after sowing (Miele et al. 2001). (ii) The extraction method. It is claimed that the yields are highest using supercritical CO_2 followed by liquid CO_2 and then water (Lachowicz et al. 1996; 1997). However, these claims oppose the results obtained by Ehlers et al. (2001). The extraction method of basil also affects the chemical composition of the major volatiles in the extract. A hydrodistilled material contains a significantly larger proportion of the lower boiling point hydrocarbons such as pinene, myrcene, terpinene, limonene and oxygenated terpenes including 1,8-cineole, fenchone, and camphor. On the other hand, the CO_2 extracts contain a large number of unidentified high boiling point constituents with retention times, measured by gas chromatography, between those of geraniol (33.5 min) and eugenol (43.73 min), which are either present in small amounts (or are undetectable) in the hydrodistilled oil.

As far as the chemical composition is concerned, there appears to be no major benefits in using supercritical CO_2 over liquid CO_2 for extracting basil; actually, the production costs for extraction with liquid CO_2 are lower. However, sensory evaluation has shown that the hydrodistilled oil and the liquid CO_2 extracts (obtained with a one-stage separator under controlled conditions) were quite different (Lachowicz et al. 1996; 1997). (iii) Different milling techniques might induce modifications in the composition of the vegetable matrix and may have an adverse effect on the content of thermally labile compounds, such as linalool, methylchavicol and methylcinnamate (Reverchon et al. 1992). (iv) Freeze drying of basil leaves may affect the concentration of linalool (Grayer et al. 1996). (v) Supplementary UV-B treatment of glasshouse-grown sweet basil may affect the levels of most major volatiles such as the phenyl-propanoids (eugenol, methyleugenol) and the terpenoids (notably linalool, 1,8-cineole and *trans*-β-ocimen) (Johnson et al. 1999). (vi) The wavelengths of light reflected from coloured mulches can affect leaf size, aroma and concentrations of soluble phenolics in sweet basil. Plants grown over yellow and green mulches contain significantly higher levels of aroma compounds (including linalool and eugenol) than those grown over white and blue covers (Loughrin and Kasperbauer 2001). (vii) The content of methylchavicol and methyl-(*E*)-cinnamate is usually higher in field-grown basil than in greenhouse plants; in contrast, linalool content falls in all field-grown basil compared to greenhouse plants (Morales et al. 1993; Vieira and Simon 2000).

2.3.2 Testing methods for the AM activity of essential oils

Different methods of AM activity determination of essential oils (or essential oil components) affect the results. Numerous studies on the AM activity of essential basil oil and its principal constituents have been reported (Prasad et al. 1986; Sinha and Gulati 1990; Lis-Balchin et al. 1998; Hammer et al. 1999; Friedman et al. 2002; Opalchenova and Obreshkova 2003). However, it is difficult to compare the results of these studies because of substantial variations in the basil essential oils, test microorganisms and test methods. There is a need for the development and validation of standard methods to accurately determine the efficacy of essential oils or oil components and to compare the published data in different studies.

2.3.2.1 Principal methods

Generally, these techniques provide preliminary information on the potential usefulness of the tested compound. The techniques represented in this category include: (i) Diffusion methods - the agar diffusion test has probably been the most widely used in the past of the end point tests. It has been often referred to as the paper disc assay. However, this description is probably too narrow (Davidson and Parish 1989). Many variations in the test exist, including the use of cylinders, cups, wells, ditch plates and agar overlays (Barry 1986; Zaika 1988). A zone of "no growth" around the disc defines the extent of AM activity. The size of this zone depends on the rates of diffusion and cell growth (Barry 1986). Results of these tests are qualitative. Microorganisms are generally termed "susceptible", "intermediate", or "resistant", depending upon the diameter

of the inhibitory zone. Quantitative results are adequate with a high degree of standardisation, but better methods are available, (ii) Dilution methods - broth and agar dilution methods are widely used to determine the minimum inhibitory concentration (MIC) of oil-based compounds. The MIC is defined as the lowest concentration of the compound that inhibits growth of a microorganism after a specified incubation period (Parish and Davidson 1993), and (iii) Micro-atmosphere method - this method allows the determination of the AM activity of essential oils or essential oil components in the vapour phase. In this method, the agent diffuses towards the agar in an inverted Petri dish (Amvam Zollo et al. 1998), or a vial (Caccioni et al. 1997). Microbial growth is monitored through visible growth detection in the case of an inverted Petri dish. In the case of a vial, this growth is monitored by the presence, at equilibrium in the headspace, of metabolic carbon dioxide produced by the microorganism.

2.3.2.2 Limitation of the test methods

The AM activity of essential oils can be demonstrated by numerous methods. Methods involving agar media such as the paper disc diffusion assay, the agar well diffusion assay and the agar dilution assay are used most frequently. Due to the low water solubility of essential oils, emulsifiers such as Tween 20 (polyoxyehtylene-2-sorbitan monolaurate), Tween 80 (polysorbate 80) and Triton X100, or solvents like ethanol, are often used to enhance the solubility of hydrophobic compounds in both solid and liquid media. However, emulsifiers as well as solvents have attracted some criticism concerning their direct action on microorganisms and their possible effect on the AM activity of essential oils.

Emulsifiers are claimed to assist in the penetration of AM agents into the bacterial cell wall and membrane (Kim et al. 1995; Hammer et al. 1999).

The quantity of an emulsifying agent can also affect the results. Generally, lipophilic molecules, including the components of the essential oil of basil, may become soluble within the micelles formed by non-ionic surfactants such as Tween 20 and Tween 80, and thereby be partitioned out from the aqueous phase of the suspension (Schmolka 1973). Kazmi and Mitchell (1978) claimed that AM agents solubilised within micelles do not contribute to the AM activity, as they do not come in direct contact with the target microorganisms. Remmal et al. (1993) found that MICs vary with the kind of emulsifying agent used. They confirmed the fact that solvents and emulsifiers often used in AM studies decrease the AM activity of essential oils.

2.3.3 Results for the AM activity of basil extracts

The published data on the antibacterial activity of the basil essential oils and their constituents is larger than that on their antifungal activity. Unfortunately, the published data on the former subject are very difficult to compare. The chemical composition of basil essential oils and extracts is known to vary with the local climatic and environmental conditions (Vieira and Simon 2000). Thus, samples of basil essential oil may have the same common name even when they are composed of different subspecies of *O. basilicum* L. (Guenther 1975; Lawrence 1988). The selection of test microorganisms, the way of exposure of the microorganisms to basil essential oils and the method used to

evaluate their AM activity, all vary amongst the different publications (Zaika 1988; Remmal et al. 1993; Hulin et al. 1998).

Thus, the contradictory conclusions reached in the early studies on the AM activity of basil essential oils are not surprising. Poor solubility and high volatility often preclude the application of traditional AM assays, such as agar diffusion or zone-of-inhibition tests. As a result, agar or broth dilution methods using variable concentrations against a variety of target species are often used (Barry 1986; Parish and Davidson 1993). The findings of some studies are summarised in **Tables 2.13** and **2.14**.

2.3.3.1 Studies involving diffusion methods

Lahariya and Rao (1979) studied the AM effectiveness of the essential oil of *O. basilicum* tested *in vitro* against 10 different microorganisms. They found that this essential oil was more active than the reference, streptomycin, in inhibiting the growth of *Bacillus pumilus*, but it had no activity against *Bacillus mycoides*, *Pseudomonas mangiferae indica*, *Staphylococcus albus* and *Vibrio cholerae*. The oil was found to be most effective against *Bacillus anthraces*, and less effective against *Bacillus substalis* and *Salmonella paratyphi*. In addition, it had certain activity against all the tested fungi, including *Microsporum gypseum*, *Aspergillus fumigatus*, *A. niger*, *Penicillium liliacinum* but was less active than the reference, griseofulvin.

Table 2.13 Range of concentrations of basil extracts reported to inhibit the growth of microorganisms

Concentration	Test condition and inhibition type	Reference
Bacteria		
10 mg mL^{-1}	Liquid medium, MIC[1]	Aboul Ela et al. (1996)
1250-5000 μg mL^{-1}	Liquid medium, MIC	Ndounga and Ouamba (1997)
200 μL mL^{-1}	Liquid medium, viable cell count	Fyfe et al. (1998)
0.05-0.1 mL mL^{-1}	Liquid medium, MIC	Smith-Palmer et al. (1998)
>2-0.5 mL mL^{-1}	Solid medium, MIC, visible growth	Hammer et al. (1999)
5-100 μL mL^{-1}	Liquid medium, viable cell count	Koga et al. (1999)
0.007-0.03 μL mL^{-1}	Liquid medium, MIC	Opalchenova and Obreshkova (2003)
Fungi		
1-1.5 μL mL^{-1}	Liquid medium, mycelial growth	Dube et al. (1989)
5000 μg mL^{-1}	Liquid medium, MIC, mycelial growth	Ndounga and Ouamba (1997)
15.6-31.2 μg mL^{-1}	Liquid medium, MIC, mycelial growth	Rai et al. (1999)
Yeast		
6.25 mg mL^{-1}	Liquid medium, MIC	Aboul Ela et al. (1996)
1250 μg mL^{-1}	Liquid medium, MIC	Ndounga and Ouamba (1997)
0.5 mL mL^{-1}	Solid medium, MIC, visible growth	Hammer et al. (1999)

[1] MIC = minimum inhibitory concentration

Table 2.14 Range of concentrations of the principal constituents of basil extracts reported to inhibit the growth of microorganisms

Concentration	Test condition and inhibition type	Reference
Bacteria		
500-750 μg mL^{-1}	E[1], solid medium, visible growth	Moleyar and Narasimham(1992)
10-20 μg mL^{-1}	E, liquid medium, MICs	Nascimento et al. (2000)
>1000 μg mL^{-1}	L, solid medium, visible growth	Moleyar and Narasimham(1992)
2.5 mg mL^{-1}	L, liquid medium, MIC	Aboul Ela et al. (1996)
>13.3-3.33 μL mL^{-1}	L, liquid medium, MICs	Pattnaik et al. (1997)
>20-10 μL mL^{-1}	L, liquid medium, MICs	Wan et al. (1998)
1.25-20 μL mL^{-1}	MC, liquid medium, MICs	Wan et al. (1998)
Fungi		
125 μg mL^{-1}	E, liquid medium, mycelial growth	Hitokoto et al. (1980)
62.5-125 μg mL^{-1}	E, liquid medium, toxin production	Hitokoto et al. (1980)
200 μg mL^{-1}	E, liquid medium, mycelial growth	Moleyar and Narasimham(1986)
6.25 mg mL^{-1}	L, liquid medium, MICs	Aboul Ela et al. 1996)
> 5-0.2 μL mL^{-1}	L, liquid medium, MICs	Pattnaik et al. (1997)
125 μg mL^{-1}	MC, liquid medium, mycelial growth	Caccioni et al. (1997)
1-2.5 μL	MC, vapor phase in vial, CO_2 production	Caccioni et al. (1997)
Yeast		
6.25 mg mL^{-1}	L, liquid medium, MIC	Aboul Ela et al. (1996)
0.2 μL mL^{-1}	L, liquid medium, MICs	Pattnaik et al. (1997)

[1] E = eugenol; L = linalool; MC = methylchavicol

Reuveni et al. (1984) investigated the fungistatic activity of essential oils from *O. basilicum* chemotypes against *F. oxysporum* f. sp. *Vasinfectum* and *Rhizopus nigricans*. They reported that both European and Reunion chemotypes showed 100% inhibition of *R. nigricans* whereas the local selection type (in Israel) exhibited 96.4% inhibition of this strain. European, Reunion and local selection types exhibited 46.8, 2.6 and 65.4% inhibition of *F. oxysporum*, respectively. Conner and Beuchat (1984) determined the effects of plant essential oils on the growth of 13 food spoilage and industrial yeasts using a standard zone-of-inhibition test on yeast extract-malt extract-peptone-glucose agar (YMPG). The basil essential oil inhibited only slightly the growth of *Kloeckera apiculata* and there was no inhibition of *Candida lipolytica*, *Debaryomyces hansenii* and *S. cerevisiae*. This result is in agreement with the findings of Ozcan and Erkmen (2001), who used a dilution method, but it is in contradiction with the findings of Meena and Sethi (1994) and Lachowicz et al. (1998). This discrepancy might be due to a different chemotypes of sweet basil. However, further work is required to explain this contradiction.

Prasad et al. (1986) studied the AM activity of essential oils of *O. basilicum* (French), *O. basilicum* (Indian), and *O. basilicum* (Niazbo) which are rich in linalool, methylchavicol, and methylcinnamate respectively, against 11 Gram-positive and 7 Gram-negative bacteria. They found that these oils were more effective against Gram-positive than against Gram-negative bacteria. For example, all the Gram-positive bacteria *Bacillus sacharolyticus*, *Bacillus stearothermophilus*, *B. subtilis*, *Bacillus thurengiensis*, *Micrococcus glutamicus* and *Sarcina lutea* were inhibited by each of these Basil essential oils. However,

the Gram-negative strain *Salmonella weltevreden* only was suppressed by all the oils. Prasad et al. (1986) also found that methylcinnamate type basil essential oil inhibited all the 13 tested fungi and only *Histoplasma capsulatum* grew in the presence of linalool type basil oil. *Candida albicans*, *H. capsulatum*, and *Sporotrichum schenckii* were found to be resistant to methylchavicol type basil oil. Sinha and Gulati (1990) found that each of these basil essential oils was also effective against *S. aureus*, *E. coli*, *Salmonella typhi*, *S. paratyphi*, *Shigella boydii*, and *Proteus vulgaris*. All basil essential oils showed also an antifungal effect on *C. albicans*, and *S. schenckii*, with methylchavicol type basil essential oil being highly effective. This effect has been predominantly associated with the main constituents, linalool and methylchavicol.

Deans and Ritchie (1987) screened 50 plant essential oils (including basil) for their antibacterial properties against 25 genera of bacteria by using the agar diffusion technique. They found that most of the bacteria, including *Aeromonas hydrophila*, *B. subtilis*, *Brevibacterium linens*, *B. thermosphacta, Erwinia carotovora*, *E. coli*, *Lueconostoc cremoris*, *S. aureus*, *Streptococcus faecalis* and *Yersinia enterocolitica*, show a reasonably broad sensitivity to undiluted basil essential oil. Gangrade et al. (1989) examined the antibacterial properties of the linalool and the methylcinnamate types of the essential oils of *O. basilicum*, in the pure state and at four dilutions (1:10, 1:100, 1:1000 and 1:10000, prepared with dimethyl sulfoxide, DMSO) against 4 major bacterial species. They found that both essential oils had an inhibitory activity against *S. aureus* and *E. coli* at all dilutions. The dilution of either oil with DMSO beyond 1:1000 resulted in no inhibition against *Streptococcus pyogens*. Basil essential oil also showed an

inhibition against *B. cereus*, *Lactobacillus acidophilus*, *A. niger* and *S. cerevisiae*, as determined by the paper disc agar diffusion method, both at ambient temperature and at 37 °C (Meena and Sethi 1994). These results were expanded and supported by Aboul Ela et al.(1996) and Elgayyar et al. (2001), who showed that basil essential oil has antibacterial and antifungal activity against *S. aureus*, *E. coli*, and *A. niger*.

Baratta et al. (1998) reported that the methylchavicol type of basil essential oil showed a significant activity against the growth of *S. aureus* food poisoning organisms. They also reported that *B. subtilis, B. thermosphacta, E. carotovora*, *B. linens* and *Pseudomonas aeruginosa* were resistant to undiluted basil essential oil. These findings contradict those obtained by Dean and Ritchie (1987) and Lachowicz et al. (1998), presumably because of the different chemotypes of basil essential oil used in the two studies. In two publications, Lis-Balchin and Deans (1997) and Lis-Balchin et al. (1998) described the relationship between the bioactivity and chemical composition of commercial essential oils, including that of the methylchavicol type basil essential oil. The authors reported that a strong bioactivity was observed when the major component was eugenol and a less pronounced one when the main constituents were geraniol, citronellol and linalool. Methylchavicol has not shown a strong AM activity. The findings of Lis-Balchin et al. (1998) contradict those of Reuveni et al. (1984), Sinha and Gulati (1990), and Baratta et al. (1998).

Essential oils extracted by hydrodistillation from 5 different varieties of *O. basilicum* L. plants (Anise, Bush, Cinnamon, Dark Opal and a commercial

sample of dried basil) in Australia, were examined by the agar well diffusion method for their AM activity against a wide range of food-borne Gram-positive and Gram-negative bacteria, yeasts and moulds. All five essential oils of basil showed AM activity against 20 out of 24 tested microorganisms including *A. hydrophila*, *B. cereus*, *B. subtilis*, *B. thermosphacta*, *E. coli*, *L. plantarum*, *L. monocytogenes*, *Mucor piriformis*, *Penicillium candidum*, *Penicillium expansum*, *S. cerevisiae*, *S. typhimurium*, *S. aureus*, *Candida colliculosa*, *Candida formata*, *Candida humicola* and *Zygosaccharomyces bailli*. In Addition, the spectrum of AM activity did not vary greatly between oils from the different varieties of basil, except for *Enterococcus faecalis* that was found to be resistant to Cinnamon basil oil but sensitive to the other 4 basil oils. *Pseudomonas* species were found to be resistant to all the tested oils (Lachowicz et al. 1998). Nascimento et al. (2000) found *P. aeruginosa* to be susceptible to basil essential oil (containing linalool, methylchavicol and eugenol). Rai et al. (1999) examined the antifungal activity of the essential oils of 10 plant species (including *O. basilicum*), grown in Chhindwara, India, against five *Fusarium* species. They found that the essential oils of basil (methylcinnamate-rich type) were active against all *Fusarium* species, and especially active against *Fusarium acuminatum*, *Fusarium solani*, *Fusarium pallidoroseum* and *Fusarium chlamydosporum*.

The AM activity of the individual principal constituents of basil essential oil (linalool, methylchavicol, eugenol and methylcinnamate) was also studied. Knobloch et al. (1989) evaluated the AM activity of essential oil components against Gram-negative bacteria (e.g. *Enterobacter aerogenas* and *P. vulgaris*), Gram-positive bacteria (e.g. *S. aureus* and *B. subtilis*), and fungi (e.g. *Aspergillus*

flavus, *A. niger*, *Aspergillus ochraceus* and *P. expansum*). They found that linalool, with its high water solubility, had a significant AM activity compared to cinnamaldehyde, citral, geraniol, eugenol and menthol whereas methylchavicol, with its lower water solubility, had a low AM activity. The ability of essential oil constituents to penetrate the cell walls of a bacterium or fungus is directly related to their solubility in water. Thus, the AM activity of essential oils is due to their solubility in the phospholipid bilayer of cell membranes (Knobloch et al. 1989). It was also reported that the antibacterial activities of monoterpene alcohols (including linalool, nerol, citronellol and geraniol) are more effective than their antifungal activity.

Meena and Sethi (1994) found that eugenol has an inhibitory effect against *A. niger*, *L. acidophilus* and *S. cerevisiae*. Kim et al. (1995) studied the antibacterial activity of some essential oil components (including linalool and eugenol) against 5 food-borne pathogens (*E. coli*, *E. coli* O157: H7, *S. typhimurium*, *L. monocytogenes* and *Vibrio vulnificus*). They found that eugenol showed a dose-related increase in the zone-of-inhibition against the five strains, whereas linalool exhibited a similar effect against all tested strains except for *L. monocytogenes*. Linalool inhibited the growth of *L. monocytogenes* but the difference in the zone size between the test concentrations (5, 10, 15 and 20% v/v) was not significant.

Pattnaik et al. (1997) studied the antibacterial properties of the aromatic constituents of essential oils. The results of the disc diffusion assays showed that linalool was the most effective compound and retarded 17 out of 18 bacterial

strains (only VR-6, a *Pseudomonas*, was found to be resistant), followed by cineole, geraniol, menthol and citral. They also found that the MIC values of the essential oils were usually lower than those of their constituents. One possible reason for this result could be the synergistic action of the constituents in the oils. Mazzanti et al. (1998) found that linalool was the active compound that completely inhibited the growth of all yeasts (seven strains of *C. albicans*, *Candida krusei* and *Candida tropicalis*), *S. aureus* and *E. coli*. Authentic pure linalool showed a similar antibacterial spectrum to that of basil essential oils. However, pure methylchavicol exhibited a much narrower antibacterial spectrum, with an activity against only 8 out of the 24 strains of organism tested (Lachowicz et al. 1998). This result is in contradiction with the findings of Wan et al. (1998), in spite of the fact that the same parameters and the same experimental technique were used. A possible explanation might be batch-to-batch variations (Lis-Balchin and Deans 1997) or a difference in sources of compounds.

Scora and Scora (1998) investigated the fungicidal effect of volatile compounds, with the main basil essential oil components, against three *Penicillium* species. It is known that phenolic compounds like carvacrol, thymol and eugenol posses a major fungicidal effect. Etherified compounds like anethole, methylchavicol and safrole exhibit less fungicidal action while monoterpene hydrocarbons like limonene and β-myrcene have almost no effect. As noted by Knobloch et al. (1989), the variation in the fungicidal action of essential oil components seems to rely on their water solubility and lipophilic properties (i.e., their ability to penetrate the chitin-based cell walls of fungal hyphae). Specific

functional groups and the interference of membrane-associated enzyme proteins may also affect the results (Kurita et al. 1981; Knobloch et al. 1989).

Recently Dorman and Deans (2000) reported on the antibacterial activity of 21 plant volatile oil components (including eugenol and linalool) against 25 bacterial strains by the agar well diffusion technique. Eugenol exhibited the widest spectrum of activity against 24 out of 25 bacteria, except for *L. cremoris*, followed by linalool (against 23 strains, except *L. cremoris* and *P. Aeruginosa*). These results contradict those obtained by Lachowicz et al. (1998) and Wan et al. (1998) who used the same technique and found that linalool inhibited *L. cremoris*. The components with phenolic structures, including eugenol, were highly active against the test microorganisms. Members of this class are known as either bacteriocidal or bacteriostatic agents, depending on the concentration (Pelczar et al. 1993). These components are strongly active despite their relatively low solubility in water (Knobloch et al. 1989; Suresh et al. 1992; Kim et al. 1995). Alcohols are known to possess bacteriocidal rather than bacteriostatic activity against vegetative cells. The tertiary alcohol, linalool, is active against the test microorganisms, potentially acting as either a protein denaturing agent (Pelczar et al. 1993) or as a solvent dehydrating agent. Knobloch et al. (1989) demonstrated the relationship between water-solubility of terpenoids and their AM activity on whole cells. The solubility of essential oils and their terpenoid compounds in water should therefore be taken into consideration when studying the action of these compounds on the membrane-catalysed functions within the cell wall that acts as a physical barrier.

2.3.3.2 Studies involving agar and broth dilution methods

Agar dilution

Dube et al. (1989) studied the antifungal activity of the essential oil of *O. basilicum* by an agar dilution method. They showed that the essential oils of basil at a concentration of 1.5 mL L^{-1} completely suppresses the mycelial growth of 22 species of fungi, including the mycotoxin-producing strains of *A. flavus* and *Aspergillus paralyticus*. In addition, the lethal dose of the oil was found to be 4 times less than that of Agrozim, Bavistin, and Emison and 6 times less than Sulphex and Celphos. The oil of *O. basilicum* is evidently a potent mycotoxic agent endowed with the ability to kill aflatoxin-producing strains. Therefore, this oil is more effective and preferable, being natural, over synthetic fungicides.

Hammer et al. (1999) studied the AM activity of a large number of essential oils and other plant extracts (including basil essential oils) against a diverse range of organisms using either the agar dilution or the broth micro-dilution method. The MICs of basil essential oils obtained by the agar dilution method ranged from 0.5 to > 2.0% v/v. The essential oils of basil inhibited all tested organisms at a concentration below 2.0% v/v except for *E. faecalis*, *P. aeruginosa* and *Serratia marcescens*.

Kurita et al. (1981) examined the antifungal activity of 47 kinds of essential oils and several related compounds against 7 fungi. The results suggest that secondary alcohols (e.g. 2-octanol, L-menthol, borneol) and tertiary alcohols (e.g. linalool) possess a markedly lower antifungal activity compared to primary alcohols such as cinnamyl alcohol, geraniol and citronellol. The antifungal

activity of eugenol (4-allyl-guaiacol), a phenolic compound, was found to be 8-10 times higher than that of guaiacol (*o*-methoxyphenol), and 3-4 times higher than that of creosol (4-methylguaiacol).

From the molecular structure, it is clear that the addition of alkyl or alkenyl group(s) to the benzene ring of either phenol or guaiacol enhances the AM activity. The activity of these phenolic compounds appeared to depend on the size of the added alkyl or alkenyl group, where the larger the size of the alkyl or alkenyl group, the stronger the AM activity (Kurita et al. 1981; Knobloch et al. 1989; Pelczar et al. 1993). Since alkyl or alkenyl groups are hydrophobic, these results indicate that a hydrophobicity above a minimum extent is required for phenolic compounds to show a potent AM effect.

Reuveni et al. (1984) studied the percentage of inhibition of principal constituents of basil on *R. nigricans* and *F. oxysporum.* They found that both linalool and methylchavicol had the highest percentage of inhibition (100%) against *R. nigricans* while a value of 38.1% only was found for eugenol. The reverse was found for *F. oxysporum* where the percentage inhibition of eugenol was highest (100%), while for linalool and methylchavicol the values were only 26.4% and 30.3% respectively. In addition, the AM activities of basil essential oils of different chemotypes were predominantly related to their main components (Reuveni et al. 1984; Farag et al. 1989). This is in agreement with the results of Pattnaik et al. (1997) working with citral, a major antifungal component in lemongrass.

The study by Karapinar and Aktug (1987) on the inhibition of food-borne pathogens by 4 components of spice showed that eugenol is the most effective inhibitor against *S. typhimurium*, *S. aureus* and *Vibrio parahaemolyticus*. Moleyar and Narasimham (1992) studied the antibacterial activity of 15 essential oil components against the food-borne pathogens: *Staphylococcus* sp., *Micrococcus* sp., *Bacillus* sp. and *Enterobacter* sp., using an agar plate technique. Cinnamic aldehyde was found to be the most active compound, followed by citral, geraniol, eugenol and menthol. Linalool was found to exhibit only a slight antibacterial activity.

Broth dilution

Different researchers used the broth dilution method reporting the following results. Hitokoto et al. (1980) claimed that basil leaves show a complete inhibition of ochratoxin A, in the production of *A. ochraceus* and a partial inhibition of the growth and toxin production of *A. flavus* and *Aspergillus versicolor*, and the growth of *A. ochraceus*. Basilico and Basilico (1999) investigated the inhibitory effects of some spice essential oils, including the essential oil of basil (*O. basilicum*), on *A. ochraceus* growth and ochratoxin A production. They reported that at a level of 1000 ppm, only basil affected the fungal growth and the production of ochratoxin A up to 7 days but permitted mould growth afterwards. This is in agreement with the work of Hitokoto et al. (1980).

Lis-Balchin et al. (1998) studied the antifungal activity of the methylchavicol type of basil essential oil against 3 fungi and found that the oil exhibited 94, 76 and 71% of inhibition on *A. niger*, *A. ochraceus*, and *Fusarium culmorum* respectively. These results are in agreement with those of Baratta et al. (1998) who worked with the agar well diffusion method and found that methylchavicol type basil essential oil shows 93.1% inhibition on *A. niger*. Amvam Zollo et al. (1998) concluded from the MIC results of the oil, as determined by the broth micro-dilution method after 7 days of incubation, that *O. basilicum* essential oil has an important antifungal activity. The oil was fungicidal against *C. albicans*, and *A. flavus* at 5000 ppm but it was not fungistatic on *Cryptococcus neoformans* up to 1250 ppm.

Recently, Ozcan and Erkmen (2001) studied the antifungal activity of basil essential oil collected in Turkey. They found the oil to be ineffective on *S. cerevisiae*, *A. niger* and *Rhizopus oryzae*, contrary to the findings of Prasad et al. (1986), Dube et al. (1989), and Meena and Sethi (1994). Again, this contradiction might be due to the different chemotype of sweet basil or due to different test methods.

Smith-Palmer et al. (1998) examined the AM properties of 21 plant essential oils and 2 essences (including basil essential oil) against 5 predominant food-borne pathogens: *Campylobacter jejuni*, *S. enteritidis*, *L. monocytogenes*, *S. aureus* and *E. coli*. The results of bacteriocidal and bacteriostatic concentrations showed that the two Gram-positive bacteria, *S. aureus* and *L. monocytogenes*, are more sensitive to inhibition by plant essential oils than the three Gram-negative

bacteria. This is in agreement with the results of Prasad et al. (1986). Thus, in general, lower bacteriostatic and bacteriocidal concentrations are required for basil essential oil against *S. aureus* and *L. monocytogenes*. It is not completely clear why Gram-negative bacteria should be less susceptible, but it may be associated with the outer membrane of Gram-negative bacteria that endows the bacterial surface with strong hydrophilicity and acts as a strong permeability barrier (Nikaido and Vaara 1985).

Fyfe et al. (1998) studied the inhibition of *L. monocytogenes* and *S. enteritidis* by combinations of plant essential oils with either benzoic acid or methyl-paraben (ester of *p*-hydroxybenzoic acid). This work highlighted the fact that the essential oil of basil at 0.2% v/v in the broth is a potent inhibitor of both strains where cells are undetectable (< 10 colony forming units (cfu) mL^{-1}) at 4, 8, 24 and 48 h. Even after 1 h only of exposure, there were only 3.4 and 1.4 $\log_{10}$ cfu mL^{-1} of the cultures *L. monocytogenes* and *S. enteritidis* present respectively. Fyfe et al. (1998) suggested that the properties of basil essential oil should be determined in both a broth and a food system.

Lachowicz et al. (1998) reported that a synergistic antibacterial effect was found when a combination of 5% w/v of sodium chloride (NaCl) and 0.1% v/v Anise basil essential oil in MRS broth (pH 6.2) was used. This system completely suppressed the growth of *Lactobacillus curvatus* up to 99 h (which was the time for growth detection) compared to the Anise basil essential oil (51.4 h) or 5% NaCl (28.3 h) alone. The work of Mejlholm and Dalgaard (2002) showed that

0.1% v/v basil essential oil resulted in over 85% reduction in the growth rate (RGR) of *Photobacterium phosphoreum* in a liquid medium at 2 and 15 °C.

Koga et al. (1999) studied the bacteriocidal activity of basil and sage essential oils against a range of bacteria, including *V. parahaemolyticus*, by viable count determination. Using this method, they were able to compare the bactericidal activity in both the exponential and stationary growth phases. Their findings show that Gram-positive bacteria exhibit higher resistance to basil essential oil than Gram-negative bacteria. *S. aureus*, *Micrococcus luteus*, and *B. subtilis* show very high resistance to the essential oil of basil. The viability of these three strains treated with 1% v/v of this essential oil was above 90%. *L. monocytogenes* and *B. cereus* were more sensitive to basil essential oil than other Gram-positive bacteria. Nearly all Gram-negative bacteria exhibited high sensitivity to basil essential oil. In particular, *Vibrio* species and *Aerobacter hydrophila* had very high sensitivities to this oil. The viability of these strains treated with 0.01% v/v basil essential oil ranged from 0.014 to 3.64% (Koga et al. 1999). There is a partial disparity between the results of Koga et al. (1999) and those of Prasad et al. (1986), Smith-Palmer et al. (1998), and Elgayyar et al. (2001).

Opalchenova and Obreshkova (2003) have recently studied the activity of basil against multi-drug resistant clinical bacterial strains by using different test methods including MICs determination and time-kill kinetics. The experimental data obtained after the application of different methods of investigation demonstrated a strong inhibitory effect of basil essential oil extracted from *O.*

basilicum L. on multi-drug resistant clinical isolates of the genera *Staphylococcus*, *Enterococcus*, and *Pseudomonas*. The chosen bacteria are widespread and pose serious therapeutic difficulties because of their high extent of resistance. For this reason, Opalchenova and Obreshkova (2003) considered that the results they obtained were encouraging.

According to Mahmoud (1994), the antifungal action and antiaflatoxigenic properties of certain essential oil constituents (including linalool and eugenol) could be determined on a toxigenic strain of *A. flavus*. Similarly myrcene, ocimene, δ-3-carene and linalool appeared to cause slight enhancement of both growth and aflatoxin production. Initially, no growth or aflatoxin production in the presence of eugenol for up to 8 days was observed. This can be attributed to the presence of the aromatic moiety and the phenolic-hydroxy group of eugenol; the latter is known to be reactive and forms hydrogen bonds with active sites on target enzymes (Farag et al. 1989). After 8 days, there was a poor vegetative growth, accompanied by a remarkably high concentration of aflatoxin. This observation is in accordance with the observation made by Basilico and Basilico (1999), in spite of the different species tested.

Kim et al. (1995) used a liquid culture assay and found that eugenol possesses a potent inhibitory/bacteriocidal activity against the 5 bacterial strains: *E. coli*, *E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes*, and *V. vulnificus*, followed by linalool. Wan et al. (1998) later determined the effect of BSL and BMC on the growth of *A. hydrophila* and *P. fluorescens*. The effect of BMC (0.1 and 1% v/v) on resting cells (10^5 cfu mL^{-1}) of *A. hydrophila* and *P. fluorescens* in

saline (0.9% w/v NaCl) was also determined after treatment at 20 °C for 10 min. The addition of either 0.1 or 1% v/v BMC caused a decrease in the viable count of *A. hydrophila* to levels below the detection limit (< 1 cfu mL^{-1}). BMC at 0.1% v/v showed no effect while at the level of 1% it was bacteriocidal also to *P. fluorescens* resting cells.

Recently, Friedman et al. (2002) have screened a broad variety of naturally occurring and potentially food compatible plant-derived oils and oil compounds for the AM activities of these against an epidemiologically relevant class of four species of bacterial food-borne pathogens, *C. jejuni*, *E. coli*, *L. monocytogenes*, and *S. enterica*. It was found that eugenol and methylchavicol (estragole) are the most active agents showing AM activity against all of these pathogens.

2.3.3.3 Studies involving the micro-atmosphere method

Caccioni et al. (1997) studied the antifungal activity of natural volatile compounds (including methylchavicol) by monitoring their vapour pressures. Methylchavicol appeared to be active against *P. expansum* and *B. cinerea*, when added to the liquid substrate or when introduced directly into the headspace. In the latter case, it was active at much lower doses. Methylchavicol was less effective in the vapour phase than hexanal at the same temperature and concentration. At the same dose in the headspace, hexanal induced fungistasis at a level approximately tenfold higher than that of methylchavicol.

The vapour pressure, at a given temperature, of a specific volatile molecule in a biological system can be used as an indirect measure of its "actual hydrophobicity". It is inversely related to its capacity to form links with the sheath of water molecules surrounding its polar groups (Caccioni et al. 1997; Guerzoni et al. 1997). The reason for it is that the tendency of a molecule to pass into the vapour phase is associated with the level of its interaction with water and various solutes (Caccioni et al. 1997; Gardini et al. 1997). At a constant concentration and temperature, the higher the vapour pressure, the lower the steric hindrance, due to the linked water molecules, and the higher is the hydrophobicity (Caccioni et al. 1997). However, the ability of a potentially active molecule to interact with the hydrophobic cell membranes can be regarded as a result of its intrinsic hydrophobicity which increases with the hydrocarbon chain length and/or with the presence of double bonds (Knobloch et al. 1989; Caccioni et al. 1997) and with its "actual hydrophobicity" (Guerzoni et al. 1997). Thus, due to its higher volatility, hexanal has proven to be biologically more active than methylchavicol, even though methylchavicol is more hydrophobic (Caccioni et al. 1997).

2.3.4 Food preservation

Arora et al. (1977) found that oranges coated with an emulsion containing an essential oil (including basil essential oil) or a volatile compound (*Citrus reticulata* Blanco), had a longer shelf life than uncoated ones. Oranges treated with geraniol were rendered almost completely free from blue mould decay ($\geq$ 95%). Other treatments, in a decreasing order of effectiveness, were found to be

mentha and basil essential oils. Montes-Belmont and Carvajal (1998) showed that basil oils cause a total inhibition of fungal (*A. flavus*) development on maize kernels. The optimal dosage for protection of maize was 5% v/v with hexane as the solvent. In addition, no phytotoxic effect on germination and corn growth was detected with this oil.

Ismail et al. (2001) studied the efficacy of the immersion of raw poultry in herb decoctions (including basil decoction) on the reduction of the population of *Yarrowia lipolytica*, predominant yeasts believed to play an important role in the spoilage of raw poultry. They found that a significant reduction in the populations of *Y. lipolytica* occurred when the yeast was inoculated into 100% basil, marjoram, sage, or thyme decoctions, but not in 100% oregano or rosemary decoctions, kept at 5 °C for 24 h. Further studies included only the treatment of chicken wings with sage or thyme decoctions. It was found that 100% sage or thyme decoctions significantly decreased the populations of *Y. lipolytica* but did not control its growth during storage at 5 °C for up to 9 days.

Lock and Board (1996) examined the effect of acidulants and oils on the autosterilisation of home-made mayonnaise. They found that the death rate of *S. enteritidis*, a major cause of human salmonellosis, differed amongst the various oils. Olive oil with garlic or basil showed the fastest rate of death of *S. enteritidis*, followed by soya, grape seed, rape seed, groundnut, sunflower, hazelnut and a blended olive oil.

Wan et al. (1998) studied the effect of washing fresh lettuce with methylchavicol on the survival of natural flora. They found that the effectiveness of washing the lettuce with 0.1 and 1% v/v methylchavicol derived from basil in regards to the total viable count and the presumptive counts of *Pseudomonas*, *Aeromonas* and Enterobacteriaceae was comparable to that of washing the lettuce with a 125 ppm chlorine solution. Since chlorine-based washing systems may produce harmful by-products (like chloramines and trihalomethanes), this result indicates that methylchavicol, from basil, could offer a natural alternative to washing of selected fresh salad produce and replace (or minimise) the need for chlorine solutions containing chlorine concentrations of up to 200-300 ppm (Beuchat 1996). Wan et al. (1998) suggested enhancing fresh salad preservation by delivering essential oils to the product in the washing solution of the vegetables. Avina-Bustillos et al. (1993) suggested incorporating these oils into an edible coating whereas Nicholson (1998) mentioned a food packaging film containing these oils. Such uses of essential oils would depend on cost considerations, the original odour and flavour of the oils and their suitability for the type of final product (Wan et al. 1998).

Lachowicz et al. (1997, 1998) assessed the AM effect of basil essential oil (Anise variety) on the growth of *L. curvatus* and *S. cerevisiae* in a tomato juice medium. They found that the growth of these microorganisms was completely inhibited by 0.1 and 1.0% v/v anise basil oil containing 44% linalool and 27% methylchavicol.

2.3.5 The future developments in food preservation

Basil essential oil and its principal constituents are not widely used as food preservatives. The published data show that these compounds have a potential use in food preservation, especially in conjunction with technologies of AM packages for food products. Further research on the AM activity of basil essential oil and its main components, and a better understanding of the mode of action are required in order to evaluate its usefulness in the shelf life extension of packaged foods like bakery, meat, poultry, seafood and cheeses. An additional challenge is in the area of odour/flavour transfer from packages containing natural plant extracts to the packaged foods. Thus, research is needed to determine whether natural plant extracts could act as both an AM agent and as an odour/flavour enhancer in packaged food.

2.4 Microencapsulation of AM additives

Cyclodextrins (CDs) are cyclic oligosaccharides that have been derived enzymatically from starch and have the ability to encapsulate other molecules within their ringed structure (**Figure 2.3**). Cyclodextrins are represented as shallow truncated cones composed of 6, 7, and 8 glucose units and termed α-, β-, and γ-CD, respectively. From the manufacturing point of view, the main product is β-CD. The physical properties of β-CD are listed in **Table 2.15**. Purification of the α-, and γ-CD considerably raises their production cost and puts them into the fine-chemical classification, i.e. very expensive.

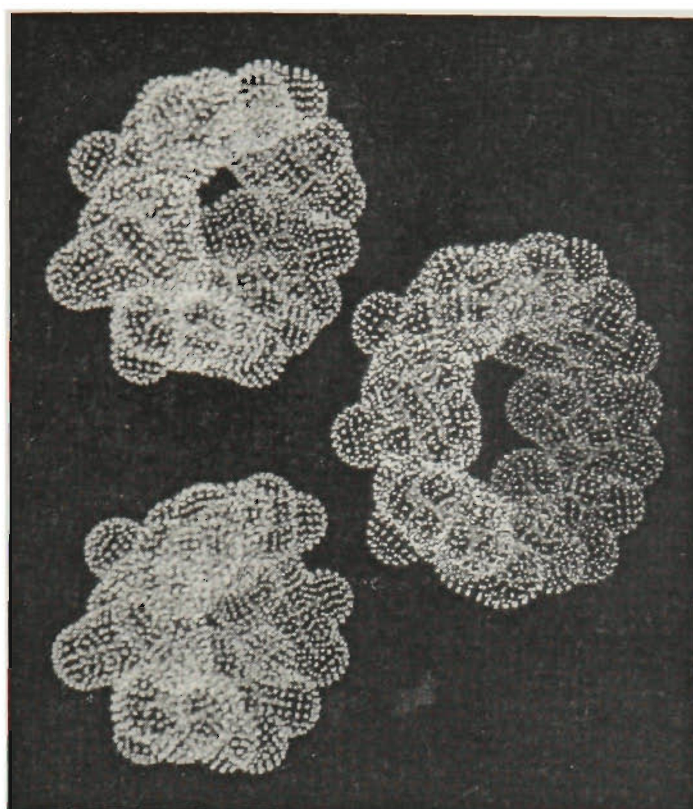


Figure 2.3 Computerised 3-D structure of cyclodextrins (Anonymous 1988)

Table 2.15 Physical properties of β-cyclodextrin

Characteristic	β-Cyclodextrin
Number of glucose units	7
Molecular weight	1135
Inner diameter of cavity/ Å	6.2
Outer diameter of cavity/ Å	15.4
Height of cavity/ Å	7.9
Volume of cavity/ $Å^3$	262
Solubility in water at 25 °C/ g $(100\ mL)^{-1}$	1.85-1.88
Specific rotation at 25 °C	162.5 ± 0.5
Melting range/ °C	255-265

Adapted from: Pszczola (1988); Szejtli (1988); Qi and Hedges (1995); Lu et al. (2001); Rusa et al. (2001)

The primary polar hydroxyl groups project from one outer edge and the secondary polar hydroxyl groups project from the other end (**Figure 2.4**). While outer surfaces (tops and bottom) are hydrophilic, an internal cavity has a relatively high electron density and is hydrophobic in nature, due to the hydrogens and glycosidic oxygens oriented to the interior of its cavity (Shahidi and Han 1993). Because of the hydrophobic nature of the cavity, the molecules of suitable size, shape, and hydrophobility enable noncovalent interaction with CDs to form stable complexes. For instance, β-CD molecules are able to form inclusion complexes with volatile compounds of typical molecular mass ranging from 80 to 250 (Rusa et al. 2001). Microencapsulation is one of the most effective techniques for protecting them against oxidation, thermal degradation, and evaporation (Szente and Szejtli 1988; Reineccius 1989; Hedges et al. 1995).

This protection occurs because volatile molecules are held tightly within the molecular structure of β-CD. The interaction between β-CD (host) and volatile molecules (guests) may involve total inclusion or association with only the hydrophobic part of the molecule (Shahidi and Han 1993). Goubet et al. (1998) state that retention of volatile compounds is a complex phenomenon in which several factors take part. With particular regard to the volatile compound, chemical function, molecular weight, steric hindrance, polarity and relative volatility have been shown to be important. For example, the higher the molecular weight, the higher the retention (Reineccius and Risch 1986; Goubet et al. 1998). Among the chemical groups reviewed, alcohols are usually the best retained compounds by carbohydrates including β-CD. The same trend, namely a higher retention of alcohols than that of other compounds, has been observed also

when encapsulating a mixture of 10 volatiles in β-CD. Linalool was the most retained compound among mixtures including five esters, two aldehydes, γ-deca-lactone, and butyric acid (Fleuriot 1991). As far as polarity is concerned, the more polar the compound the less of it is retained (Voilley 1995). According to Saravacos and Moyer (1968) and Bangs and Reineccius (1981), the higher the relative volatility of a compound, the lower is its retention. When the carrier is considered, it has been shown that retention is influenced by its chemical functions, its molecular weight and its state. Based on literature data, Goubet et al. (1998) summarised that the amorphous state of the carrier is the most efficient for retention of volatiles, the collapsed state results in a loss of volatiles and crystallisation leads to the largest release of the encapsulated compounds.

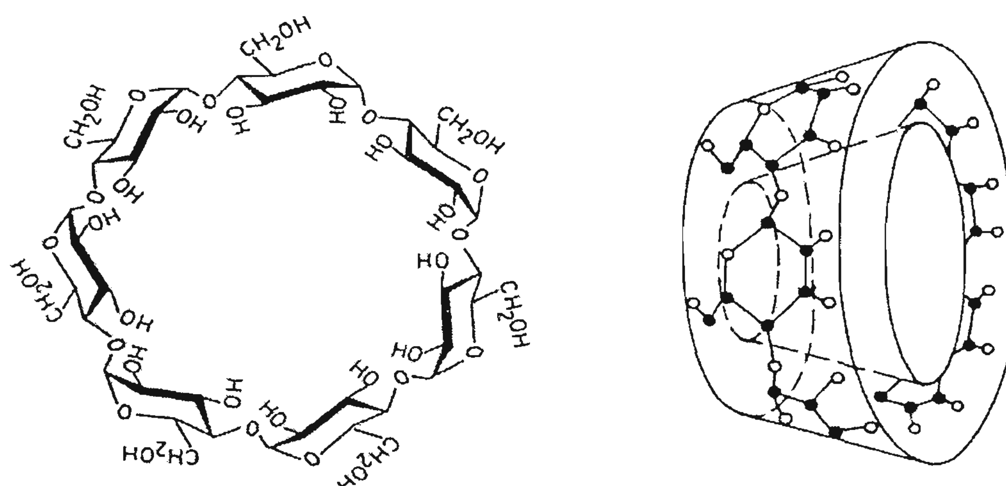


Figure 2.4 Molecular structure of β-cyclodextrin (Pszczola 1988)

Recently, Reineccius et al. (2002) reported that γ-CD generally functioned best in terms of initial flavour retention. On storage, however, losses of volatiles were greater for γ-CD than for α-CD. Cyclodextrins were found to stabilise several problematic, less stable flavour compounds, with α- and β-CD showing the best overall retention during spray-drying and storage. Cyclodextrins may well prove to be useful in stabilising flavour compounds that cannot be effectively stabilised using other techniques.

Trotta et al. (2000) studied the thermal degradation of CDs and substituted β-CDs. They found that in an inert atmosphere they all decompose in one major step (252-400 °C) leaving a residue (char), that continues to decompose at a low rate when the temperature is further increased. However, the decomposition temperature, the char yield and the thermal stability depend on the type of substituent in the cyclodextrin.

Currently, β-CD is widely used in the food, pharmaceutical, medical, chemical, and textile industries. Huang et al. (1999) proposed using an inclusion compound (IC) between an antibiotic and β-CD in biodegradable/bioabsorbable film for medical applications. Antibiotic ICs have been incorporated into bandage, dressing, and sutures. Later, Lu et al. (2001) reported a promising result for Irgasan DP300 (Triclosan)-β-CD-IC embedded in biodegradable/ bioabsorbable poly(ε-caprolactone) films. These are rendered resistant to the growth of *E. coli*.

2.5 Estimation of population size of microorganism

The accurate estimation of the population size of a microorganism in relation to microbial growth parameters, particularly lag time (λ) and maximum specific growth rate (μ_{max}), is important in many areas of microbiology; for instance, in investigating the effects of AM agents for optimising microbial media and developing kinetic models for application in food technology and biotechnology. Turbidimetric estimation from absorbance measurements has the benefits of being rapid, non-destructive, inexpensive and relatively convenient compared to other techniques, particularly viable count methods.

Turbidity is the result of light scattering by a colloidal suspension and can be related to viable counts by a linear relationship (Hudson and Mott 1994; Begot et al. 1996; Dalgaard and Koutsoumanis 2001) Many microbial suspensions are colloidal since the cells do not settle out quickly from the suspension. These suspended cells scatter light, causing turbidity (Ball 1997). Differential light scattering measurement depends on the number of cells and their morphology (i.e. size, shape, and structure of the cells) (Berkman and Wyatt 1970). As the number of cells rises a proportional increase in turbidity occurs. However, the relationship between turbidity and cell concentration is different for each species. However, this technique may have some problems. In the optical density (OD) measurement, the initial inoculum size must be large enough for the instrument to measure accurately the OD and quantify the turbidity. If the inoculum is too small, the measured lag time becomes the time required for the culture to reach a sufficiently high turbidity to be measured by the instrument, as demonstrated by Shida et al. (1975). Absorbance measuring devices have typically detection

thresholds in the range of 10^6-10^7 cells mL^{-1} (Perry and Staley 1997). Such an initial inoculum size will result in a linear relationship between optical density and cell count over a narrow range of 10^7-10^9 cells mL^{-1} (Rattanasomboon et al. 1999).

2.6 Release of AM additives

Apart from AM activity of the films, understanding the release rate which is controlled by diffusion is also an essential aspect in developing AM food packaging materials. Several studies on the slow release of AM agents (acetic acid, allyl isothiocyanate, benzoic anhydride, benzoyl chloride, hexamethylenetetramine, nisin, potassium sorbate, propionic acid, propyl paraben, sorbic acid) from packaging films into food simulants have been reported (Weng and Hotchkiss 1993; Redl et al. 1996; Lim and Tung 1997; Han and Floros 1998, 2000; Devlieghere et al. 2000a, b; Ouattara et al. 2000b; Choi et al. 2001; Chung et al. 2001a, b; Kim et al. 2002a; Teerakarn et al. 2002; Cha et al. 2003b; Ozdemir and Floros 2003).

Diffusivity is an important parameter in predicting the rate of migration of an AM agent from a polymeric AM film onto the food. The release profile from an AM film is in the opposite direction to that of sorption (such as flavour scalping) (Sadler and Braddock 1991). The relationship between the sorption and the desorption, or releasing action, is given in **equation 2.1**:

$$[M_t/M_\infty]_{desorption} = 1 - [M_t/M_\infty]_{sorption} \qquad \textbf{(2.1)}$$

where M_t is the total amount of small molecules migrated after time t, and M_∞ is the maximum amount of molecules that can migrate after infinite time.

Several methods for the measurement of diffusion have been reported (Fox 1980; Naesens et al. 1981; Giannakopoulos and Guilbert 1986; Lim and Tung 1997). Redl et al. (1996) suggested a relatively convenient and rapid method for determining additive release from AM films by immersing them in food simulants such as distilled water, buffer solution, iso-octane, ethanol, acetic acid, rectified olive oil (Feigenbaum et al. 2000; McCort-Tipton and Pesselman 2000).

2.7 Microorganisms in foods

Microbial activity is often the primary mode of deterioration and loss of quality and safety of many foods (Hotchkiss 1997). Microorganisms require certain conditions for growth and reproduction. In packaged foods, these conditions are either determined by intrinsic properties of the food such as pH and water activity (A_w) or extrinsic factors related to the storage environment (Parry 1993). The latter factors can be dealt with by using AM packaging to retard spoilage and extend shelf life.

Microbial food spoilage causes undesirable sensory changes in colour, texture, flavour or odour and ropiness which can render the food unsaleable or inedible. Spoilage is an important safeguard in preventing food poisoning outbreaks by providing warning signs of deterioration to the consumer.

Some microorganisms like *Salmonella* spp., *E. coli* O157:H7 and *Cryptosporidium parvum* are responsible for food poisoning, and have caused recently outbreaks in the US and other parts of the world which, in turn, has raised public concerns. With the emergence of food-borne organisms with a low infective dose, food poisoning cases are sporadic in nature and detectable primarily *via* surveillance. *Campylobacter* spp. is recognised as a common cause of food poisoning in developed countries. *Y. enterocolitica* food poisoning may not have been reported in full. Another organism which is capable of low-temperature (4 °C) multiplication is *L. monocytogenes*. There have been notable outbreaks in Western Europe and in the US. Food poisoning caused by *Clostridium perfringens* is usually associated with mass catering, notably an inadequate cooling of large quantities of contaminated cooked meat products (Eley 1996a; Christian et al. 2003).

New methods of food preservation include the use of natural AM additives, microwave processing, hydrostatic pressure, electrical resistance heating, high-voltage pulse techniques, microbial decontamination, aseptic processing and modified atmosphere packaging (MAP) and active packaging (AP) (Gould 1995). Microorganisms may still survive such treatments and some of the processes (e.g. MAP) may even enhance potential growth of food-borne pathogens due to temperature abuse by retailers and consumers (Parry 1993).

Hazard Analysis Critical Control Points (HACCP)-based approach to food safety in achieving food safety outcomes may have a number of limitations

related to microbiological limits. Microbiological criteria may be used by regulators for both domestic and border control, to provide assurance that a particular food is safe to be eaten. Microbiological criteria used for compliance testing however, generally provide poor assurance of safety, especially when there is a low extent of microbiological contamination. This is often the case for pathogens such as enterohaemorrhagic *E. coli* (EHEC) and *L. monocytogenes*. Moreover, when the shelf life of a food product is short, by the time an evaluation is made as to whether the food contains a suspected pathogen, the bulk of the food may have already been consumed and the adverse events are not prevented (De Buyser et al. 2001; Christian et al. 2003).

It is clear that as far as microbial contamination in foods is concerned, there are still challenges that have to be surmounted and that food packaging requires additional innovative solutions. One way of controlling the growth of undesirable microorganisms on foods is by incorporating AM agents into the packaging materials (Suppakul et al. 2003a).

Several researchers reported on the inactivation of microbial growth on different food products by AM films or coatings. The food products include curled lettuce, soybean sprout, cucumber, strawberries, ground beef, chicken breasts, oysters, Tilapia fillets, hot dogs, Bologna, summer sausage, ham, bread, orange juice, milk and Chinese Oolong tea (Baron 1993; Ishitani 1995; Huang et al. 1997; An et al. 1998; Chung et al. 1998; Lee et al. 1998; Devlieghere et al. 2000b; Andres et al. 2001; Ha et al. 2001; Cagri et al. 2002, 2003; Kim et al. 2002a, b; Soares et al. 2002).

2.8 Cheddar cheese

Cheese is the most diverse class of dairy product that has been investigated considerably and still is most interesting and challenging for studies. As a result of the dynamic activity of enzymes and microorganisms, cheeses are inherently unstable. Throughout manufacture and ripening, cheese production demonstrates a finely orchestrated series of consecutive and concomitant biochemical synchronised and balanced reactions, resulting in products with highly desirable aroma and flavours (Fox 1993). The final sensory attribute of cheese is affected by the type of milk treatment such as pasteurisation, irradiation, micro-filtration, and high-pressurisation (Kheadr et al. 2002; Pandey et al. 2003). The safety aspect is the driving force for making cheese from pasteurised milk, as the most common pretreatment. Some pathogens like *Salmonella, E. coli* O157: H7 and *L. monocytogenes* are of high concern in raw milk and they are normally destroyed during pasteurisation. Despite the safety concern, there is still a large demand for cheeses made from raw milk as they possess strong and unique flavours. These characteristic flavours are derived from the native enzymes and bacteria present in raw milk and are not obtainable in the heat-treated milks. In the US, the government continues to view raw-milk products as a health threat to the public since raw-milk cheese products have been associated with outbreaks of diseases in recent years (De Buyser et al. 2001).

The occurrence of yeasts in cheese is not unexpected due to the low pH, low moisture content, elevated salt concentration and low storage temperatures (Fleet 1990). Yeast population counts of 10^4 - 10^6 cfu g^{-1} are frequently found in cheeses (Fleet and Mian 1987; Welthagen and Viljoen 1999). The yeasts, being

natural contaminants in the cheese making process, contribute to the ripening by metabolising lactic acid (Lenoir 1984) and contribute to the sensory quality of the cheese. However, an increase in pH due to lactic acid utilisation may encourage the growth of bacteria that affect the flavour and texture but may also pose a risk to public health (Viljoen 2001). *D. hansenii* and *Y. lipolytica* are typical food-borne yeast species prevailing in dairy products and these are capable of predominating the yeast composition in such systems. Ferreira and Viljoen (2003) reported recently that application of both *D. hansenii* and *Y. lipolytica* as part of the starter culture for the production of matured Cheddar cheese is recommended because of the good strong flavour achieved after a ripening period of 4 months. The cheese prepared in this way had a clean, slightly sweet, pleasant taste and retained its good, strong flavour after 9 months.

The growth of filamenteous fungi is the main spoilage problem on cheese. There are only a few fungi species (e.g. *Penicillium commune*, *Penicillium nalgiovense*, *Penicillium verrucosum*, *Penicillium roqueforti*, *Penicilium solitum*, and *A. versicolor*) that cause problems in cheese. (Lund et al. 1995; Nielsen and Haasum 2000). Lund et al. (1995) reported that the most important spoilage fungi of hard, semi-hard and semi-soft cheeses not containing preservatives are *P. commune* and *P. nalgiovense*. In hard cheese (Cheddar type), a variety of moulds were found as environment and surface contaminants, but only *Pencillium glomerata* was present in all cheeses that showed dark stains (Basilico et al. 2001).

Cheddar is a hard natural cheese. It varies in colour from pale to deep yellow, and the flavour ranges from mild and creamy (mild Cheddar) to strong and biting (mature Cheddar) (Robinson 1995). The flavour characteristics develop largely as a result of enzymatic activity over a variable period of maturation (O'Riordan and Delahunty 2003). Cheddar cheese is essentially produced by a microbial fermentation process of cow-milk by selected lactic acid bacteria. Mesophilic cultures, with an optimum temperature of ~30 °C, are used in the production of Cheddar. Defined mesophilic cultures consisting of two or more strains of *Lactococcus lactis* are generally used for the manufacturing of most Cheddar cheese worldwide. *Streptococcus thermophilus*, a thermophilic strain, may be used as an adjunct culture. Undefined or mixed-strain mesophilic cultures, mainly composed of *L. lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis*, that may include citrate metabolising strains (for flavour production) are also used to some extent (Beresford et al. 2001; Cogan and Beresford 2002).

Since adjunct starters are used in the manufacturing of Cheddar, the strains of *S. thermophilus* and/or *Lactobacillus helveticus* is quite common. Kiernan et al. (2000) demonstrated that *L. helveticus* autolyzed very rapidly in Cheddar and resulted in significantly higher levels of free amino acids and an improved flavour profile of the cheese.

During ripening, secondary flora also develop. These include non-starter lactic acid bacteria (NSLAB), enterococci, yeasts and moulds (Rehman et al. 2000; Cogan and Beresford 2002; Ferreira and Viljoen 2003; Giraffa 2003). The role of NSLAB in the development of Cheddar flavour has been a contentious

issue for many years. Numerous studies have shown that lactobacilli, used as adjuncts, can affect flavour development. It would appear that selection of the adjunct strain is crucial, because certain strains of *Lactobacillus casei* produced high quality Cheddar, wile other strains of these species resulted in acidic cheese with bitter flavour defects (Lawrence and Gilles 1987).

Twenty species of enterococci are recognised, but the most common ones are *E. faecalis* and *Enterococcus faecium*. *E. faecium* PR88, in cheese, and were recently used as probiotic adjunct culture in Cheddar cheese (Hunter et al. 1996; Stanton et al. 1998). Yeasts are also involved in the ripening process of cheese and partake in microbial interactions and contribute to texture changes and the biosynthesis of aromatic compounds like volatile acids and carbonyl compounds (Fleet and Mian 1987; Welthagen and Viljoen 1999).

Rindless cheese, including Cheddar, which is mass produced and subjected to long-term storage, is frequently packaged in pouches and vacuum sealed. The pouches are commonly made from Polyvinylidene chloride copolymer (PVDC) (Robertson 1993). In spite of the protection that PVDC films offer, cheese blocks are subject to development of mould on their surfaces and/or corners. In many cases, the surface of the cheese is contaminated after it has been formed into blocks. The films provide a good physical barrier but may not be effective in retarding incipient spoilage, especially as a result of loss of vacuum or improper handling. Nielsen and Haasum (2000) suggested that AM releasing films could be considered for this type of active cheese.

Contamination of cheese by foodborne pathogens and spoilage microorganisms is of great concern in the cheese industry. Apart from incipient culture, microbial contamination of cheese may occur in post-processing or in the packaging and distribution stages as well as in multiple opening in reclosable packaging. *L. monocytogenes* is a facultative anaerobic, Gram-positive, psychrotrophic, motile, non-spore-forming, small rod-shaped bacterium, growing under temperature conditions ranging from 1 to 44 °C (Adams and Moss 2000; Ray 2001). The cells are relatively resistant to freezing, and are harmed by pH ≤ 5.0 (Ray 2001). *Listeria monocytogenes* is often present in nature and has been found in soft and semi-soft cheese (Farber et al. 1987). Previous work indicated that *L. monocytogenes* survives for more than a year in Cheddar cheese (Ryser and Marth 1987). For more than 50 years it has been recognised as being a human and animal pathogen. It causes Listeriosis - a disease that primarily causes miscarriage in pregnant women, meningitis in newborn infants, and affects adults with weakened immune systems (Ryser and Marth 1989). However, the behaviour of the pathogen depends mainly on the strain of *L. monocytogenes* (Ryser and Marth 1987) and on the conditions of the cheese during the manufacturing, ripening and storage period (Erkmen 2003). *Listeria monocytogenes* continues to pose a major threat to the food industry as a post-processing contaminant. Commonly found in home refrigerators, this psychotrophic pathogen can readily contaminate refrigerated foods and grow in some products to potentially hazardous levels. An identical strain of *L. monocytogenes*, *L. innocua* is often selected for inhibition studies because it is not pathogenic, but closely related to *L. monocytogenes*, as results of DNA-DNA

hybridisation, multilocus enzyme analysis, and 16S rRNA sequencing experiments have shown (Swaminathan 2001).

Escherichia coli is a Gram-negative, facultative anaerobic, non-spore-forming, mobile, rod-shaped bacterium, and a normal inhabitant of the intestinal tract of humans and warm-blooded animals and birds. It has been used as an index organism of fecal contamination and of the presence of enteric pathogens in food and water. Since the mid-1940s, evidence has been accumulated showing that certain *E. coli* strains cause diarrhea, particularly in infants. Some strains can produce enteric, urinary tract and wound infections as well as food poisoning, and occasionally septicaemia and meningitis (Eley 1996b; Ray 2001). Recently, they have been divided into nine groups: (i) enteropathogenic *E. coli* (EPEC), (ii) entertoxigenic *E. coli* (ETEC), (iii) enteroinvasive *E. coli* (EIEC), (iv) enterohaemorrhagic *E. coli* (EHEC), (v) enteroaggregative *E. coli* (EAEC), (vi) diffuse-adhering *E. coli* (DAEC), (vii) necrotoxigenic *E. coli* (NTEC), (viii) *E. coli* producing cytolethal-distending toxin (CDT), and (ix) Shiga toxigenic *E. coli* (STEC). The strains in EHEC group (a principal serogroup is O157: H7) have been recognised recently as the cause of severe bloody diarrhea (haemorrhagic colitis) and haemorrhagic uremic syndrome (HUS) in humans (Meng et al. 2001; Ray 2001; Bell 2002; Desmarchelier and Fegan 2003). In all cheeses, *E. coli* is considered to be the organism of primary concern (Hasell and Salter 2003). The emerging public health problems of listeriosis and haemorrhagic colitis, as well as HUS prompted this investigation into the inhibition of *L. innocua* and *E. coli* during the storage period of Cheddar cheese.

2.9 Food contamination from packaging material

From time to time, contamination in food from the packaging material becomes an important issue to be considered. Lau and Wong (2000) reviewed the current state of knowledge about contamination in food products from packaging materials. Packaging materials have been found to present a source of contamination due to the migration of substances that are categorised into three major classes: (i) additives in polymers, (ii) monomers and oligomers, and (iii) contaminants from processing additives and decomposition products. Such contamination may cause not only safety but also flavour issues.

One of the most common reasons for consumer rejection of food products is an undesirable and/or unacceptable flavour. Every year the food industry receives complaints from consumers considering off-flavour or taints in fresh, processed and packaged foods. Flavour problems usually stem from consumer complaints. Hence, apart from the immediate financial costs resulting from having to dispose of a batch of tainted food, the loss of consumer confidence in the product has very crucial long-term consequences (Mottram 1998).

One of the most frequent causes to food taints results from the transfer of residual solvents used in printing or lamination processes during package manufacturing (Whitfield 1986; Reineccius 1991; Ewender et al. 1995; Tice 1996; Mottram 1998). Traces of some monomers and oligomers may also cause off-flavours. For instance, off-flavours were detected in multi-layered polystyrene containers from residual styrene monomer, and ethylbenzene that had diffused into yoghurt (Durst and Laperle 1990; Ehret-Henry et al. 1994; Tang et al. 2000).

Andersson et al. (2002) studied the volatile compounds present in smoke from extrusion of LDPE film in a coating line. They found and identified 40 aliphatic aldehydes and ketones, together with 14 different carboxylic acids. The film thickness affected the concentrations of the degradation products, with a thicker film giving higher amounts of by-products. Since many of the identified compounds have a very characteristic taste and smell, this subject is of interest in food packaging applications.

Possible off-flavours should be considered also when dealing with AM packages that have been designed to slowly release active substances onto food products. (Suppakul et al. 2003a).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Polymers

The polymers used in the present studies included linear low-density polyethylene (LLDPE) (Dowlex 2045 E, Dow Chemical, Australia), low-density polyethylene (LDPE) (Alkathene XJF 143, Qenos Pty. Ltd., Australia) and ethylene vinyl acetate copolymer (EVA) (Escorene™ Ultra LD 318, ExxonMobil Chemical, USA).

3.1.2 AM additives

The AM additives used in the experiments were linalool (L260-2, Aldrich Chemical Company, Inc., USA) and methylchavicol (AUSTL 21320, Aurora Pty. Ltd., Australia) with the purity of 97% and 98%, respectively.

3.1.3 Chemicals

The chemicals used in this research were methylcellulose (MC) (18,804-2), hydroxypropyl methylcellulose (HPMC) (42,321-1), polyethylene glycol (PEG) (20,236-3) supplied by Aldrich Chemical Company, Inc., USA. Ethanol was supplied by CSR Ltd., Australia. Iso-octane (Unichrom 2516-2.5L GL) was purchased from APS Chemicals, Australia. β-cyclodextrin (β-CD) (C-4767) of 98% purity was supplied by Sigma-Aldrich, Australia. Sodium dihydrogen orthophosphate (NaH_2PO_4. $2H_2O$) (30132), di-Sodium hydrogen orthophosphate

(Na_2HPO_4) (30158.5000) and Sodium chloride (NaCl) (10241.AP) were purchased from BDH Chemical Australia Pty. Ltd.

3.1.4 Media

The media used in the present studies were nutrient broth (CM 1), nutrient agar (CM 3), and potato dextrose agar (CM 139) purchased from Oxoid, USA. Bacteriological agar (RM 250), plate count agar (AM 144), trytone soya broth (AM 185) and YM broth (RM 229) were obtained from Amyl, Australia.

3.1.5 Count plates

The count plates used in the experiments were 3M Petrifilm™ aerobic count plates, 3M Petrifilm™ *E. coli* and coliform count plates and 3M Petrifilm™ yeast and mould count plates. All these count plates were supplied by 3M Microbiology Products, USA.

3.1.6 Microorganisms

The microorganisms used in this research were *Staphylococcus aureus* (FSA 3601), *Listeria innocua* (FSA 2305), *Escherichia coli* (FSA 1301) and *Saccharomyces cerevisiae* (FSA 3301). All of the test strains were obtained from the Culture Collection of Food Science Australia, Werribee, Victoria, Australia.

3.2 Methods

3.2.1 Preliminary study of AM films

3.2.1.1 Preparation of AM LLDPE films

Linear low-density polyethylene (LLDPE) films of 45-50 μm thickness containing either linalool or methylchavicol as well as films without these additives were prepared from LLDPE pellets. Additive-free LLDPE pellets were ground and the powder was doped in linalool or methylchavicol dissolved in isooctane. A pre-blended master batch powder containing approximately 15% w/w linalool or methylchavicol was mixed with additive-free LLDPE pellets and manufactured into films by the extrusion film blowing process using a single screw extruder with a diameter of 50 mm (Telford Smith, Australia). The target additive concentration was approximately 1.0% w/w linalool or methylchavicol. The temperature profile in the extruder was 195, 205, 210, 200, 210 °C from the first barrel zone to the die. Films without linalool or methylchavicol were also manufactured and were used as controls as well as the substrate for solution-coating and absorption methods for preparing AM films.

In the preparation of AM films by coating, the coating solution was made from 7.0 g methylcellulose, 3.0 g hydroxypropylmethylcellulose, 100 mL distilled water, and 200 mL 95% v/v ethanol. Methylcellulose and HPMC were slowly added to the distilled water and stirred until they completely dissolved. Ethanol was slowly added to the solution and 6 mL of polyethylene glycol was added as a plasticiser. Linalool or methylchavicol was then added to the coating solution at a level that rendered the final coated films with the appropriate

concentration of the extract in the coating solution. The coating medium was applied to the LLDPE film using a roller and the film was then dried in air at room temperature for 24 h. The thickness of the coating was determined by the difference in thickness between the non-coated and the coated film measured with a hand-held micrometer with a precision of 0.001 mm (Hahn and Kolb Stuttgart, Germany).

For preparing films *via* the absorption technique, linalool or methylchavicol was dissolved in isooctane at a level appropriate to achieve the correct concentration of the extract (after absorption) in the film, on a mass basis. Film pieces, 2.5 × 2.5 cm, were then immersed in this solution at room temperature overnight in order to absorb the agent. Subsequently, excess isooctane was evaporated at room temperature in a fume cabinet.

3.2.1.2 Additive quantification in LLDPE film

The actual concentration of linalool or methylchavicol in the prepared samples was determined by gas chromatography (GC). The procedure was as follows: 5 g of film was extracted for 18 h by Soxhlet extraction using 150 mL of isooctane. An aliquot of the extract of a precisely known volume was sampled for GC analysis. A Varian Star 3400-CX GC equipped with a fused silica capillary column DB-5 (30 m × 0.25 mm i.d., film thickness 0.25 μm, J & W Scientific, USA) was used. The following conditions were applied: injected volume, 1.0 μL; initial column temperature, 80 °C, heating rate: 5 °C min^{-1} up to 180 °C, kept at this temperature for a additional 5 min; injector temperature, 250 °C, split ratio,

1:100; FID detector temperature, 300 °C; carrier gas, nitrogen. The linalool and methylchavicol contents of the samples were calculated from prepared standard curves.

3.2.1.3 AM activity of LLDPE films in solid media

The films were tested for their inhibition against the selected microorganisms: *Staphylococcus aureus* (Gram-positive bacteria), *Escherichia coli* (Gram-negative bacteria) and *Saccharomyces cerevisiae* (yeast) by using an agar disc diffusion method (Acar and Goldstein 1986; Parish and Davidson 1993) (**Table 3.1**).

All microorganisms used in the microbiological assay were twice-passaged 15 h cultures grown in either nutrient broth or YM broth. Optical densities of all inocula were measured at 620 nm using a Pharmacia LKB Novaspec II spectrophotometer. Cell densities were estimated from standard curves and confirmed by the "pour plate" method on plate count agar for bacteria and on potato dextrose agar for yeast (Swanson et al. 1992).

Each film sample was cut into a circle of 5 mm in diameter and sterilised with UV light for 2 min (Cooksey 2000) prior to being placed on an agar plate surface seeded with 1 mL of test culture consisting of 10^6 organisms. The plates were incubated for 1-2 days at the required temperature for each culture. The clear zone formed around the film disc in the media was recorded as an indication

Table 3.1 Experimental-conditions used for the agar disc diffusion assay on AM films in the preliminary study

FSA No.[1]	Test organism	Medium[2]	Time/ h	Temperature/ °C
3601	*Staphylococcus aureus*	Nutrient Agar	24	37
1301	*Escherichia coli*	Nutrient Agar	24	37
3301	*Saccharomyces cerevisiae*	YM Agar	48	30

[1] Culture collection of the Food Science Australia (FSA), Werribee, Victoria, Australia

[2] Nutrient Agar, prepared from Nutrient Broth (Oxoid, CM1) and bacteriological agar (Amyl, RM250); YM Agar, prepared from YM Broth (Amyl, RM229) and bacteriological agar

of inhibition of the microbial species. The evaluation of inhibitory activity was carried out in quadruplicate, by measuring the diameter of the inhibition zone with a Vernier caliper with a precision of 0.02 mm (Mitutoyo, Japan). An average of four diameter measurements, taken 45° apart from each other, was used as the result of each test.

3.2.1.4 Data analysis

Individual experiments in solid media were performed in quadruplicate. Data points were represented by the mean. Data sets were subjected to a variance (ANOVA) analysis and the Tukey test at the 0.05 level of significance using KyPlot 2.0 for Windows (Kyence Inc., Japan).

3.2.2 Microencapsulation of AM additives

3.2.2.1 Precipitation method

The compounds β-CD, linalool and methylchavicol were used as raw materials in the microencapsulation process. The moisture content of the β-CD was 9.59% w/w (dry weight basis) as determined by an air-oven method (AOAC 1999). A precipitation method (Reineccius 1989) was employed to prepare the β-CD-linalool complex. 10 gram of β-CD was dissolved in 100 mL of an ethanol/water mixture (1:2) maintained at 55 °C on a hot plate. A predetermined quantity of linalool dissolved in ethanol (10% w/v) was then slowly added to the warm β-CD solution. The heating was stopped and the mixture was covered and stirred for 4 h. The final solution was cooled overnight at 4 °C. The precipitated β-CD-linalool complex was recovered by filtration. This precipitate was dried in an oven at 50 °C for 24 h. The powder was then removed from the oven, allowed to dry in air for an additional 24 h and weighed. The amount of complex recovered was calculated on the basis of dry weight. Finally, the β-CD-linalool complex was stored at 25 °C in an airtight container.

The starting mass ratio of core material (linalool) to β-CD was 15:85. The β-CD-methylchavicol complex was prepared by the same method as that used to produce the β-CD-linalool complex. Each treatment was performed in duplicate. The statistical differences between the means were determined using the Student *t* test at $p \leq 0.05$ (least-squares difference).

3.2.2.2 Moisture determination

The moisture contents of β-CD-linalool and β-CD-methylchavicol complexes were analysed by the air-oven method (AOAC 1999), at 105±2°C for 3 h. The required drying operation was repeated until the difference in weight between two successive measurements was not greater than 0.1% of the weight of the sample.

3.2.2.3 Additive quantification in β-CD complex

The amount of linalool or methylchavicol in the samples was determined by gas chromatography (GC). The procedure was as follows: 5 g of inclusion compound was Soxhlet extracted with isooctane. An aliquot of the extract with a precisely known volume was then sampled for GC analysis as described in **Section 3.2.1.2**.

3.2.3 Sensitivity enhancement of OD measurement

3.2.3.1 Design of the spectrophotometer

A UV-VIS spectrophotometer (Cary 300, Varian Australia Pty., Ltd.) was used to measure the amount of light transmitted transversely through a path length cuvette (rectangular, 17.5 mL; Cary UV-Varian Australia Pty., Ltd.). The path length of the cuvette was 50 mm. The accuracy of the measurement is ± 0.005 unit of optical density (UOD) and the photometric reproducibility ± 0.002 UOD. This apparatus was used for OD measurements, at wavelengths of 400, 470, and 540 nm.

3.2.3.2 Microorganism and medium

The reference strain, *Escherichia coli* was used in this study. Stock culture was stored at –80 °C in nutrient broth. Working culture was grown at 37 °C for overnight on Nutrient agar and subcultured twice at 37 °C in nutrient broth for 18 h in order to obtain cells in the stationary growth phase. Cells were harvested by centrifugation at 6,000 × g for 2 min and washed once with a 5 mM NaCl solution. The supernatant was discarded and the cells were washed again. Cells were re-harvested and suspended in fresh nutrient broth. Cell densities of approximately 1×10^3 cfu mL^{-1} were calculated and prepared from cultures of approximately 7.50×10^8 cfu mL^{-1} for *E. coli* by dilution with 0.1 M sodium phosphate buffer (pH 7.0).

3.2.3.3 Growth experiments

All experiments were conducted at a constant temperature of 37 °C and cultures were shaken continuously at 100 rpm in an incubation shaker (Innova™ 4230, New Brunswick Scientific, U.S.A.). The initial concentration of bacteria (as indicated by viable count) was 10^3 cells mL^{-1}. Growth data were obtained intermittently and comparisons were made between turbidimetry and plate count. For the plate count method using 3M Petrifilm™ aerobic count plate, samples were diluted in 0.1 M sodium phosphate buffer (pH 7.0), after which 1 mL aliquots were spread in duplicate on 3M Petrifilm™ aerobic plates. Viable numbers were estimated from colony counts after an incubation period of 24 h at

37 °C. Growth curves and correlation between optical density and viable cell count were generated.

3.2.4 Study of AM films

3.2.4.1 Preparation of AM LDPE-EVA films

Low-density polyethylene (LDPE)-ethylene vinyl acetate (EVA) films of 45-50 μm thickness with and without linalool or methylchavicol were prepared from LDPE pellets. Pre-blended master batch EVA powder containing approximately 15% w/w linalool or methylchavicol was mixed with virgin LDPE pellets and manufactured into films with a concentration of 1.5% w/w linalool or methylchavicol by extrusion film blowing in a single screw extruder (Telford Smith, Australia). The temperature in the extruder was approximately 160 °C (all zones). Low-density polyethylene films were used as controls and were prepared at similar conditions to the films containing the active agents, since EVA was only applied for the purpose of active compound encapsulation; not as a functional component for film performance. Hence, the non-AM control film should not include EVA.

3.2.4.2 Additive quantification in LDPE-EVA film

The amount of linalool or methylchavicol in the samples was determined by gas chromatography (GC). The procedure was as follows: 5 g of film was extracted for 18 h by Soxhlet extraction using 150 mL of isooctane. An aliquot of

the extract with a precisely known volume was then injected into the GC as described in **Section 3.2.1.2**.

3.2.4.3 AM activity of LDPE-EVA films in solid media

The films were tested for their inhibition against the target microorganisms: *Listeria innocua*, *Staphylococcus aureus* (Gram-positive bacteria), *Escherichia coli* (Gram-negative bacteria) and *Saccharomyces cerevisiae* (yeast) by using an agar disc diffusion method described in the literature (Acar and Goldstein 1986; Parish and Davidson 1993) (**Table 3.2**).

Table 3.2 Experimental-conditions used for the agar disc diffusion assay on AM films in the study

FSA No.[1]	Test organism	Medium[2]	Time/ h	Temperature/ °C
2305	*Listeria innocua*	Tryptone Soya Agar	24	37
3601	*Staphylococcus aureus*	Nutrient Agar	24	37
1301	*Escherichia coli*	Nutrient Agar	24	37
3301	*Saccharomyces cerevisiae*	YM Agar	48	30

[1] Culture collection of the Food Science Australia (FSA), Werribee, Victoria, Australia

[2] Tryptone Soya Agar, prepared from Tryptone Soya Broth (Amyl, AM185) and bacteriological agar (Amyl, AM250), Nutrient Agar, prepared from Nutrient Broth (Oxoid, CM1) and bacteriological agar (Amyl, RM250); YM Agar, prepared from YM Broth (Amyl, RM229) and bacteriological agar

All test cultures used in the microbiological assay were twice-passaged 15 h cultures grown in tryptone soya broth, nutrient broth or YM broth. Cell

densities of 10^6 organisms were calculated and prepared from cultures of approximately 7.50×10^8 cfu mL^{-1} for *L. innocua* and *E. coli*, 4.85×10^8 cfu mL^{-1} for *S. aureus*, and 4.75×10^8 cfu mL^{-1} for *S. cerevisiae*, as described in **Section 3.2.3.2**. Cell densities were also confirmed by the "pour plate" method on plate count agar for bacteria and on potato dextrose agar for yeast (Swanson et al. 1992). Each film sample was cut into a circle of 4 mm in diameter and sterilised with UV light for 2 min (Cooksey 2000) prior to being placed on an agar plate surface seeded with 1 mL of test culture. The plates were incubated for 1-2 days at the appropriate temperature for each culture. The clear zone formed around the film disc in the medium was recorded as an indication of inhibition of the microbial species. The evaluation of inhibitory activity was carried out in quadruplicate by measuring inhibition zones with a Vernier caliper (Mitutoyo, Japan). An average of four measurements, taken 45° apart from each other, was used as the result of each test.

3.2.4.4 AM activity of LDPE-EVA films and additives in liquid media

Escherichia coli was used in this study. The stock culture was stored at –80 °C in nutrient broth. The working culture was grown at 37 °C overnight on nutrient agar and subcultured twice at 37 °C in nutrient broth for 18 h in order to obtain cells in the stationary phase of growth. Cell densities of approximately 1×10^3 cfu mL^{-1} were prepared, as described in **Section 3.2.3.2**. The AM films were cut into small pieces (1×10 cm). The number of pieces of each film used was such that the ratio of film area/medium volume was either 1 or 2 cm^2 mL^{-1} as suggested by Appendini and Hotchkiss (2002). The film pieces were added to

300 mL of the cell suspension in a 1 L Erlenmeyer flask and incubated at a constant temperature of 37 °C in an incubation shaker (Innova™ 4230, New Brunswick Scientific, U.S.A.) with a continuously rotating speed of 30 rpm, simulating agitation during transportation (Hong et al. 2000; Appendini and Hotchkiss 2002). Samples were collected from the cell suspensions at various time intervals and diluted appropriately with 0.1 M sterile sodium phosphate buffer solution (pH 7.0) (Spencer and Ragout de Spencer 2001), after which 1 mL aliquots were spread in duplicate on 3M Petrifilm™ aerobic count plates. Viable numbers were estimated from cell counts after an incubation period of 24 h at 37 °C.

The AM activity of pure linalool or methylchavicol was tested by the same procedure. Linalool (1.50 or 10.0 mg mL^{-1}) or methylchavicol (10.0 mg mL^{-1}) was added to 300 mL of the cell suspension in a 1 L Erlenmeyer flask, instead of the AM film.

3.2.4.5 Data analysis

Individual experiments in the liquid media were carried out in duplicate. Data points were represented by the mean. Data sets were subjected to a variance (ANOVA) analysis and the Tukey test at the 0.05 level of significance using KyPlot 2.0 for Windows (Kyence Inc., Japan).

3.2.5 Characterisation of AM films

3.2.5.1 Water vapour transmission rate measurement

A Lyssy Vapour Permeation Tester L80-4000 J was used to determine the water vapour transmission rate of the AM LDPE-EVA films. The measurements were conducted in accordance with ASTM D 895 at 38 °C and 90% RH. Experiments were repeated eight times.

3.2.5.2 Oxygen transmission rate measurement

An Oxygen Permeation Tester, Illinois 8000 was used to measure oxygen transmission rate of the AM LDPE-EVA films. The measurements were conducted in accordance with ASTM D 3985-95 at 23 °C and 0% RH. Experiments were repeated eight times.

3.2.5.3 Ultraviolet-visible spectroscopy

A UV-VIS spectrophotometer (Cary 300, Varian Australia Pty, Ltd) was used to measure the transparency of the AM LDPE-EVA films according to ASTM D1746-92.

3.2.5.4 Infrared spectroscopy

AM LDPE-EVA film samples were cut into 3 × 3 cm squares and were attached to a sample holder of a Bruker Vector 22 infrared spectrophotometer for their IR spectra determination according to ASTM D5576-94. A total of 64 scans

were recorded at wave numbers in the range 4000-400 cm^{-1} to confirm the presence of the AM agent. In addition, special interest was focused on the carbonyl region (1740-1700 cm^{-1}) from a thermooxidative degradation point of view. The carbonyl index was calculated as the ratio between the difference in absorbance at the peak in the carbonyl region and the baseline at this peak and the difference in absorbance of the peak at 1463 cm^{-1} (as an internal standard) and the baseline of this peak (Khabbaz et al. 1999; Zhao et al. 2003).

3.2.5.5 Differential scanning calorimetry

A Perkin-Elmer differential scanning calorimeter DSC-7 equipped with a flow-through cover and calibrated with indium and zinc was used to obtain the differential scanning calorimetric data in accordance with ASTM D3417-83. Thermograms for the AM LDPE-EVA film samples (5-6 mg) were recorded in three consecutive runs: (a) heating from 50 to 180 °C, followed by (b) cooling from 180 to 50 °C, and (c) second heating from 50 to 180 °C. The experiments were performed at heating and cooling rates of 10 °C min^{-1} under a nitrogen atmosphere, to avoid a thermooxidative degradation. The degree of crystallinity (χ) was determined from the ratio between the melting enthalpy (or heat of fusion) of the sample and that of a 100% crystalline polyethylene (PE), assumed to be 293 J g^{-1} (Wunderlich 1973; Teh et al. 1994). The melting temperature was taken at the maximum of the endothermic peak.

3.2.5.6 Thermogravimetric analysis

A Perkin-Elmer thermogravimetric analyzer TGA-7 equipped with a flow-through cover was used to obtain the thermogravimetric (TG) data, according to ASTM D3850-94. The mass-loss curves for AM LDPE-EVA film samples (5-6 mg) were recorded when heating from 50 to 600 °C at a heating rate of 20 °C min^{-1}. TG curves were obtained also in the form of the first derivative of the mass change with time.

3.2.5.7 Scanning electron microscopy

Scanning electron microscopy (SEM) was conducted to observe the morphology of AM LDPE-EVA films. The instrument used in this work was a PHILIPS XL-30. Cryogenic microtoming could not be used in this work and therefore the possibility of blade damage to the samples cannot be excluded. All specimens were coated with a thin layer of gold to eliminate charging effects (Loretto 1994).

3.2.5.8 Data analysis

Data points were represented by the mean of the measured values. The data were subjected to an analysis of variance (ANOVA) and the Tukey test at the 0.05 level of significance using KyPlot 2.0 for Windows (Kyence Inc., Japan).

3.2.6 Release of AM additive from AM films into a food simulant

3.2.6.1 Film thickness measurement

A hand-held micrometer (Hahn & Kolb, Stuttgart, Germany) was used for measuring film thickness. Five readings were taken for each sample, one at the sample center and four around the perimeter.

3.2.6.2 Release experiments

The release of linalool and methylchavicol from the AM LDPE-EVA films was investigated by immersing 4 pieces (5 × 5 cm) of the tested film in 100 mL of iso-octane, as an alternative fatty food simulant, in a closed system, and storing at 4, 10 and 25 °C in an incubation shaker (Innova™ 4230, New Brunswick Scientific, U.S.A.) with a continuously rotating speed of 30 rpm. An aliquot was sampled at various times. Experiments were performed in triplicate.

3.2.6.3 Additive quantification in aliquot

The actual concentration of linalool or methylchavicol in the aliquot was determined by gas chromatography (GC). An aliquot of the extract with precisely known volume was injected into a GC for analysis as described in **Section 3.2.1.2**.

3.2.6.4 Data analysis

The initial part of the diffusion curves ($M_t/M_\propto < 0.6$ was tested for linearity with regard to $t^{1/2}$ using a linear correlation procedure (KyPlot 2.0 for Windows, Kyence Inc, Japan). since such a linearity would indicate compliance with the general law of diffusion (Crank 1975). The diffusion coefficients D ($m^2\ s^{-1}$) of linalool and methylchavicol were later calculated using the half-time method (**equation 3.1**) (Lim and Tung 1997; Han and Floros 2000):

$$D = 0.0491 L^2/t_{0.5} \qquad \textbf{(3.1)}$$

where L is the film thickness, and $t_{0.5}$ is the time required to reach one half of the migrant concentration ($M_t = 0.5M_\infty$)

Theoretical values of the fractional mass release as a function of time were calculated assuming an exponential rise to the maximum level as shown in **equation 3.2** (Schwartzberg 1975; Lim and Tung 1997):

$$M_t/M_\infty = 1 - \exp(-kt) \qquad \textbf{(3.2)}$$

where k is the empirically obtained rate constant (s^{-1}) that dependents on the mass transfer properties, geometry and other conditions of the film material (Han and Floros 2000).

In order to determine the temperature dependence of the diffusion coefficient, an Arrhenius activation energy equation (**equation 3.3)** was used (Crosby 1981; Naylor 1989; Chatwin 1996):

$$D = D_0 \exp(-E_a/RT) \quad \textbf{(3.3)}$$

where D_0 is a pre-exponential factor, E_a is the activation energy, R is the ideal gas constant (8.314 J $mole^{-1}K^{-1}$), and T is the absolute temperature. The parameters D_0 and E_a can be obtained by curve fitting of the experimental data (Helmroth et al. 2002).

The data were also analysed by the time response function using the Hill coefficient (**equation 3.4**):

$$M_t/M_\infty = 1/(1 + (k/t)^n) \quad \textbf{(3.4)}$$

where k is a rate constant, and n is the Hill coefficient, indicating the degree of cooperativity of the agent (Hill 1909; Barcroft and Hill 1910).

The kinetic results were analysed using a time-response function with the Hill coefficient to determine the rate constant of the kinetic equation. Values of the rate constant at different temperatures were subjected to applied cluster analysis using Ward method (Jacobsen and Gunderson 1986). Finally, the two-way ANOVA with replication procedure was applied to evaluate the significance of the main effects of temperature and time, and their interaction.

3.2.7 Storage of AM films

3.2.7.1 Long-term storage of AM LLDPE films

The AM LLDPE films from the preliminary study (**Section 3.2.1**) were used in this experiment. The rolls of approximately 100 m films containing

linalool or methylchavicol were kept at ambient temperature for 1 year (long-term storage). Samples were then used to evaluate their AM activity in solid media.

3.2.7.2 AM activity of LLDPE films after long-term storage

For determining the effect of the worse case storage scenario, film samples taken from the outside and side regions of the rolls were tested for their inhibition of *Escherichia coli* (Gram- negative bacteria) by the agar disc diffusion method described in the literature (Acar and Goldstein 1986; Parish and Davidson 1993). This is because the loss of active agent over time is expected to be greater from the exposed outside and side regions of the roll than the inside and centre regions. The test culture used in the microbiological assay was a twice-passaged 15 h culture grown in nutrient broth. Cell densities of 10^6 organisms were calculated and prepared from cultures of approximately 7.50×10^8 cfu mL^{-1} for *E. coli.* Cell density was also confirmed by the "pour plate" method on plate count agar for bacteria (Swanson et al. 1992).

Each film disk (4 mm diameter) sample was sterilised with UV light for 2 min (Cooksey 2000) prior to being placed on the surface of an agar plate seeded with 1 mL of test culture. The plates were incubated for 1-2 days at the appropriate temperature for each culture. The clear zone formed around the film disc in the medium was recorded as the inhibition of the microbial species. The evaluation of inhibitory activity was carried out in quadruplicate. The measurement of the inhibition zones was performed with a Vernier caliper

(Mitutoyo, Japan). An average of four measurements, taken 45° apart from each other, was used as the result of each test.

3.2.7.3 Accelerated storage of AM LDPE-EVA films

The AM LDPE-EVA films (**Section 3.2.4**) were used in this work for the study of accelerated storage conditions. The rolls of approximately 100 m films containing linalool or methylchavicol were stored at 25 and 35 °C. The outside and side regions of the rolls were periodically sampled and quantified for the residual amount of linalool or methylchavicol.

3.2.7.4 Additive quantification in AM film

The actual concentration of linalool or methylchavicol in the prepared samples was determined by gas chromatography (GC). The procedure was as follows: 5 g of film was extracted for 18 h by Soxhlet extraction using 150 mL of isooctane. An aliquot of the extract with precisely known volume was then injected into a GC for analysis as described in **Section 3.2.1.2**.

3.2.7.5 Data analysis

The experiments in solid media were performed in quadruplicate. The data points were represented by the mean. The data sets were subjected to analysis of variance (ANOVA) and the Tukey test at the 0.05 level of significance using KyPlot 2.0 for Windows (Kyence Inc., Japan).

3.2.8 Application of AM films on Cheddar cheese

3.2.8.1 Inhibition of microbial growth on cheese using AM films

Cheese preparation and storage

Cheddar cheese was purchased from a local retail outlet. For the AM packaging experiments, cubes (3 × 4 × 2 cm) weighing ca. 20 g were cut. During the storage test, the cheese was randomly divided into three lots for different packaging treatments (control film, 0.34% (w/w) linalool-LDPE-EVA film and 0.34% (w/w) methylchavicol-LDPE-EVA film) and packaged by wrapping the film (8 × 12 cm), which was previously sterilised with UV light for 2 min (Cooksey 2000), tightly around the cheese. The packaged cheese samples were stored at 4 °C for 21 days and periodically analysed for their microbiological quality during storage.

Microbiological analysis

Cubes of Cheddar cheese were examined for levels of total aerobic mesophilic bacteria, coliform bacteria and yeasts/moulds. Three packages from each treatment were aseptically opened on the sampling days, then a 20 g of cheese cube was aseptically transferred to a sterile stomacher bag. 180 mL of 0.1 M sterile sodium phosphate buffer solution (pH 7.0) was added and the sample homogenised for 1 min in a laboratory blender (Seward Stomacher® 400, Seward Medical, UK). A series of decimal dilutions was carried out according to recommended microbiological protocols (Spencer and Ragout de Spencer 2001). In order to determine total aerobic mesophilic and coliform bacterial counts and yeast/mould counts, 1 mL of serially diluted samples were plated in duplicate on

3M Petrifilm™ aerobic total count plates, 3M Petrifilm™ *E. coli* and coliform count plates, and 3M Petrifilm™ yeast and mould count plates, respectively. Aerobic total count plates and *E. coli* and coliform count plates were incubated aerobically for 24 h at 37 °C, while yeast and mould count plates were incubated for 5 days at 30 °C. Colonies were counted and results expressed as cfu g^{-1}.

3.2.8.2 Inhibition of bacterial growth on cheese using AM films

Inoculation of bacteria on cheese and storage

Storage at refrigerated temperature

Commercial, sliced Cheddar cheese was purchased from a local supermarket. For the packaging experiments, the slices (9 × 9 × 0.2 cm) weighing ca. 20 g were sterilised by UV light for 1 h on each side in a laminar flow cabinet (Taniwaki et al. 2001). During the storage test, the cheese was randomly divided into 2 sets for different bacterial inoculations (*E. coli* and *L. innocua*). Each set of the cheese was also randomly divided into three lots for different packaging samples (LDPE as control film, 0.338% w/w linalool-LDPE-EVA and 0.344% w/w methylchavicol-LDPE-EVA films). The slices of Cheddar cheese were then surface inoculated with *E. coli* or *L. innocua* at a level of 10^4 cfu g^{-1} and packaged by wrapping the film (12 × 24 cm), which was previously sterilised with UV light for 2 min (Cooksey 2000), tightly around the cheese and heat-sealing the three open sides. The packaged cheese samples were stored at 4 °C for 35 days and periodically collected for analysis of bacteriological quality during storage.

Storage subjected to temperature abuse

The second set of experiments was prepared in a similar way as described in the previous section. The packaged cheese samples were stored at 4 °C for 1 day and then subjected to a temperature abuse (Cutter 1999; Siragusa et al. 1999) by storing them at 12 °C for 15 days, and periodically performing a bacteriological analysis.

Bacterial enumeration

Slices of Cheddar cheese were tested at appropriate intervals for numbers of *E. coli* and *L. innocua*. Two packages from each treatment were opened aseptically on the sampling days, and a 20 g cheese sample was aseptically transferred to a sterile stomacher bag. A 180 mL aliquot of 0.1 M sterile sodium phosphate buffer solution (pH 7.0) was added and the sample homogenised for 1 min in a laboratory blender (Seward Stomacher® 400, Seward Medical, UK). A series of decimal dilutions were performed according to recommended bacteriological protocols (Spencer and Regout de Spencer 2001). In order to examine *E. coli* and *L. innocua* populations, 1 mL of serially diluted samples was plated in duplicate on 3M Petrifilm™ *E. coli* and coliform count plates and tryptone soya agar count plates, respectively. Both count plates were incubated aerobically for 24 h at 37°C. Colonies were counted and the results were expressed as cfu g^{-1}.

3.2.8.3 Sensory analysis of cheese wrapping with AM films

Cheese wrapping and storage

Cheddar cheese was purchased from a local retail outlet. For the packaging experiments, cubes (3 × 2 × 2 cm) weighing ca. 10 g were cut and then surface sterilised by exposure to UV light for 1 h in a laminar flow cabinet (Taniwaki et al. 2001). During the storage test, the cheese was randomly divided into three lots for different packaging material samples (LDPE as control film, 0.338% w/w linalool-LDPE-EVA and 0.344% w/w methylchavicol-LDPE-EVA films) and packaged by wrapping the film (6 × 8 cm), which was previously sterilised with UV light for 2 min (Cooksey 2000), tightly around the cheese. The packaged cheese samples were stored at 4 °C for 42 days and were subjected to periodic sensory evaluation.

Sensory evaluation

A panel of twelve un-trained members was employed for evaluation of the presence of basil's constituents (or basil tainted flavour) in the Cheddar cheese wrapped in AM LDPE-EVA films containing linalool or methylchavicol. The standard triangle test method (Stone and Sidel 1993) was used. In this study, each panelist was presented with samples that had been subjected to two sets of treatments: (i) six cube samples of Cheddar cheese, in two sets: one set comprising two cubes wrapped in the control film and one wrapped in the linalool-LDPE-EVA film, and in the second set: one cube wrapped in the control film and two cubes wrapped in the linalool-LDPE-EVA film, and (ii) six cube samples of Cheddar cheese in two sets: one set comprising two cubes wrapped in

the control film and one wrapped with methylchavicol-LDPE-EVA film, and in the other set: one cube wrapped in the control film and two cubes wrapped in the methylchavicol-LDPE-EVA film. Panelists were asked to choose the odd sample in each set of the three. The result will indirectly indicate whether linalool or methylchavicol could be detected as a tainted flavour. Cheddar cheese samples and controls were tested in this manner after 1, 2, 3, 4 and 6 weeks of storage. Probability tables were employed to determine levels of significant difference between samples and controls as described by Roessler et al. (1978).

4 RESULTS AND DISCUSSION

4.1 Preliminary study of AM films

4.1.1 Concentration of AM additive in LLDPE film

Extrusion film blowing was considered as one option for manufacturing of such a sophisticated product as antimicrobial (AM) packaging by direct incorporation of naturally derived AM compounds into the film. For this purpose, several AM films were prepared by master batch blending of the additives followed by extrusion film blowing. At the beginning of the study, LLDPE resin was pulverised and then soaked overnight in the AM agent dissolved in isooctane. After drying, the master batch was added to LLDPE pellets. The entire mixture was then extruded to form the film. Through this approach, the additive could be properly diluted and dispersed in the polymer melt, leading to a film with a uniformly dispersed AM agent (**Figure 4.1**). This result was observed and confirmed by scanning electron microscopy (**Section 4.5**). This finding is consistent with the study by Hong et al. (2000) on AM films incorporating clove extract.

Upon incorporating the linalool or methylchavicol agents in the present study, the extruded film, which contained 0.05% w/w additive, attained a characteristic, slightly light brown colour. The linalool or methylchavicol incorporated films had therefore a slightly reduced transparency compared to the additive-free LLDPE film and so these AM films may have a slight disadvantage

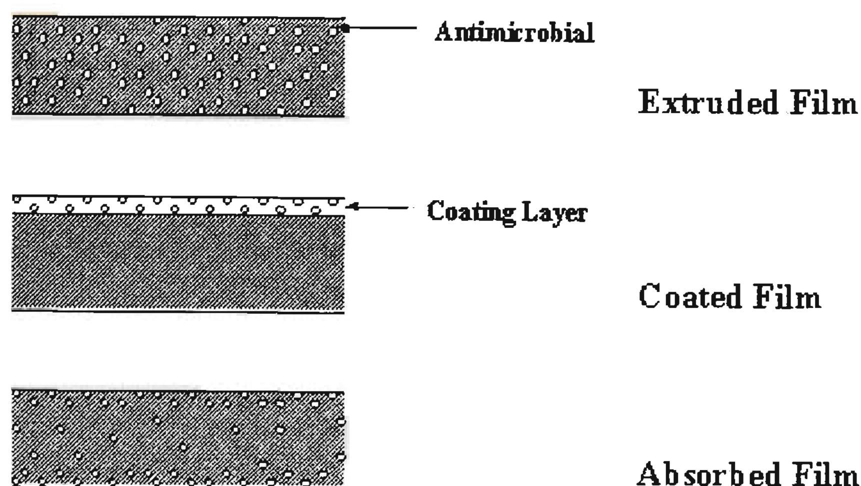


Figure 4.1 Distribution of AM additive in various film matrices

in applying them in see-through packaging systems (Han and Floros 1997; Hong et al. 2000). In addition, due to the higher melting temperature of LLDPE compared to LDPE, a temperature profile of 90-95 °C as previously applied by Han and Floros (1997), or of 160-190 °C as previously used by Ha et al. (2001) could not be used in the present study for the production of the AM films by extrusion film blowing. Lower manufacturing temperatures are preferable in order to minimise the loss of active agent by evaporation. The temperature profile of 195-210 °C, required for manufacturing the film resulted in a high loss of active agent. The limitations of the single screw extruder available for our experiments further affected the expected results. The residual amount of linalool or methylchavicol in the extruded films was found to be approximately 0.05% w/w (target concentration was 1.0% w/w in blend).

In the films prepared by solution-coating, the thickness of the coating was found to be in the range of 3 to 6 μm which corresponds to a mean coating on a weight basis of 8.77% w/w (standard deviation 0.54). The concentrations of the active agents (either 1.0% or 0.05% w/w) in the coating were calculated on the basis of the film weight gain.

The 1.0% w/w methylchavicol-absorbed film prepared by the absorption method was slightly sticky after the excess iso-octane was evaporated compared to linalool-absorbed films. A residual deposit of methylchavicol on the film surface may have been the cause of this behaviour.

4.1.2 AM activity of LLDPE films in solid media

All prepared LLDPE films containing linalool or methylchavicol showed a significantly positive AM activity against *E. coli* in the agar disc diffusion test (**Table 4.1**). The size of the zone is taken as a quantitative measure of AM activity. It should be noted, however, that the size of the zone may be limited not by activity but by diffusion from the polymer and through the agar. Colonies of *E. coli* could not be viewed in the circular region directly above the film samples containing the constituents of basil, while such colonies were formed all over the control plates. The microbial inhibition indicates that a portion of either linalool or methylchavicol was released from the 0.05% w/w extruded or both of the 1.00% w/w and 0.05% w/w coated film samples, and diffused into the agar layer, retarding the development of microbial cells in the agar. Although linalool and methylchavicol are almost insoluble in pure water, they may be slightly soluble in

Table 4.1 AM activity of LLDPE films against *E. coli* as observed by agar disc diffusion assay

Method	Target Conc./ %w/w	Actual Conc./ %w/w	Zone of Inhibition/ mm[1]
Extrusion			
LLDPE	-	-	-[2]
linalool	1.0	0.0559	11.3 ± 1.9[3]ab
methylchavicol	1.0	0.0529	8.8 ± 0.4a[4]
Coating[5]			
Coated without agent	-	-	-
linalool	1.0	0.9108	15.3 ± 1.4b
	0.05	0.0477	12.2 ± 0.7ab
methylchavicol	1.0	1.0259	13.5 ± 0.6ab
	0.05	0.0492	11.3 ± 1.0ab
Absorption			
linalool	1.0	0.9085	11.9 ± 1.0ab
	0.05	0.0528	10.2 ± 1.0a
methylchavicol	1.0	1.0161	15.3 ± 0.7b
	0.05	0.0534	13.7 ± 0.8ab

[1] no antimicrobial activity against *Staphylococcus aureus* and *Saccharomyces cerevisiae*
[2] -, no reaction
[3] Values for zone of inhibition are represented as mean ± SEM (standard error of the mean)
[4] The treatment with same letter within row is not statistically significant difference ($p > 0.05$)
[5] Active compound only present in the coating layer

the water held by the agar due to the presence of some hydrophobic substances from beef extract and peptone (Chung et al. 2003).

Figure 4.2 shows a symmetrical zone of inhibition. The clear zones around the film discs on the agar medium plate inoculated with *E. coli* were wider in the LLDPE film that had absorbed 1.0% w/w methylchavicol as well as in the LLDPE film that was coated with 1.0% w/w linalool than for any of the other treatments. However, no statistically significant difference ($p > 0.05$) was

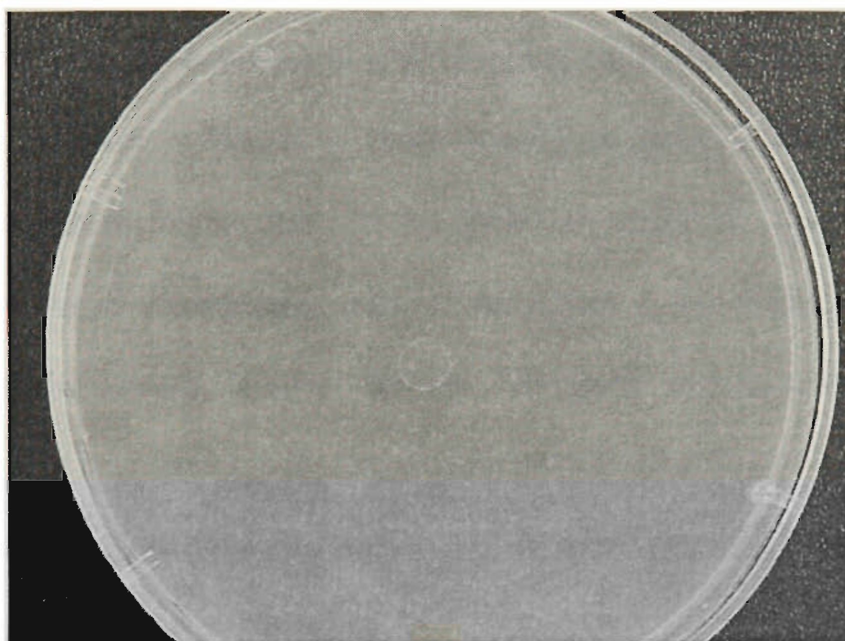


Figure 4.2 Growth inhibition of *E. coli* by LLDPE film containing 0.05% w/w linalool or methylchavicol

found between the films with different concentrations, active agents, or preparation methods. Linalool showed a higher level of inhibition than methylchavicol in the AM films prepared by extrusion or coating, in spite of the fact that methylchavicol possesses a greater extent of AM activity than linalool (Reuveni et al. 1984; Wan et al. 1998; Friedman et al. 2002). The reason may stem from the faster diffusion of linalool and its greater solubility in water (and subsequently its more pronounced presence in the aqueous-based agar media), compared with methylchavicol (Knobloch et al. 1989). In contrast,

methylchavicol exhibited a slightly higher level of inhibition than linalool, when the absorption method was used, probably due to the higher concentration of active agent closer to the film surface as a result of this method of preparation (**Figure 4.1**) and the higher AM effect. The higher diffusion rate of linalool is perceived to be the determining factor for showing higher inhibition than methylchavicol in the extrusion film blowing and coating methods (Knobloch et al. 1989). However, it should be noted that higher diffusion efficiency is not always required for higher AM activity. Diffusion should be matched with the growth kinetics of the microorganism. If the diffusion rate of the AM agent is faster than the growth kinetics then the AM agent will be diluted and its efficiency will be reduced. This emphasizes the role of diffusion in the size of zone of inhibition and highlights the fact that diffusion rate might not necessarily be a quantitative measure of efficacy.

Table 4.2 shows that basil extracts seem to be better AM agents against *E. coli* (a Gram-negative bacterium) than other natural extracts. The results for *E. coli* with LLDPE films containing the very low level of 0.05% w/w linalool or methylchavicol were better than those for poly(ε-caprolactone) (PCL) film embedded with 5% w/w triclosan-β-cyclodextrin-inclusion compound (triclosan-β-CD-IC) (Lu et al. 2001) and poly(*L*-lactic acid) (PLLA) film impregnated with 10% w/w of the antibiotic neomycin sulfate-β-CD-IC (Huang et al. 1999).

Table 4.2 AM activity of PE films incorporated with basil extract in comparison with some other AM films

AM additive	Geometry[1]	Area/ mm^2	*S. aureus*	*E. coli*	*S. cerevisiae*
Bacteriocin					
1% Lacticin BH5-coated LDPE (An et al. 2000)	Square	100.00	–	–	Not tested
1% Lacticin NK24-coated LDPE (An et al. 2000)	Square	100.00	–	–	Not tested
1% Nisin-coated LDPE (An et al. 2000)	Square	100.00	–	–	Not tested
Plant extract					
Basil (*Ocimum basilicum* L.) extract (BE)					
0.05% linalool-extruded LLDPE (Suppakul et al. 2002)	Circle	19.64	–	+ +	–
0.05% BE-methylchavicol-extrude LLLDPE (Suppakul et al. 2002)	Circle	19.64	–	+	–
Clove extract (*Eugenia caryphyllata*) (CE)					
1% CE-extruded LDPE[2]	Square	100.00	Not tested	–	–
Grapefruit seed extract (*Citrus paradisi*) (GFSE)					
0.1% GFSE-casted Na-alginate (Cha et al. 2002)	Circle	19.64	–	–	Not tested
0.1% GFSE-EDTA-casted Na-alginate (Cha et al. 2002)	Circle	19.64	+	+	Not tested
0.1% GFSE-extruded LDPE (Lee et al. 1998)	Square	100.00	+	+	–
1% GFSE-coated LDPE (Ha et al. 2001)	Square	100.00	+ +	+ +	+
1% GFSE-co-extruded multiplayer LLDPE (Ha et al. 2001)	Square	100.00	–	–	–

Table 4.2 (Continued)

AM additive	Geometry[1]	Area/ mm^2	*S. aureus*	*E. coli*	*S. cerevisiae*
Plant extract					
Huanglian (*Coptis chinensis*) extract (HE)					
1% HE-extruded LDPE (An et al. 1998)	Square	100.00	–	–	–
Rhubarb (*Rheum palmatum*) extract (RE)					
1% RE-extruded LDPE (An et al. 1998)	Square	100.00	–	–	–
Metal ion					
1% Ag-zirconium-extruded LDPE (An et al. 1998)	Square	100.00	–	–	–
Organic acid					
1% Sorbic acid-extruded LLDPE (An et al. 1998)	Square	100.00	+	+	–

[1] Square: Film size 10 × 10 mm; For inhibition zone: –, no reaction; +, 0.5-2.5 mm; + + 2.5-7.0 mm (substracting the length from the centre of film in 0/90 degree)
Circle: Film diameter 5 mm; For inhibition zone: –, no reaction; +, 0.5-2.5 mm; + + 2.5-7.0 mm (substracting the radius of film disc)
[2] Expectation from the result of Hong et al. (2000)

4.2 Microencapsulation of AM additive

4.2.1 Complexation

The recovery of linalool and methylchavicol from their respective β-CD complexes after reaching equilibrium is presented in **Table 4.3**. In each case the recovery of the complex material was less than the amount originally incorporated. One factor that may contribute to a lower recovery of these additives might be an operational loss since some of the linalool and methylchavicol were observed to remain in the solution after the complexes were formed. Furthermore, some loss of the volatile additive may have occurred *via* evaporation during the long complexation process. Also, due to partitioning, an equilibrium concentration of the volatile compounds remains in the liquid phase. Furthermore, it is expected that a certain amount of volatile compound may have been lost by evaporation from the surface of the complex during the drying stage.

The results, given in **Table 4.4**, are in agreement with those obtained by Bhandari et al. (1998). In that study as well as the present study, a higher recovered mass was found as a result of higher amounts of β-CD co-crystallising from the solution, since the co-crystallised product is expected to be less soluble than pure β-CD. A significantly higher amount of β-CD-linalool complex was found relative to the amount of β-CD-methylchavicol complex. It is evident that in both studies, the amount of complex increased with an increase in the availability of β-CD (**Table 4.4**).

Table 4.3 Recovery of linalool and methylchavicol complexes

Inclusion Compound	Initial Material (β-CD + volatile)/ g[1]	Mass Recovered (complex)/ g	% Recovery[2]
β-CD-linalool	10.8114±0.0126	9.9402±0.1588	91.94[b]
β-CD-methylchavicol	10.8069±0.0202	9.4996±0.2053	87.90[a]

[1] Dry weight basis; mean±standard deviation
[2] Treatments having different superscripts are significantly different ($p \leq 0.05$)

Table 4.4 Complexation on co-crystallisation of β-cyclodextrin and the recovery of inclusion compound

Inclusion Compound	β-CD used/ g	Theoretical co-crystallized β-CD[1] / g	Volatile used/ g	Theoretical Total/ g	Mass recovered/ g	Difference[2]/ g
β-CD-linalool	9.0455	7.1955	1.7659	8.9614	9.9402	+0.9788
β-CD-methylchavicol	9.0407	7.1907	1.7662	8.9569	9.4996	+0.5427

[1] Assumed amounts of co-crystallised β-CD, result from total β-CD used less its soluble amount in 100 mL of water (1.85 g)
[2] Difference in weight between the mass recovered and the theoretical amounts of co-crystallised β-CD plus volatile used

4.2.2 Concentration and additive retention in β-CD complex

The results in **Table 4.5** show that the actual concentrations of linalool and methylchavicol in the complexes were 3.34 and 2.67 mg g^{-1} respectively. The retained linalool and methylchavicol in the β-CD was found to be, by extraction, 1.88 and 1.43% respectively of the original quantities of the compounds. Linalool, a tertiary alcohol, showed a higher concentration and retention than methylchavicol, an ether. This is in agreement with the findings of Fleuriot (1991). These values of the actual concentration of linalool and methylchavicol in the complex are, however, very low compared to the findings of Sadafian and Crouzet (1988). The latter researchers reported linalool retention of 9.2-13.6% in the complex in a microencapsulation by extrusion process. In a precipitation process, the results may be explained by droplet stripping (Rosenberg et al. 1990). Above the solubility limits, volatile compounds can associate to form small drops in the matrix that may be lost by stripping. Preparation of β-CD complexes using a precipitation methods appears very simple but, in practice, it is not. Taking into consideration the high cost of β-CD and the low inclusion efficiency of β-CD for linalool and methylchavicol encapsulation, this technique may not be adequate for the preparation of linalool and methylchavicol concentrates to be used in AM films. However, it may be of interest for other kinds of AM agents, especially natural extracts, which might be appropriate for microencapsulation such as eugenol (Reineccius et al. 2002) that can be used in AM film fabrication.

Table 4.5 Actual concentration and retention of volatile content in complexes

Inclusion Compound	Actual concentration of volatile/ % w/w	Retention of volatile/ %[1]
β-CD-linalool	0.334	1.88
β-CD-methylchavicol	0.267	1.43

[1] volatile as a percentage of volatile content used in the complexation process

4.3 Sensitivity enhancement of OD measurement

4.3.1 Bacterial growth

No contamination was shown in the cuvette of a non-inoculated medium used as a reference. At the beginning of the experiment, perturbation was noted on the average curves at different wavelength, as depicted in **Figure 4.3**. These could be associated with the changes in temperature inside the cuvette.

In the optical density measurements using pathlength cuvette, the lag times at all tested conditions were approximately 4.25 h. In **Figure 4.4**, the change in the numbers of *E. coli* cells as a function of time is shown for the direct plate count method. The lag time was about 1.33 h. These findings indicate that the optical density technique with pathlength cuvette is not a suitable method for measuring an inoculum size in the 10^3 cfu mL^{-1} range.

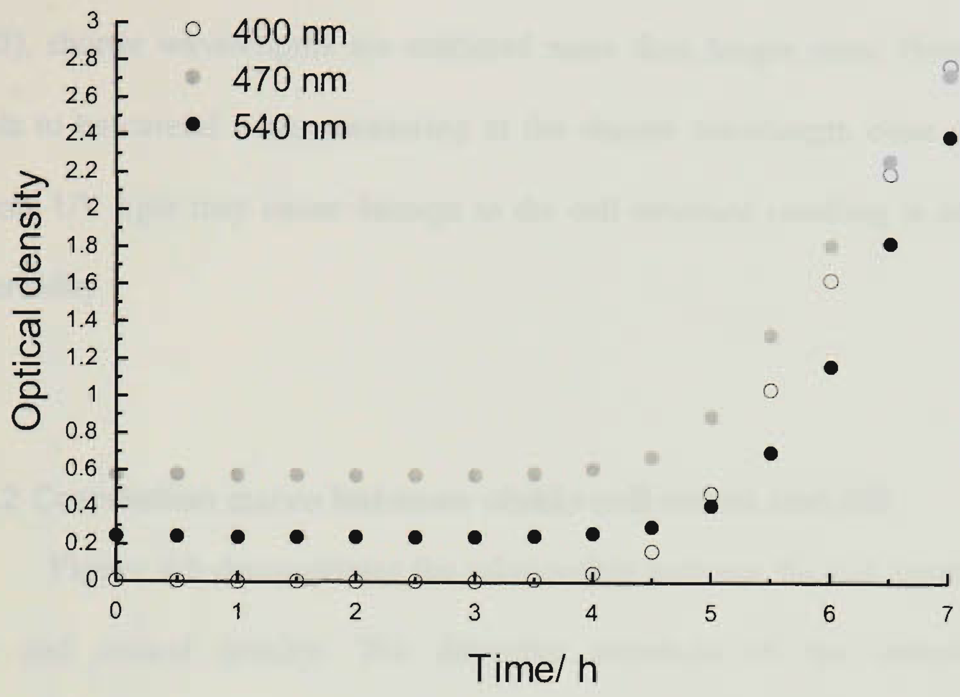


Figure 4.3 Growth curves of *E. coli* using turbidimetry at different wavelengths

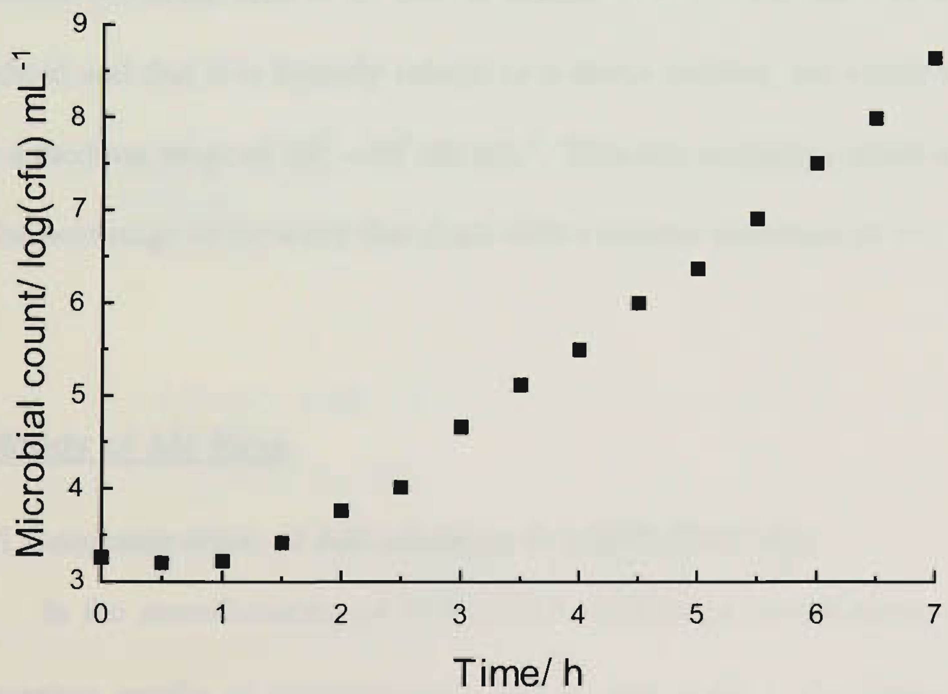


Figure 4.4 Growth curve of *E. coli* using viable cell count

In general, according to the Rayleigh scattering (Bohren and Huffman 1983), shorter wavelengths are scattered more than longer ones. However, one needs to be careful when measuring at the shorter wavelength close to the UV region. UV light may cause damage to the cell structure resulting in an increase in turbidity.

4.3.2 Correlation curve between viable cell count and OD

Figure 4.5 demonstrates the relationship between the cell numbers of *E. coli* and optical density. The detection threshold of the optical density measurements could be enhanced by using a pathlength cuvette and/or operating at shorter wavelengths. The results of this study suggest that the possible minimum inoculum size of *E. coli* is around 5×10^5 cfu mL^{-1} as a detection threshold and that it is linearly related to a direct method, the viable cell count, over a medium range of $10^5 - 10^8$ cfu mL^{-1}. Thus this technique could not be used for the next stage of the work that deals with a smaller inoculum of 10^3 cfu mL^{-1}.

4.4 Study of AM films

4.4.1 Concentration of AM additive in LDPE-EVA film

In the manufacturing of LDPE-EVA AM films by extrusion, a constant temperature profile of approximately 160 °C was used in the present work in order to minimise the loss of active agent by evaporation, as recommended in the literature (Han 2000; Ha et al. 2001). A loss of the active agent was observed during the extrusion process but it was significantly lower than the losses

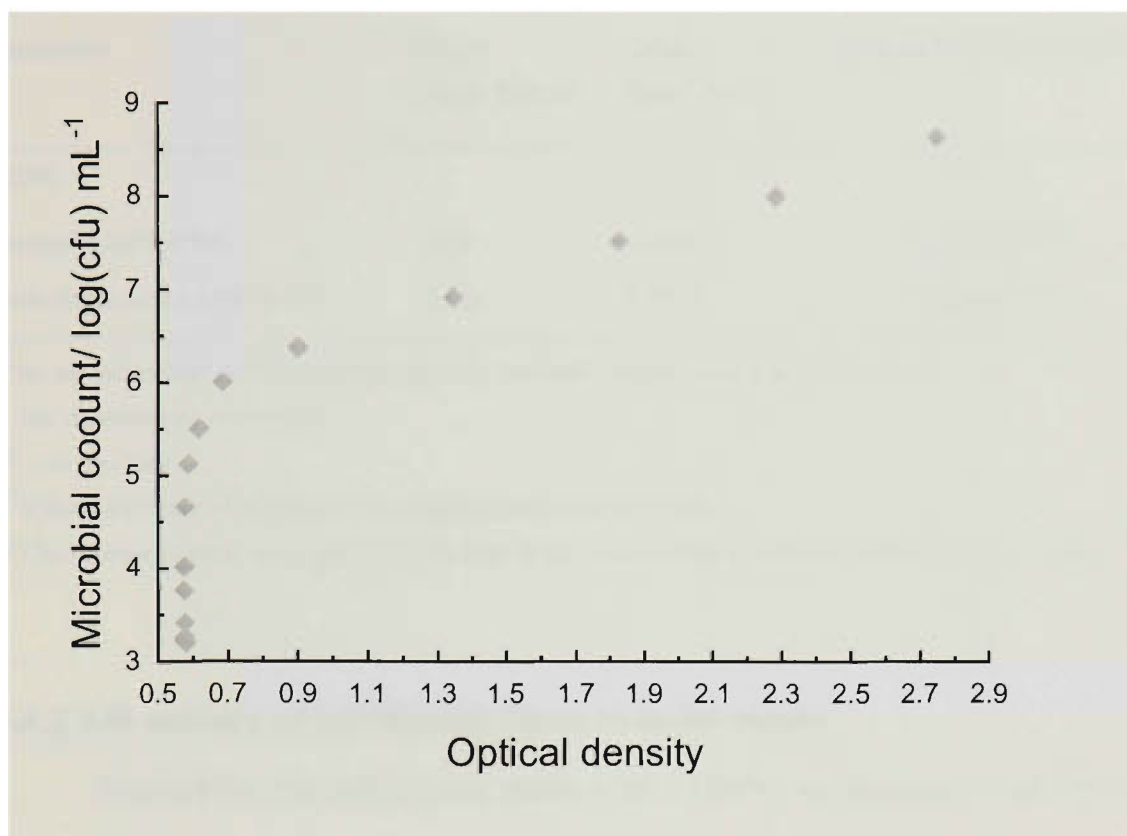


Figure 4.5 Relationship between viable cell count and optical density at 470 nm

observed in a previous study with LLDPE alone (Suppakul et al. 2002). The actual amount of linalool or methylchavicol in the extruded films was found to be 0.34%w/w (**Table 4.6**). This increased retention of the active agent might be attributable to the lower temperature in the extruder and to the interaction of the active agent with the EVA copolymer. Ethylene vinyl acetate may assist in "solubilizing" the molecules of the active agents within the polyethylene matrix.

Table 4.6 AM activity of LDPE-EVA films against *E. coli* as observed by agar disc diffusion assay

Treatment	Target Conc./ %w/w	Actual Conc./ %w/w	Zone of Inhibition/ mm[1]
LDPE	-	-	-[2]
linalool-LDPE-EVA	1.00	0.3381	11.52±0.19[3] b
methylchavicol-LDPE-EVA	1.00	0.3445	10.09±0.18a[4]

[1] no antimicrobial activity against *Listeria innocua*, *Staphylococcus aureus* and *Saccharomyces cerevisiae*

[2] -, no reaction

[3] Values for zone of inhibition are represented as mean±SEM

[4] The treatment with same letter within row is not statistically significant difference ($p > 0.05$)

4.4.2 AM activity of LDPE-EVA films in solid media

Similarly to the preliminary study with LLDPE, all prepared LDPE-EVA films containing linalool or methylchavicol represented positive AM activity against *E. coli*. (**Table 4.6** and **Figure 4.6**). Furthermore **Figure 4.7**, clearly suggests there is an eccentric characteristic AM activity leading by the extrusion direction consistent with an anisotropic material (Lagaroni 2003). Film cutting at 0/90 degree to extrusion direction (**Figure 4.7a**) showed a zone of inhibition in agreement with the study of Cutter et al. (2001). However, no explanation of this kind of eccentric characteristic was given. A statistically significant difference ($p \leq 0.05$) was found between the films with different active agents. According to Elgayyar et al. (2001), the present results show that all the films can be qualitatively characterised as having between "mildly inhibitory" and "moderately inhibitory" characteristics against *E. coli*.

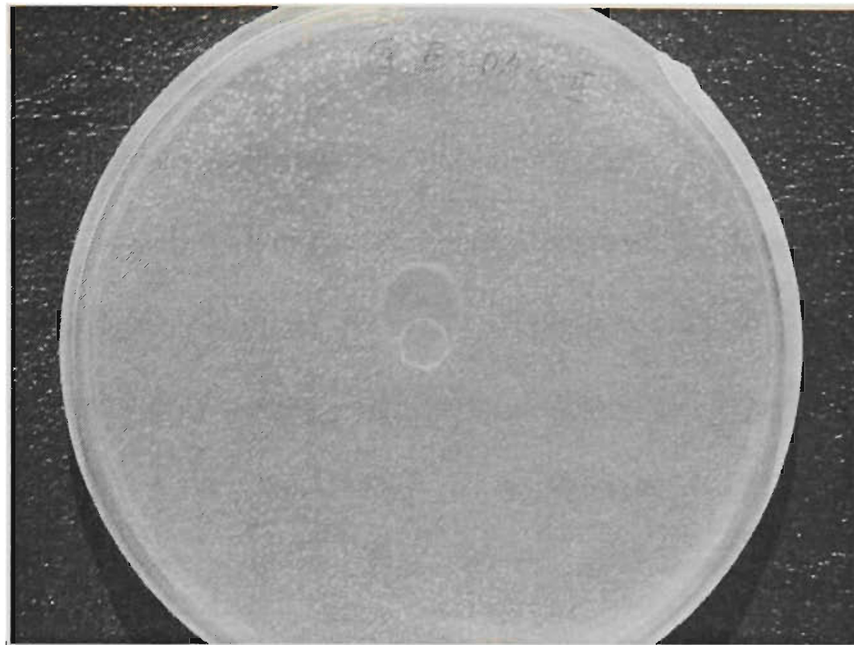
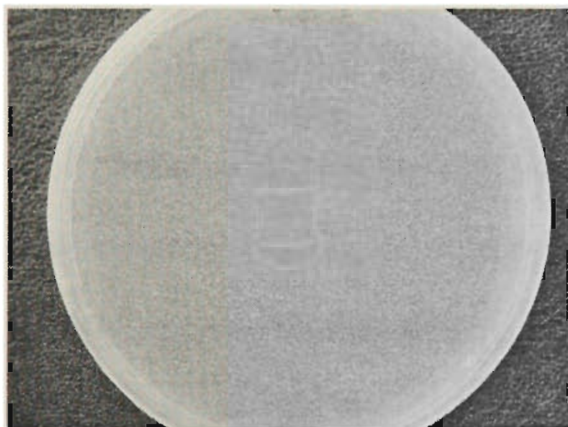
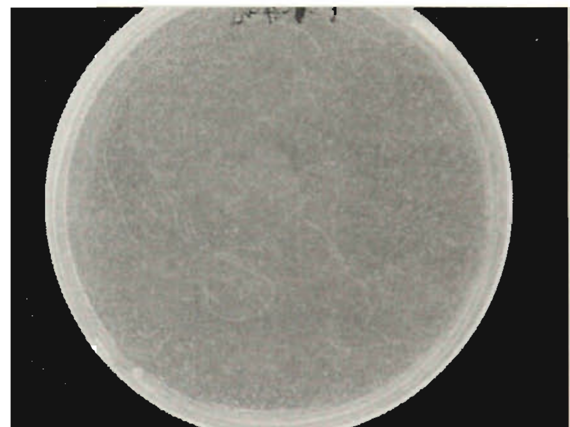


Figure 4.6 Growth inhibition of *E. coli* by LDPE-EVA film containing 0.34% w/w linalool or metyhlchavicol





(a) (b)

Figure 4.7 Growth inhibition of *E coli* by LDPE-EVA film containing linalool or methylchavicol with different film cutting

(a) Film cutting at 0/90 degree of extrusion direction

(b) Film cutting at 45/45 degree of extrusion direction

Linalool and methylchavicol are known to have a broad spectrum of AM activity against a variety of microorganism such as *Aeromonas hydrophila*, *Bacillus cereus*, *E. coli*, *Listeria monocytogenes*, *S. aureus*, *S. cerevisiae*, *Aspergillus* sp., and *Penicillium* sp. (Suppakul et al. 2003b). As shown in **Table 4.6**, the lack of inhibitory action of linalool or methylchavicol against *L. innocua*, *S. aureus* and *S. cerevisiae* probably stems from the fact that the concentration of linalool or methylchavicol in the films was much below the values required for demonstrating inhibitory effects. Additionally, a lower concentration of basil extract in a film matrix will result in a decrease in either linalool or methylchavicol in the aqueous phase of the agar layer where microbial proliferation takes place. However, it is interesting to note that LDPE-EVA films containing such a low concentration (0.34% w/w) of linalool or methylchavicol still inhibit the activity of *E. coli*, a Gram-negative bacterium.

Variation in diffusion rates, partition coefficients and the like might affect relative activity to some extent; however will not likely affect overall trend. In order to compare the results with other species of AM additives, normalised inhibitory of AM films against *E. coli* has been calculated by dividing the area of zone of inhibition by the total film area. Linalool and methylchavicol manifestly showed a promising inhibitory effect against *E. coli* as depicted in **Figure 4.8**. Some other AM additives failed to retard the growth of *E. coli* even at higher concentrations. This might be because Gram-negative bacteria are generally more resistant to the growth inhibition and killing effects of various antibiotics and AM agents (Salton 1994) due to the strong hydrophilicity of their surface that acts as

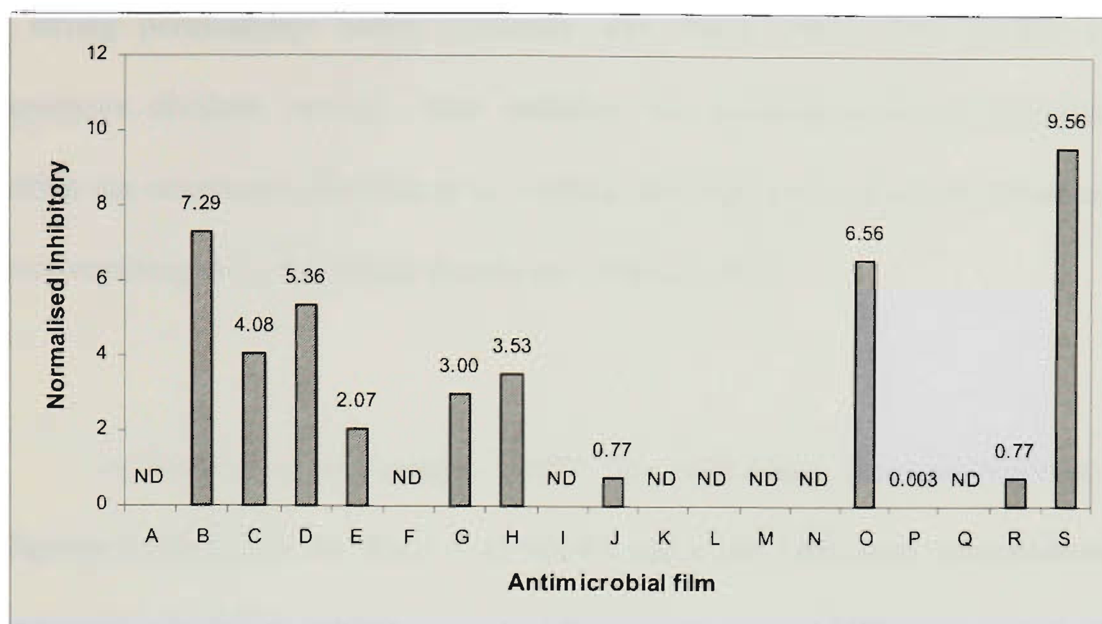


Figure 4.8 Normalised[1] inhibitory of AM films against *E. coli*

(a) 1% Ag-zirconium-extruded LDPE (An et al. 1998)

(b) 0.34% linalool-extruded LDPE-EVA (Suppakul et al. 2003c)

(c) 0.05% linalool-extruded LLDPE (Suppakul et al. 2002)

(d) 0.34% BE-methylchavicol-extruded LDPE-EVA (Suppakul et al. 2003c)

(e) 0.05% BE-methylchavicol-extruded LLDPE (Suppakul et al. 2002)

(f) 1% CE-extruded LDPE (Expectation from the result of Hong et al. (2000))

(g) 0.1% GFSE-casted Na-alginate (Cha et al. 2002)

(h) 1% GFSE-coated LDPE (Ha et al. 2001)

(i) 1% GFSE-co-extruded multilayer LLDPE (Ha et al. 2001)

(j) 0.1% GFSE-extruded LDPE (Lee et al. 1998)

(k) 1% HE-extruded LDPE (An et al. 1998)

(l) 1% Lacticin BH5-coated LDPE (An et al. 2000)

(m) 1% Lacticin NK24-coated LDPE (An et al. 2000)

(n) 6.6% Lysozyme-EDTA-casted corn zein (Padgett et al. 1998)

(o) 10% Neomycin sulfate-β-CD-IC-melt-pressed PLLA (Huang et al. 1999)

(p) 1% Nisin-coated LDPE (An et al. 2000)

(q) 1% RE-extruded LDPE (An et al. 1998)

(r) 1% Sorbic acid-extruded LDPE (An et al. 1998)

(s) 5% Triclosan--β-CD-IC-melt-pressed PCL (Lu et al. 2001)

[1] Due to non-linear relationship between concentration of AM additive and zone of inhibition, normalised inhibitory is the ratio of area of clear zone and film area.

a strong permeability barrier (Nikaido and Vaara 1985). The surface also possesses divalent cations that stabilise the lipopolysaccharide association within the membrane (Stevens et al. 1992a), and may prevent active compounds from reaching the cytoplasmic membrane (Russel 1991).

In contrast to our results, LDPE film containing chitosan polymer or oligomer investigated by Hong et al. (2000) and a cast corn zein film containing Lysozyme studied by Padgett et al. (1998) as well as the LDPE film coated with Nisin tested by An et al. (2000) exhibited no reduction of *E. coli* cells. For chitosan, Lysozyme and Nisin, penetration through the outer membrane can be accomplished by the use of a chelating agent such as EDTA or by an osmotic shock. Removal of the stabilizing effect of the cations by chelating agents will increase cell permeability to AM additives (Hancock 1984; Stevens et al. 1991, 1992b; Padgett et al. 1998). The advantage of using linalool and methylchavicol is that a chelating agent is not required. Our results also demonstrate that non-ionic surfactants such as Tween 20 or 80 are not required in order to improve the distribution of linalool or methylchavicol within aqueous media even though the latter has limited solubilities in such media.

4.4.3 AM activity of LDPE-EVA films and additives in liquid media

From viable cell count data, growth curves were generated and the maximum specific growth rate (μ_{max}) was estimated by using the logistic growth model (Kono 1968; Baranyi et al. 1993; Rosso et al. 1996; Augustin et al. 1999; Annadurai et al. 2000):

$x(t) = x_0$, for $t \leq t_{lag}$

$x(t) = x_{max} / [1 + ((x_{max} / x_0) - 1) \times \exp(-\mu_{max} \times (t - lag))]$, for $t > t_{lag}$ **(4.1)**

where $x(t)$ is the bacterial concentration (cfu mL^{-1}) at time t (h),

x_0 is the initial bacterial concentration (cfu mL^{-1}),

x_{max} is the maximum bacterial concentration (cfu mL^{-1}), and

μ_{max} is the maximum specific growth rate (h^{-1}).

The effect of the maximum growth rate of the AM film on the growth of *E. coli* was expressed by (Mejlholm and Dalgaard 2002) as the percentage reduction in the growth rate (% RGR):

$\% RGR = [(\mu_{max, na} - \mu_{max, a}) \times 100] / \mu_{max, na}$ **(4.2)**

where $\mu_{max, a}$ and $\mu_{max, na}$ are the values of μ_{max} with and without agent respectively.

For all film treatments, a slight decrease in microbial count was observed in the exponential growth phase compared to the control (**Figure 4.9**). The AM activity was observed to be a function of the film area-to-medium volume ratio. Film area-to-medium volume ratios from 1 cm^2 mL^{-1} to 2 cm^2 mL^{-1} were required to prevent detectable growth of *E. coli* (**Table 4.7**) which correspond to a

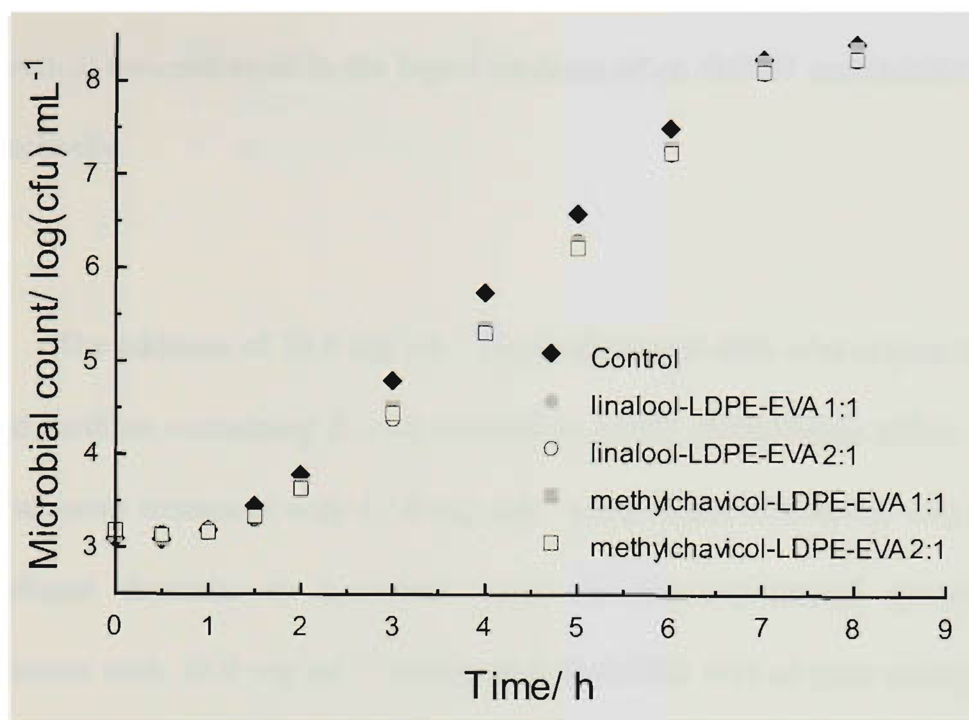


Figure 4.9 Growth curve of *E. coli* after addition of pieces of films containing 0.34 %w/w linalool or methylchavicol at different ratios of film area/medium volume

Table 4.7 AM activity of LDPE-EVA films on the maximum specific growth rate of *E. coli* in liquid medium

Treatment	Area to Volume Ratio/ cm^2 mL^{-1}	μ_{max}/ h^{-1}	% RGR[1]
LDPE	1: 1	2.182[2]c	-
	2: 1	not tested	-
linalool-LDPE-EVA	1: 1	2.118b	2.92a
	2: 1	2.042a	6.41b
methylchavicol-LDPE-EVA	1: 1	2.128b	2.92a
	2: 1	2.062a	5.53b

[1] Expressed as the percentage change in growth rate compared to the growth rate achieved for *E. coli* in the liquid medium with control treatment

[2] Values are the mean of two replicates. Any two means in same column followed by same letter are not significantly different ($p > 0.05$) by Tukey test

theoretical concentration in the liquid medium of ca. 0.0141 and 0.0282 mg mL^{-1}, respectively.

The addition of 10.0 mg mL^{-1} (equivalent to 0.46% v/v) of pure linalool in liquid medium containing *E. coli* resulted in 100% of inhibition effect within 30 min whereas treatment with 1.50 mg mL^{-1} (equivalent to 0.069% v/v) showed a significant decrease in microbial count in the exponential growth phase. Treatment with 10.0 mg mL^{-1} (equivalent to 0.38% v/v) of pure methylchavicol showed a dramatic reduction in microbial count in the exponential phase (**Figure 4.10**). This result is consistent with the work of Coma et al. (2002) involving the addition of 10% v/v of chitosan or acetic acid in liquid medium containing *L. monocytogenes*.

All films containing linalool or methylchavicol (**Table 4.7**) marginally reduced the growth rate of *E. coli* compared to pure linalool or methylchavicol (**Table 4.8**). These agents caused approximately 3-6% RGR compared to the additive-free LDPE film. The reason for this is the significantly lower concentrations (i.e. 0.0141-0.0282 mg mL^{-1} (equivalent to $6.49 \times 10^{-4} - 1.30 \times 10^{-3}$ and $5.36 \times 10^{-4} - 1.07 \times 10^{-3}$% v/v for linalool and methylchavicol, respectively) of these active agents in the film. The latter concentration range was calculated assuming that the active agent diffuses completely to produce a corresponding maximum effective level, and that the time to reach this maximum concentration is short. In regards to the maximum specific growth rate and the

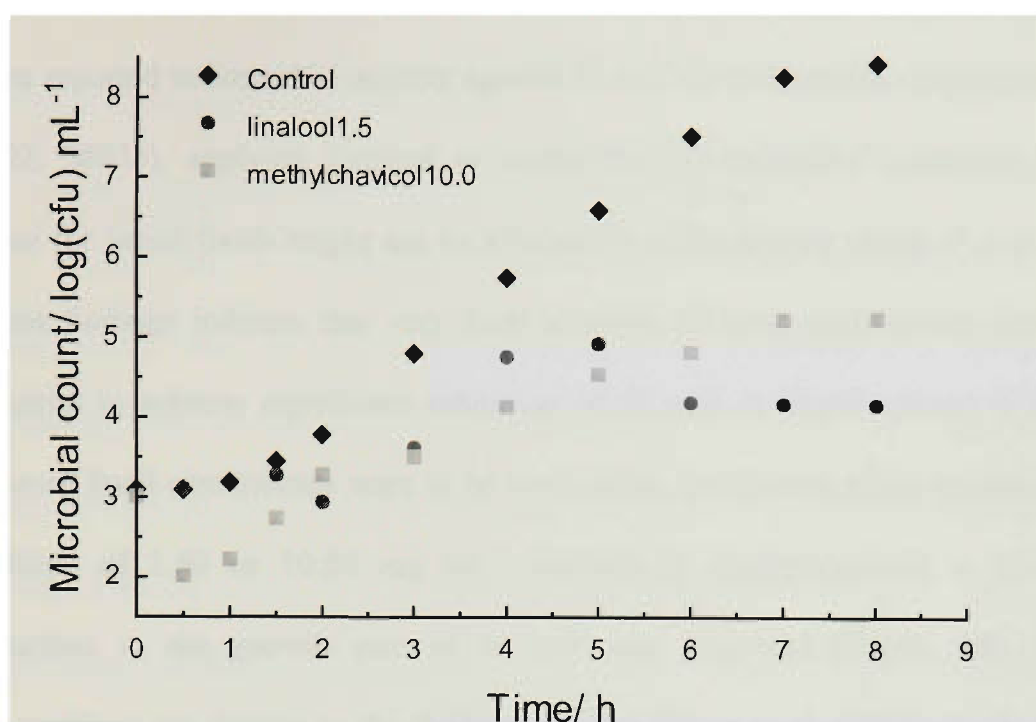


Figure 4.10 Growth curve of *E. coli* after addition of linalool or methylchavicol at particular concentration (mg mL^{-1})

Table 4.8 AM activity of linalool and methylchavicol on the maximum specific growth rate of *E. coli* in liquid medium

Treatment	Conc./mg mL^{-1}	μ_{max}/ h^{-1}	%RGR[1]
Control (without additive)	-	2.197[2]d	-
linalool	1.50	0.396b	81.97b
	10.00	0.000a	100.00c
methylchavicol	1.50	not tested	-
	10.00	1.194c	45.68a

[1] Expressed as the percentage change in growth rate compared to the growth rate achieved for *E. coli* in the liquid medium with control treatment

[2] Values are the mean of two replicates. Any two means in same column followed by same letter are not significantly different ($p > 0.05$) by Tukey test

percentage reduction in growth rate, no statistically significant differences were found between the films containing different species of active agents.

Although PE and PE-based films containing linalool or methylchavicol were reported to have AM activity against *E. coli* on solid media (Suppakul et al. 2002, 2003c), applying linalool or methychavicol-embedded packaging films alone for liquid foods might not be efficient in reducing the viable *E. coli* cells. These findings indicate that very high amounts of basil constituents would be required to achieve significant inhibition of *E. coli* in liquid culture if slowly released basil constituents were to be used alone. In the case of an instantaneous addition of 1.50 or 10.00 mg mL^{-1} linalool or methylchavicol, a 45-100% reduction in the growth rate of *E. coli* was observed (**Table 4.8**). These observations are similar to the findings of Chi-Zhang et al. (2004) dealing with nisin in a broth system. Linalool showed a higher level of inhibitory effect than methylchavicol in liquid media. This finding is in agreement with the experiment in solid media described in **Section 4.1.2**.

Linalool, a tertiary alcohol, showed an inhibition effect on the growth of *E. coli*, potentially acting as either a protein denaturing agent or as a solvent dehydrating agent (Pelczar et al. 1993). In general, the mechanism by which microorganisms are inhibited by an active compound might involve a sensitization of the phospholipid bilayer of the cell membrane, causing an increase in permeability and leakage of vital intracellular constituents (Juven et al. 1994; Kim et al. 1995), or impairment of bacterial enzyme systems (Wendakoon and Sakaguchi 1995).

4.5 Characterisation of AM films

4.5.1 Water vapour and oxygen transmission rates

There was a significant reduction in the water vapour and oxygen transmission rates of linalool-LDPE-EVA and methylchavicol-LDPE-EVA containing films in comparison with additive-free LDPE film (see **Table 4.9**). In comparison with the control LDPE film, the methylchavicol-LDPE-EVA film showed a much lower WVTR than the linalool-LDPE-EVA film. This might result from the higher hydrophobicity of methylchavicol compared to linalool. Methylchavicol showed not only AM activity but also imparted hydrophobicity, therefore acting as a barrier to water vapour. The EVA copolymer contained in these AM films also played a role in decreasing the OTR. This is because the acetate group in EVA lowers the oxygen permeability (Hernandez et al. 2000).

Table 4.9 Barrier and optical properties of AM LDPE-EVA films

Treatment	Thickness/ μm	WVTR/ g m^{-2} day^{-1}	OTR × 10^3/ cc m^{-2} day^{-1}	Transparency/ log (T_{600}) mm^{-1}
LDPE	46.8	13.72	9.17	41.30
linalool-LDPE-EVA	48.6	10.50	6.09	39.56
methylchavicol-LDPE-EVA	49.8	5.20	4.73	38.54

4.5.2 Transparency

A spectrophotometer enables the transparency of a material to be determined by light transmittance measurements. The Beer-Lambert law defines the relationship between the amount of light absorbed and the nature of the light absorbing material according to the following equation (Tinoco et al. 1995):

$$\log (I_0/I) = \log (1/T) = A = \varepsilon cL \quad \textbf{(4.3)}$$

where I_0 is the incident radiant energy, I is the transmitted radiant energy, T is defined as the transmittance of light, A is the absorbance, ε is molar absorptivity, c is the concentration of absorbing species and L is the optical path length.

The transparency at unit light path length was calculated from **equation 4.4**, which can be related to **equation 4.3**.

$$\text{Transparency} = (\log T_{600})/L \quad \textbf{(4.4)}$$

The transparency of the plastic film was determined by measuring the percentage transmittance of light at a wavelength of 600 nm (T_{600}). There was a statistically significant decrease in transparency of AM LDPE-EVA films compared to the plain LDPE film as shown in **Table 4.9**. Methylchavicol had a larger effect on the transparency than linalool. These findings are in agreement with the study of Han and Floros (1997) who investigated the optical properties of K-sorbate incorporated-LDPE and LDPE films. The transparency of AM films in the present study, containing either linalool or methylchavicol, is in the

acceptable range for plain film. Thus there would be no problems, for example, in film commercialisation, whereas the transparency of LDPE film containing 0.5-1.0% w/w K-sorbate in the work of Han and Floros (1997) decreased from approximately 40 (additive-free LDPE film) to 10 log(T_{600}) mm^{-1} in the films containing the additive. Such an effect may result in a serious drawback in applying the latter film in transparent packaging.

4.5.3 IR spectra as polymer 'fingerprint'

As shown in **Figure 4.11**, the spectrum of LDPE was consistent with published data (Haslam et al. 1972). LDPE, linalool-LDPE-EVA and methylchavicol-LDPE-EVA films exhibited similar, very low, levels of terminal vinyl groups absorbing at 990 and 908 cm^{-1}. This is comparable to the characteristics of LLDPE and metallocene polymers. In HPDE end-chain vinyl groups have also been observed in these regions (Allen et al. 2000). Comparable levels of vinylidene groups also appear to be present in all three types of films, absorbing at 888 cm^{-1}. As expected, all films exhibited a methyl absorption at 729 cm^{-1} which is absent in both the HDPE and metallocene polymers (Allen et al. 2000). Similar results have been also found with sorbic anhydride-PE film (Weng and Chen 1997). Both linalool-LDPE-EVA and methylchavicol-LDPE-EVA films revealed detectable levels of vinyl acetate groups that absorb at 1019 cm^{-1} and which are absent in LDPE film. The peaks representing EVA copolymer are also seen at 1240 cm^{-1} as acetate stretch and at 1740 cm^{-1} as ester stretch (Silverstein et al. 1991). At a longer wave number of 3602 cm^{-1}, there is a weak absorption due to non-hydrogen bonded hydroxyl groups. These are normally a

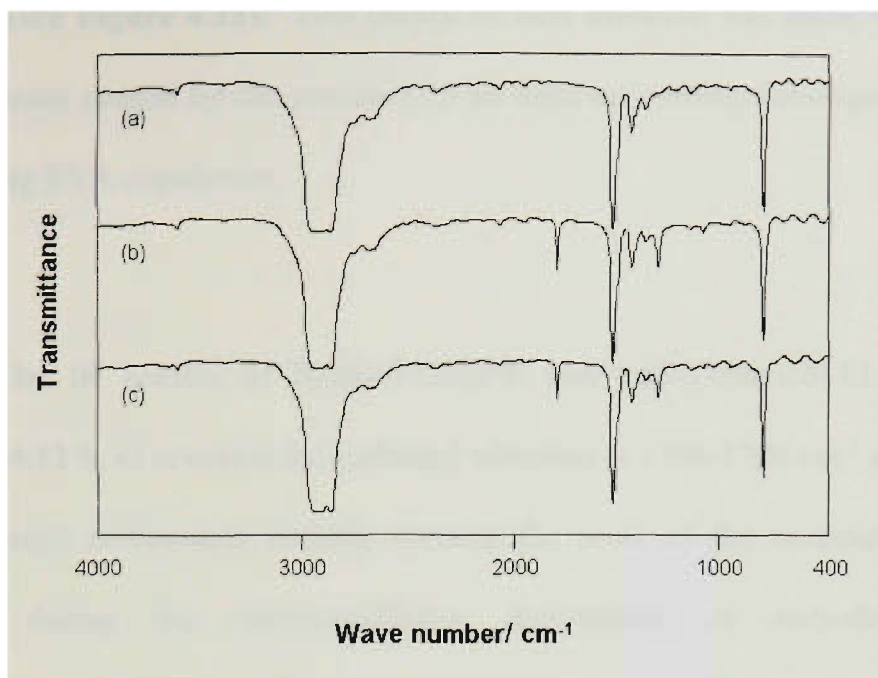


Figure 4.11 IR spectra[1] of AM LDPE-EVA films
(a) LDPE, (b) linalool-LDPE-EVA and
(c) methylchavicol-LDPE-EVA film

[1]IR spectra of pure linalool and methylchavicol are included in the **Appendix D**

measure of peroxidic species. As the IR absorption spectrum is the sum of the absorption by the additive and by the virgin polymer (and possibly also by very low concentrations of other additives in polymer), it is very difficult to distinguish both linalool and methylchavicol peaks in the spectrum of the AM films, as predominant absorption due to the polymer.

4.5.4 Carbonyl group detection

The effect of linalool or methylchavicol on the oxidative stability of the polyethylene substrate was investigated by carbonyl index measurements on films that included LLDPE as control, linalool-LLDPE and methylchavicol-

LLDPE (see **Figure 4.12**). This choice of film substrate was made to avoid the complication caused by the presence of an inherent carbonyl absorption in films containing EVA copolymer.

The IR spectra of linalool-LLDPE and methylchavicol-LLDPE films (**Figure 4.12 b, c**) revealed the carbonyl vibration at 1740-1700 cm^{-1} as expected. As carbonyl compounds usually account for most of the oxidation products formed during the thermooxidative degradation of polyethylene, the concentration of carbonyl groups can be used to monitoring the progress of degradation (Gugumus 1996). The carbonyl absorption is composed of different overlapping bands corresponding to esters (at 1743 cm^{-1}), aldehydes (at 1733 cm^{-1}), ketones (at 1720 cm^{-1}) and acids (at 1712 cm^{-1}). Therefore, four carbonyl indices are commonly defined:

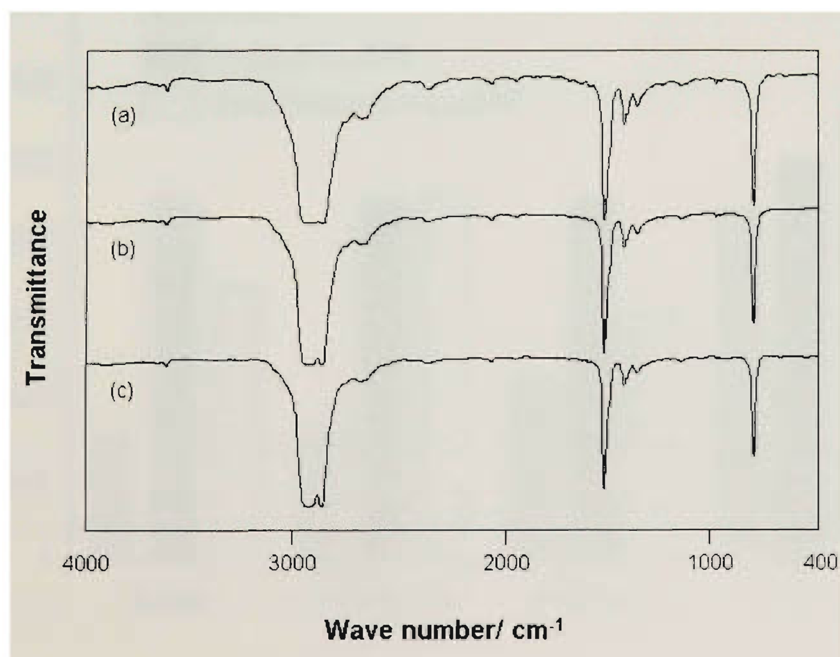


Figure 4.12 IR spectra of AM LLDPE films
(a) LLDPE, (b) linalool-LLDPE and (c) methylchavicol-LLDPE

$$I_1 = \Delta A_{1743}/\Delta A_{1463} \quad (4.5)$$

$$I_2 = \Delta A_{1733}/\Delta A_{1463} \quad (4.6)$$

$$I_3 = \Delta A_{1720}/\Delta A_{1463} \quad (4.7)$$

$$I_4 = \Delta A_{1712}/\Delta A_{1463} \quad (4.8)$$

where ΔA is the difference between the absorbance at the peak and the baseline at this peak.

Figure 4.13 shows the carbonyl absorbance for the studied materials. The LLDPE control film exhibits the lowest concentration of oxidised products, as determined by IR spectroscopy. An increase of carbonyl absorbance can be detected for linalool-LLDPE and methylchavicol-LLDPE films compared to the

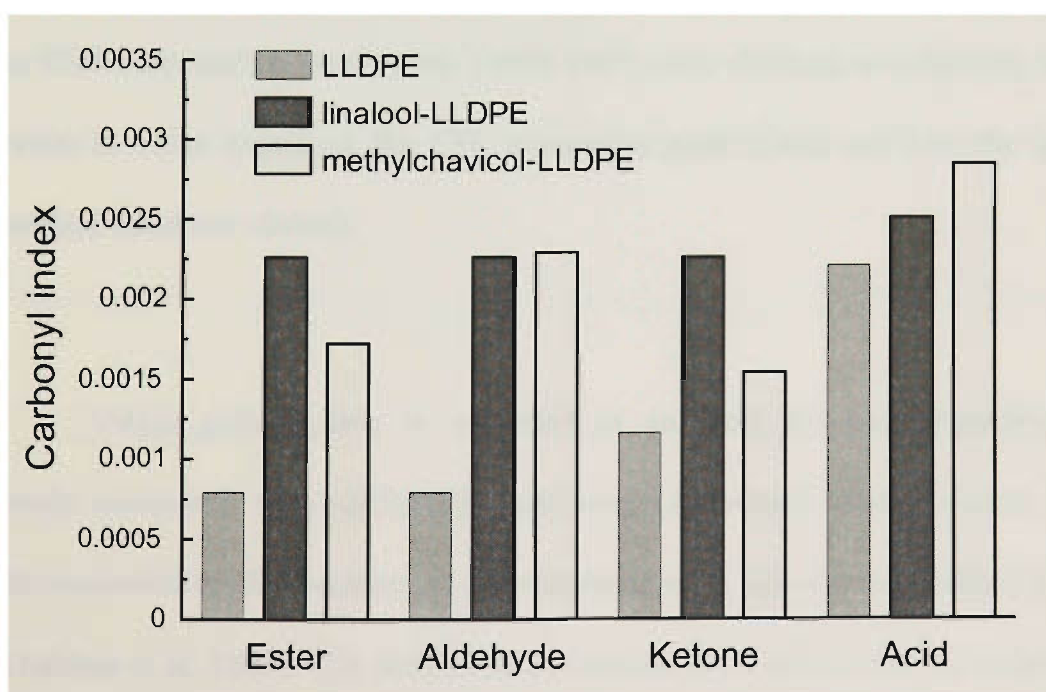


Figure 4.13 Changes in carbonyl index corresponding to ester, aldehyde, ketone and acid with CH_2 scissoring peak for AM LLDPE films

plain LLDPE. The linalool-LLDPE film was found to be more prone to thermooxidation than the methylchavicol-LLDPE film. However, these carbonyl indices are almost negligible for all the currently studied samples compared to those found by Khabbaz et al. (1999) for LDPE.

Iring et al. (1986) concluded that branched polyethylene is more rapidly oxidised than linear analogue polyethylene due to the presence of the tertiary carbon site at each branch point. The relatively low value of carbonyl index found in the present study may indicate that our polyethylene samples were more linear. The peak at 1898 cm^{-1}, which corresponds to C-H deformation modes in polyethylene is used as an alternative internal standard (Amin and Scott 1974). The carbonyl index calculated on the basis of the other internal standards such as the C-H deformation mode peak (1898 cm^{-1}) also showed, as expected, similar results to those based on the CH_2 scissoring peak (1463 cm^{-1}) as the internal standard (data not shown).

Virgin polyethylene is regarded as an inert polymer degrading very slowly compared to hydrolysable polymers (Albertsson and Karlsson 1988). Thermooxidative degradation of polyethylene is an abiotic degradation process (Khabbaz et al. 1999). The sensitivity of polyolefins towards thermal oxidation is largely due to the presence of impurities like hydroperoxides and carbonyl groups introduced during high-temperature manufacturing processes (Grassie and Leeming 1975; Carlsson and Wiles 1976; Chew et al. 1977). These groups of

impurities affect the abiotic oxidation rate to be followed by biodegradation (Albertsson et al. 1998). Thermal and thermooxidative degradation of polyethylene are complex reactions that are affected by many factors such as oxygen pressure, temperature, initiation rate, sample thickness, supermolecular structure, etc. (Michal et al. 1976).

4.5.5 Degree of crystallinity and melting temperature

Table 4.10 lists the thermal and morphological properties of the AM films. In this table ΔH_f is the melting enthalpy, T_m the peak melting temperature and χ is the degree of crystallinity. The data serve to illustrate that the incorporation of the AM agents into the films does not affect significantly the thermal or morphological properties of the films. These results are in agreement with the study of Khabbaz et al. (1999). However, the degree of crystallinity found in the present work is slightly lower than the published data for PE, generally in the range of 30-35% (Khabbaz et al. 1999; Khabbaz and Albertsson

Table 4.10 Thermal properties and polymer morphology of AM LDPE-EVA films

Treatment	ΔH_f/ J g^{-1}	T_m/ °C	χ/ %
LDPE	82.37	106.48	28.11
linalool-LDPE-EVA	83.88	106.31	28.63
methylchavicol-LDPE-EVA	80.10	106.64	27.34

2001). Corrales et al. (2002), however, reported very low degrees of crystallinity of HDPE, LLDPE and metallocene PE being 44, 18 and 14 %, respectively. Although it was not significant from a statistical point of view, the apparent slight increase in the crystallinity of the linalool-LDPE-EVA film might be explained by the creation of new intermolecular polar bonds due to carbonyl groups (Sebaa et al. 1993) whereas the reduction in crystallinity of the methylchavicol-LDPE-EVA film is probably due to the overriding disruption of the crystalline order (Billingham et al. 1976) caused by methylchavicol. In addition, as the vinyl acetate content increases, the crystallinity generally decreases (Hernandez et al. 2000). However, its effect in the present study was minor because of very low content of vinyl acetate in the linalool-LDPE-EVA and methylchavicol-LDPE-EVA films.

The results of DSC experiments showed two melting endothermic transitions, the second being associated with sample melting temperatures (Allen et al. 2000). **Figure 4.14** shows DSC thermograms of film samples obtained during a second heating run. The results of the first heating reflect the thermal history (data not shown) whereas the second heating, after controlled annealing conditions, represents the characteristics of the material. The shape of the curves, the position of the peak and the heat of melting depend on perfection of the lamellar crystals and phase aggregation (Khabbaz et al. 1999). Linalool-LDPE-EVA and methylchavicol-LDPE-EVA exhibited an endothermic peak of a first-order transition in the range of 100 and 112 °C, similar to the LDPE film, known

as semicrystalline polymer (Brydson 2000). The melting temperature of all films was about 106 °C.

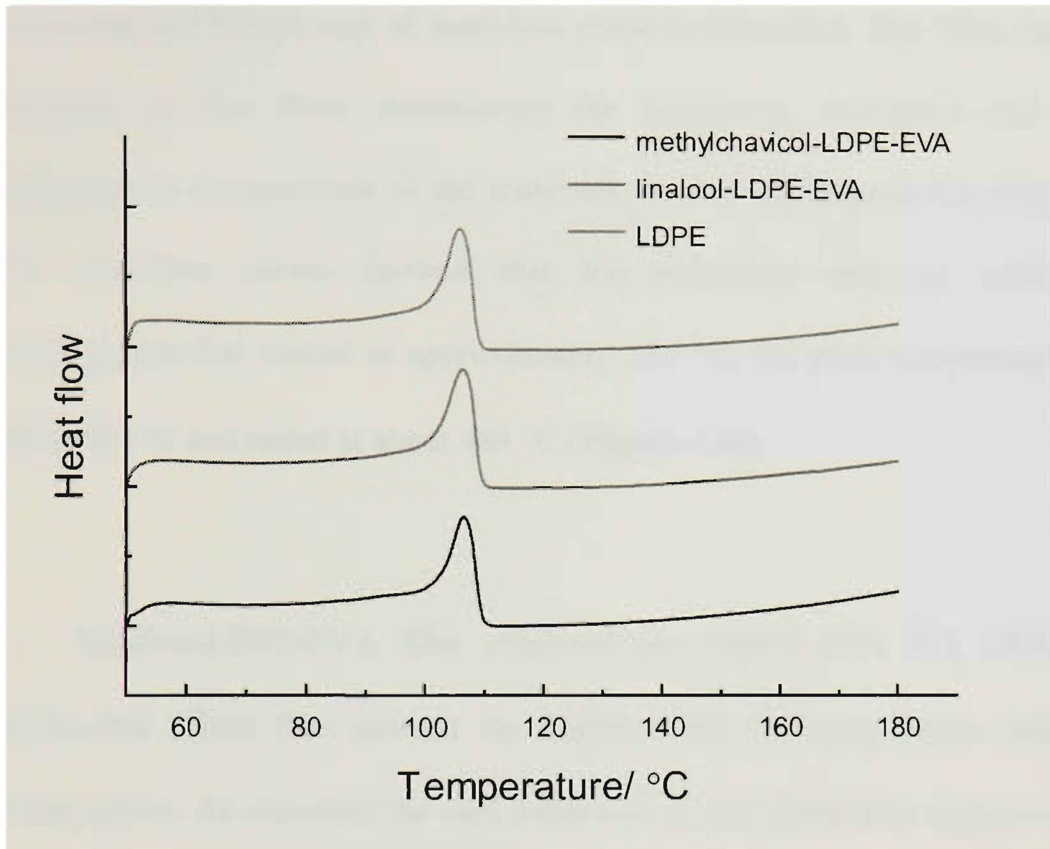


Figure 4.14 DSC thermograms of AM LDPE-EVA films

During thermal degradation of polymers as the temperature increases, chemical bonds with low-energy values are broken first while the more thermally stable bonds require higher energies for thermal degradation. Consequently, degradation of multi-component materials leads to multiple peaks (Hernandez 1997). This indicates that linalool-LDPE-EVA and methylchavicol-LDPE-EVA might not have been subjected to thermal degradation during the extrusion film blowing. This result is also reflected in the carbonyl index data discussed in the previous section.

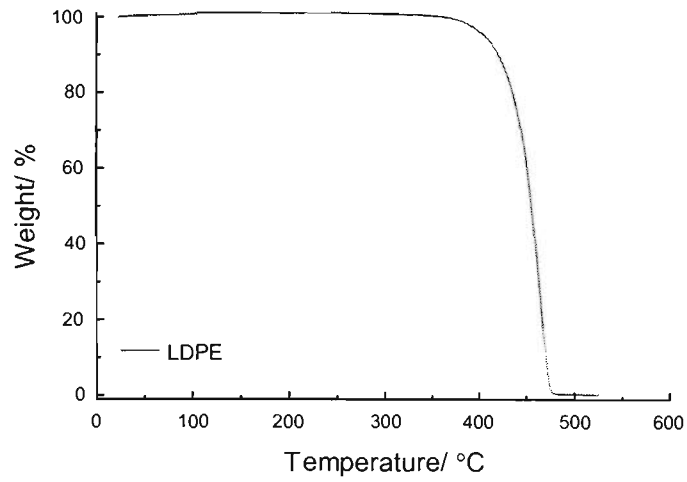
4.5.6 TGA mass-loss and peak temperature

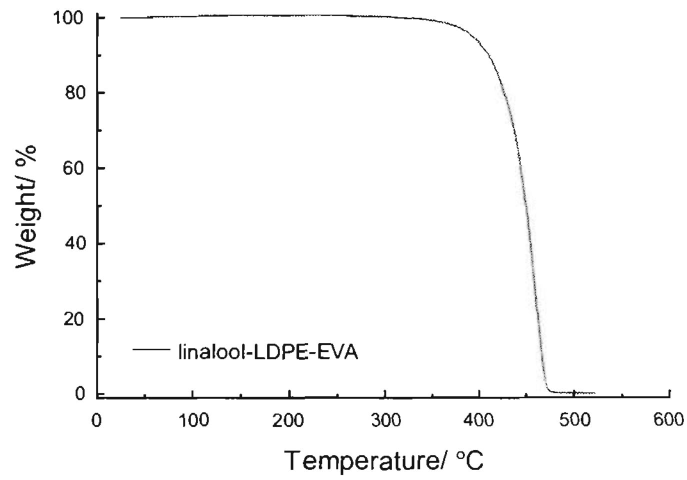
Figure 4.15 shows TGA mass-loss curves of the film samples. Because of the very low concentration of either linalool or methylchavicol which are volatile compounds, no distinct step of mass-loss could be identified. The TGA data are interesting in that these demonstrate the beginning, maximum and final decomposition temperatures of the materials. In this experiment, the derivative TGA mass-loss curves showed that the polymeric structure underwent decomposition that started at approximately 231 °C, the peak temperature was around 463 °C and ended at about 484 °C (**Figure 4.16**).

Linalool-LDPE-EVA film exhibited the lowest (461 °C) while the additive-free LDPE film showed the highest (466 °C) temperature peak for decomposition. As expected, the melt behaviour of the LDPE film suggested that it is slightly more stable than the other two films.

4.5.7 Electron micrograph

The microscopic structures of the films were examined using scanning electron microscopy. The micrographs are shown in **Figure 4.17**. Note the micrographs show some damage at the film cross-sections due to the use of mechanical sample cutting rather than cryogenic microtoming. Upon inspection of the electron micrographs, films containing linalool or methylchavicol (**Figure 4.17 b, c**) exhibited no evidence in the microstructures to suggest that linalool and methylchavicol were not evenly distributed in the film.





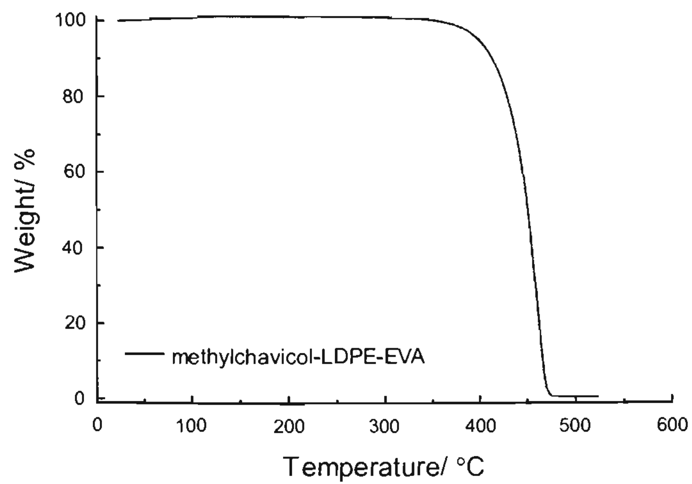
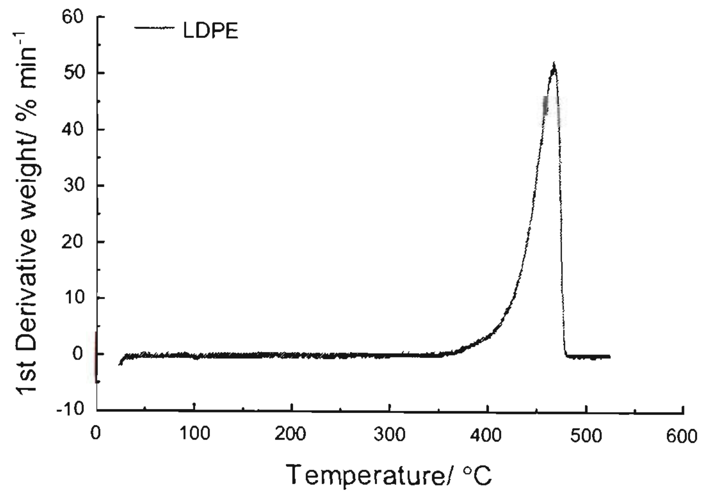
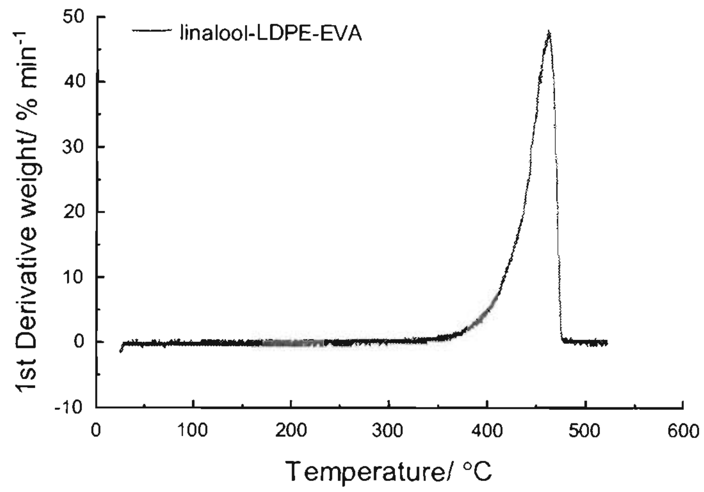


Figure 4.15 TGA mass-loss curves of AM LDPE-EVA films





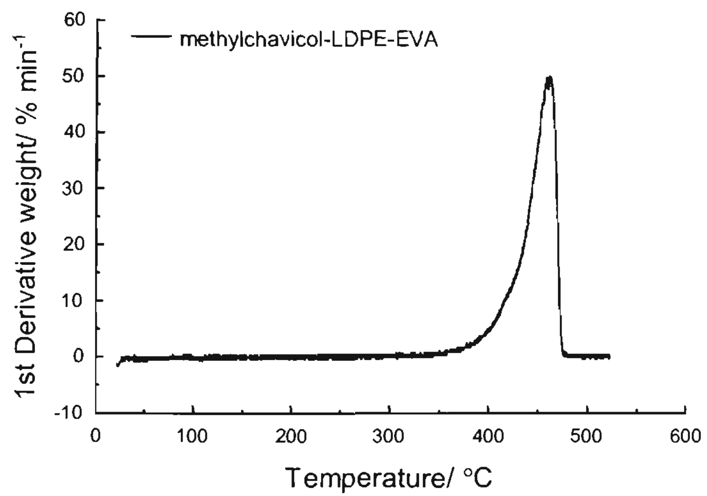
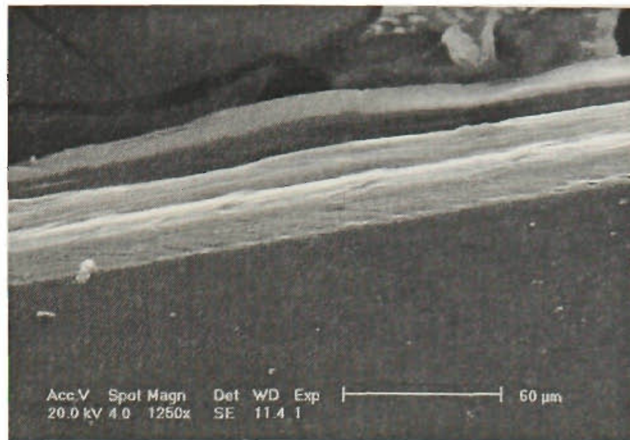
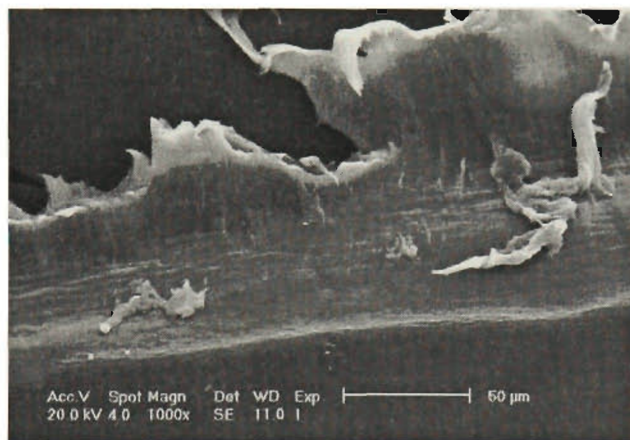


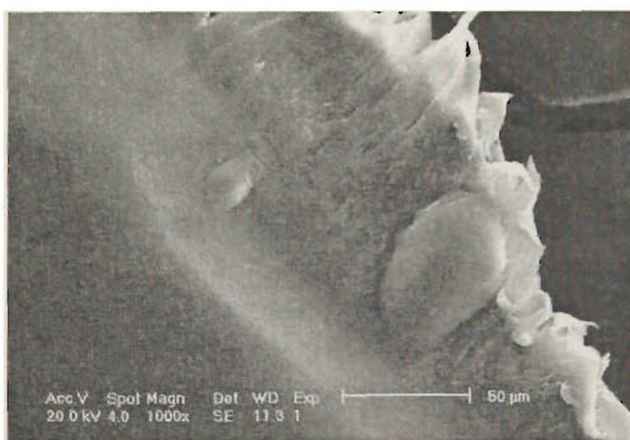
Figure 4.16 First derivative TGA mass-loss curves of AM LDPE-EVA films



(a)



(b)



(c)

Figure 4.17 SEM micrographs of the edge surfaces for AM LDPE-EVA films,
(a) LDPE, (b) linalool-LDPE-EVA and
(c) methylchavicol-LDPE-EVA

4.5.7 Electron micrograph

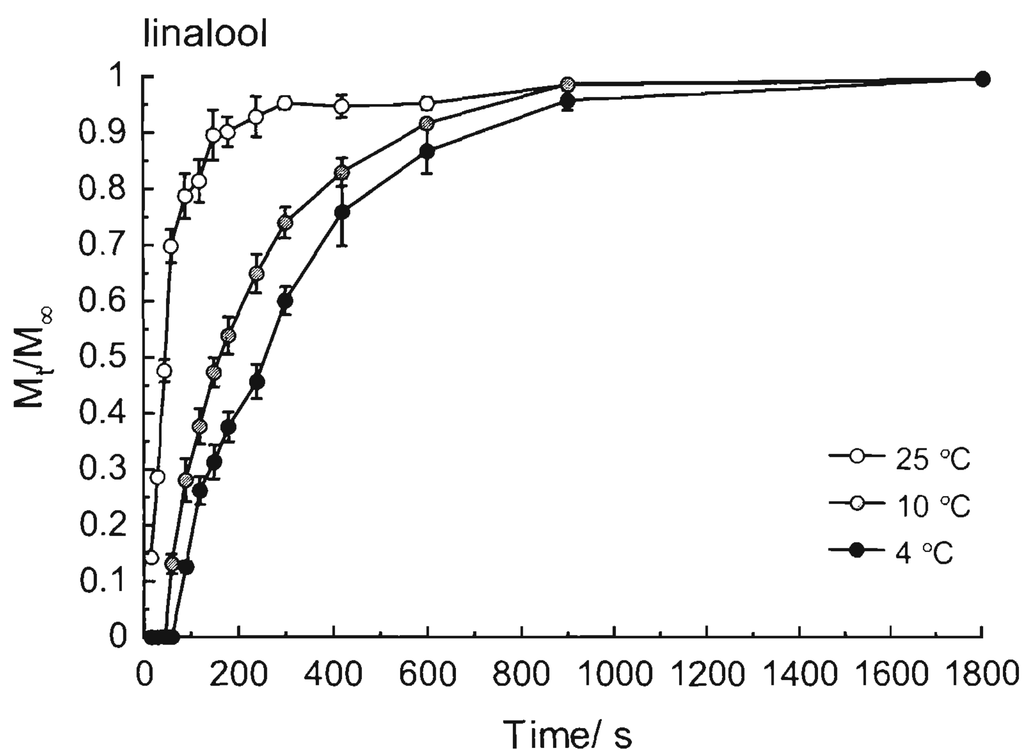
The microscopic structures of the films were examined using scanning electron microscopy. The micrographs are shown in **Figure 4.17**. Note the micrographs show some damage at the film cross-sections due to the use of mechanical sample cutting rather than cryogenic microtoming. Upon inspection of the electron micrographs, films containing linalool or methylchavicol (**Figure 4.17 b, c**) exhibited no evidence in the microstructures to suggest that linalool and methylchavicol were not evenly distributed in the film.

4.6 Release of AM additive from films into a food simulant

4.6.1 Kinetics of additive released from LDPE-EVA films

The fractional mass release, M_t/M_∞, is the mass of agent released into iso-octane at time t, M_t, expressed as a proportion of the maximum mass of agent that could be released after an infinite time, M_∞. The question of whether or not M_t/M_∞ is directly proportional to $t^{1/2}$ was firstly analysed.

The migration curves of linalool and methylchavicol from LDPE-EVA films immersed in iso-octane used as a fatty food simulant at different temperatures are shown in **Figure 4.18**. **Figure 4.19**, at 4°C, has been selected as an example to show the regression curve (predicted line) as a continuous line. The migration rate is at a maximum immediately after a lag time of immersion of ca. 60 s, and progressively reduces thereafter, until it becomes nearly complete after at about 1800 s for both agents. In all cases, linearity with respect to $t^{1/2}$ of the initial portion of the curve ($M_t/M_\infty < 0.6$ (Miltz 1987)) was quite good (r^2



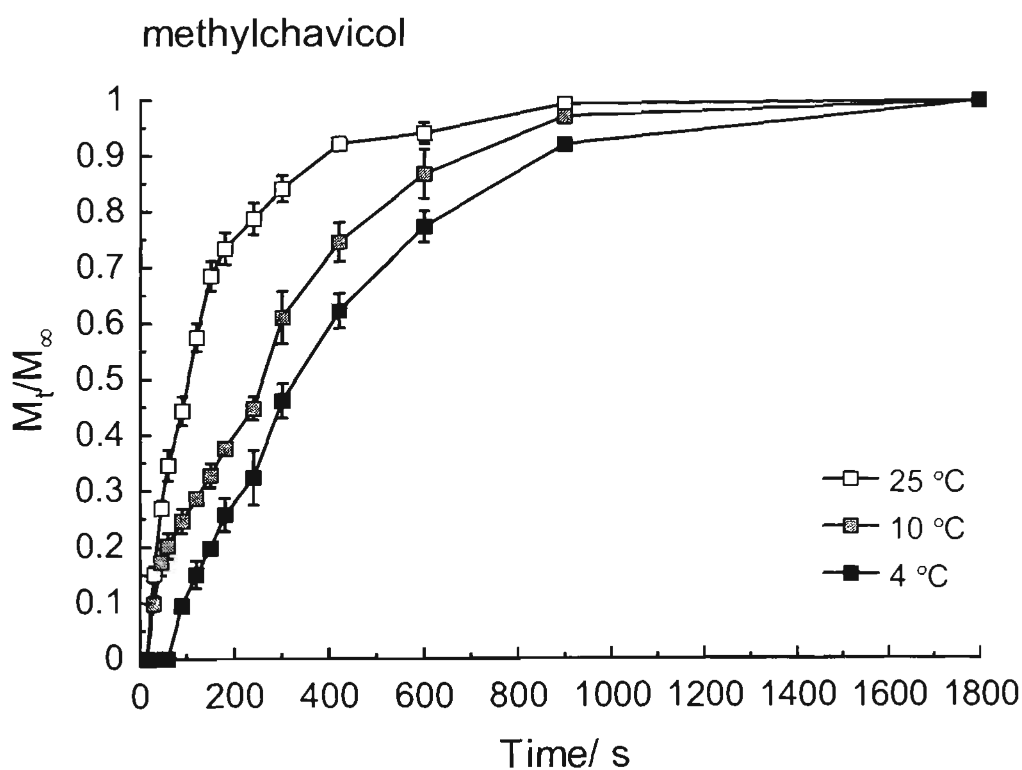
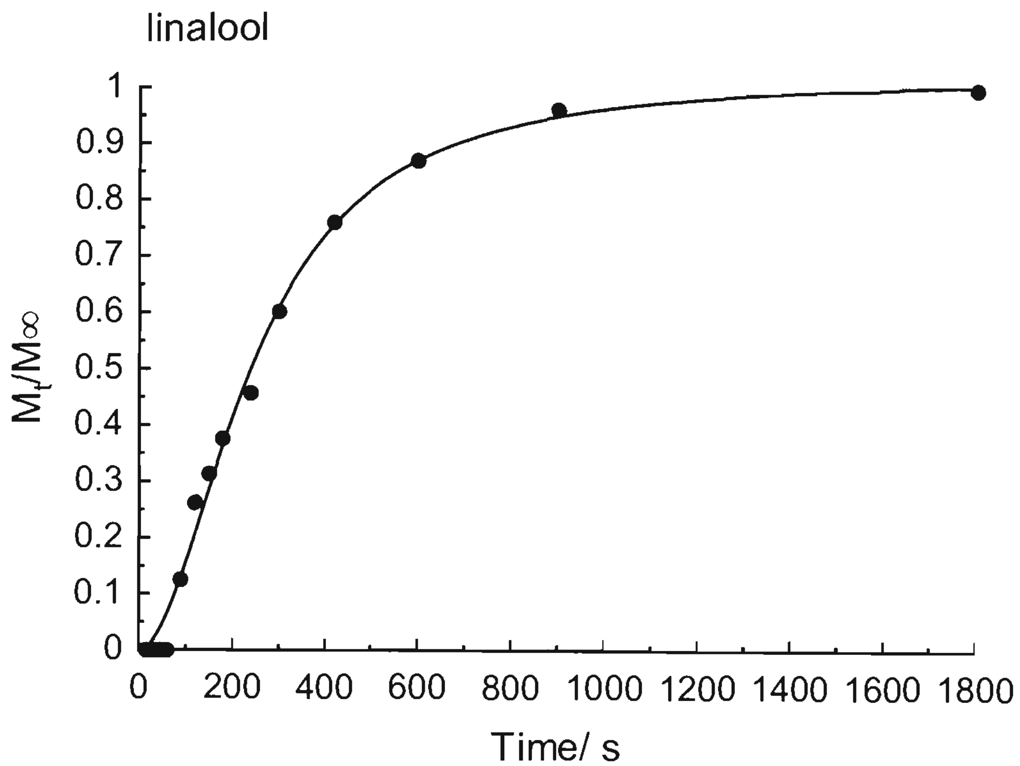


Figure 4.18 Migration curves of linalool and methylchavicol from LDPE-EVA films into iso-octane at different temperatures
Bars represent standard error around the mean



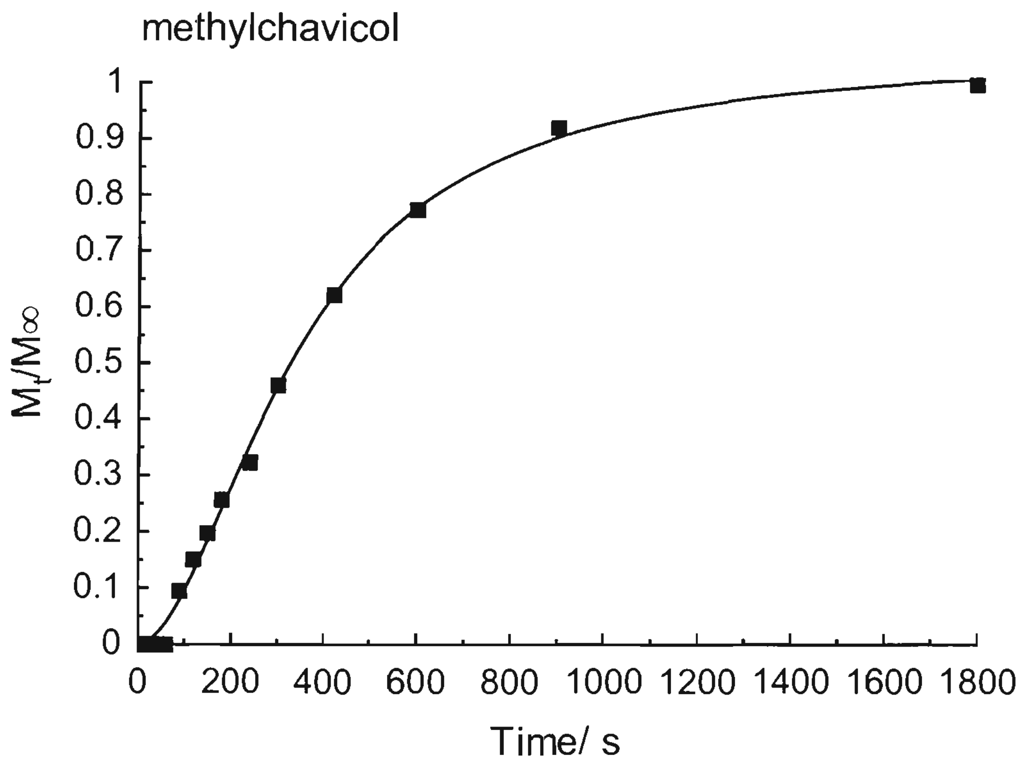


Figure 4.19 Migration curves of linalool and methylchavicol from LDPE-EVA films into iso-octane at 4 °C. Continuous line represents the sigmoid that better fits experimental data from time-response function with Hill coefficient

ranging from 0.8993 to 0.9845). In addition, the kinetics of linalool and methylchavicol release from the films were better fitted ($r^2 = 0.9944 \pm 0.0017$ and 0.9930 ± 0.0054, respectively) with a nonlinear, least-squares fit of the time-response function using Hill coefficient (**equation 3.4**).

4.6.2 Effect of temperature on migration

In accordance with the Arrhenius equation, the migration data showed a significant effect of temperature on the migration of linalool and methylchavicol, as illustrated in **Figure 4.18**. Raising the temperature from 4 to 25 °C leads to a faster rate of migration for both agents. Iso-octane is a standard fatty food stimulant that clearly behaves as one in which extraction (migration) efficiency is high. In particular the time required to release half the amount of linalool initially contained in the LDPE-EVA film decreases from 238 s at 4 °C to 165 s at 10 °C and 42 s at 25 °C, whereas the corresponding times for methylchavicol at the respective temperatures are 327 s, 231 s, and 97 s. Furthermore, the diffusion coefficient D of linalool calculated with the half-time method (**equation 3.1**) increases from 4.22×10^{-13} m^2 s^{-1} to 2.46×10^{-12} m^2 s^{-1}, and the corresponding rate constant k from **equation 3.4** decreases from 251 to 44 s^{-1}, when the temperature is increased from 4 to 25 °C (**Table 4.11**). Similar behaviour was observed in the case of methylchavicol. In the case of linalool, Hill coefficients were in the same range (1.87-1.93) at different temperatures. This is in agreement with the principle that the Hill coefficient of a given system is temperature-independent. However, at 10 °C, Hill coefficient of methylchavicol was 1.35, lower than the expected range (1.67-1.72). This might be because of

incompletion of cooperativity according to West (2000). The data show that linalool is more mobile in the polymer matrix than methylchavicol, presumably due to its lower compatibility with the polymer substrate compared with methylchavicol. This behaviour is also reflected, to some extent, in the Hill coefficients where the values of n obtained for linalool are greater than those for methylchavicol possibly indicating the greater departure from idealised behaviour exhibited by linalool compared with methylchavicol.

Table 4.11 Effect of temperature on the migration of linalool and methylchavicol from LDPE-EVA films into iso-octane

Agent	Temperature/ °C	$L \times 10^6$ [1]/ m	$D \times 10^{12}$ [2]/ $m^2\ s^{-1}$	k[3]/ s^{-1}	n[4]
linalool	4	47.0	0.42 (0.41-0.44)	250.67±21.27[c]	1.92±0.10
	10	47.3	0.68 (0.65-0.71)	167.21±10.16[b]	1.87±0.07
	25	48.4	2.46 (2.43-2.48)	44.50±2.08[a]	1.93±0.30
methylchavicol	4	48.0	0.35 (0.34-0.36)	345.99±9.82[c]	1.72±0.15[b]
	10	48.7	0.44 (0.43-0.46)	296.72±22.58[b]	1.35±0.17[a]
	25	47.5	1.10 (1.07-1.14)	99.12±3.74[a]	1.67±0.10[b]

[1] Film thickness

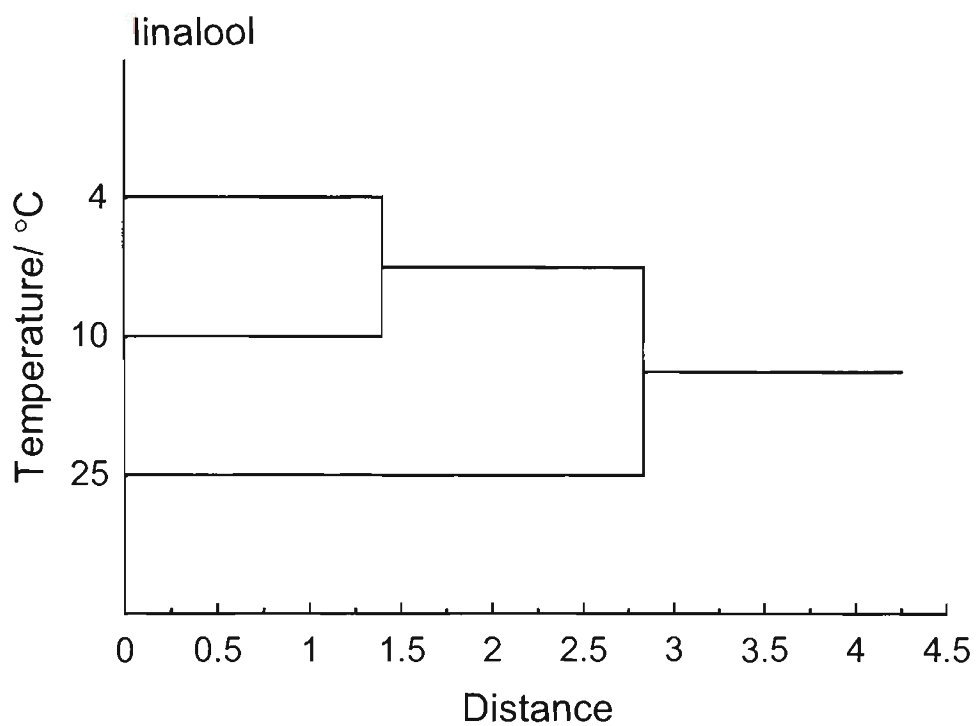
[2] Diffusion coefficient. Values in parentheses are lower and upper limits for D.

[3] Rate constant obtained by nonlinear regression. For each agent, k ±SEM. values with different letters are significantly different ($p \leq 0.01$).

[4] Hill coefficient. For each agent, n ±SEM. values with different letters are significantly different ($p \leq 0.05$).

An applied cluster analysis using Ward method is shown by the dendrogram in **Figure 4.20**. It can be seen that the rate constant for linalool (distance = 1.4) is more temperature-dependent than that of methylchavicol (distance = 0.8) in the temperature range from 4 to 10 °C. In the case of linalool there is no statistically significant difference ($p > 0.05$) in Hill coefficient in this temperature range from 4 to 25 °C. At all temperatures, both linalool and methylchavicol showed positive affinity ($n > 1$) for iso-octane. Furthermore, the temperature dependence of the diffusion coefficient is well described by an Arrhenius relation (**Figure 4.21**) with activation energies of 836 J $mole^{-1}$ and 619 J $mole^{-1}$ for linalool and methylchavicol, respectively, obtained by a time-response function with Hill coefficient (**Table 4.12**). These values are close to the activation energies derived from the half-time method equation which is normally used for the diffusion coefficient calculation (Lim and Tung 1997; Han and Floros 1998; Ouattara et al. 2000b; Kim et al. 2002a; Teerakarn et al. 2002).

The release of linalool and methylchavicol from LDPE-EVA films immersed in iso-octane, might be described by the "swelling-controlled" model for drug release that was previously reported by Armand et al. (1987) and Malley et al. (1987). Regarding this model, iso-octane first enters the polymer matrix and dissolves the agents, thus allowing their subsequent release from the polymer. The migration of linalool and methylchavicol is thus expected to escalate with increasing iso-octane penetration into LDPE-EVA film, to ultimately approach a plateau when the matrix is saturated with iso-octane (Armand et al. 1987); the experimental results obtained in the current study are consistent with this model.



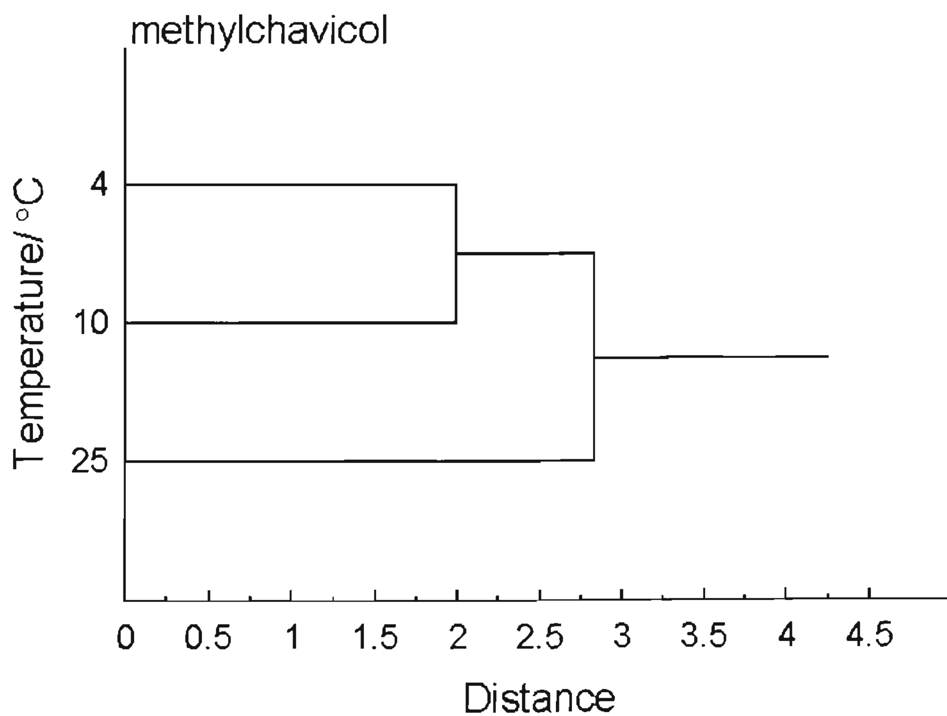


Figure 4.20 Ward clustering of the rate constant at different temperatures

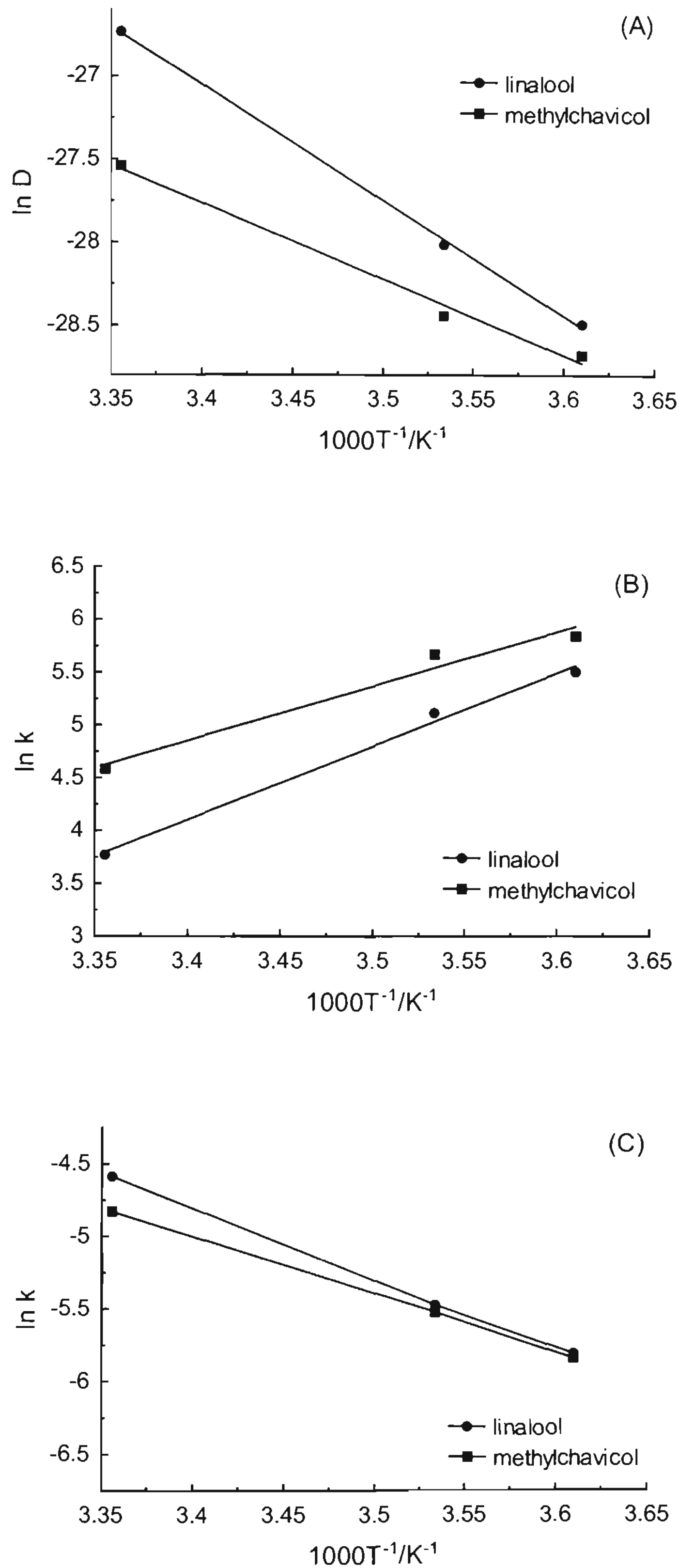


Figure 4.21 Arrhenius plots of linalool and methylchavicol incorporated in LDPE-EVA films (A) Half-time method equation (see **equation 3.1**), (B) Time-response function with Hill coefficient (**equation 3.4**), and (C) Exponential rise to maximum function (**equation 3.2**)

Table 4.12 Activation energies of linalool and methylchavicol incorporated in LDPE-EVA films

Agent	Activation energy/ J mole^{-1}	
	Half-time	Hill
linalool	839 (0.04)[1]	836 (0.10)
methylchavicol	553 (0.08)	619 (0.16)

[1] Standard error of the estimate, around the line of regression

In reality, the situation is more complex. Many interactions take place during the migration of species from polymers to liquids. In particular, iso-octane uptake generally causes polymer swelling (Feigenbaum et al. 2000). This is because the solubility parameter of iso-octane is close to the solubility parameter of LDPE (Brydson 2000). Furthermore, Lim and Tung (1997) reported a time-dependent relaxation process resulting from the swelling stress that occurs during the diffusion of liquid into polymers. As a consequence, release rates change continuously, and migration is difficult to analyse mathematically (Gnanasekharan and Floros 1997).

In this study, the initial portion of the migration curves was found to be in accordance with the predictions of Fick law for diffusion. However, evidence of the non-Fickian nature of the diffusion phenomenon is provided by the sigmoidal shape of the migration curves, especially at low temperatures. This indicates cooperativity that causes the migration curve to display sigmoidal kenetics.

The upward curvature of the experimental sorption curve shows a constant increase in the diffusion coefficient. The penetration of iso-octane molecules facilitates further penetration by the plasticisation of the polymer matrix, until the plateau is reached (Feigenbaum et al. 2000). This suggests that the release of linalool and methylchavicol from LDPE-EVA films is not determined only by diffusion (Peppas 1985). Moreover, the fractional mass release, plotted as a function of time, was better fitted by a time-response function with Hill coefficient (**equation 3.4**) than by an exponential rise to a maximum level (**equation 3.2**). These findings are in agreement with those of Ouattara et al. (2000b) who firstly reported a non-Fickian behaviour for the diffusion of acetic and propionic acid from chitosan-based films into buffer solution. Consequently, the non-Fickian behaviour observed in the present study is most likely due to simultaneous swelling (due to iso-octane uptake) and outward diffusion of linalool or methylchavicol (Ouattara et al. 2000b).

The dependency of the rate of diffusion of linalool and methylchavicol from LLDPE-EVA films is generally explained by temperature effects on the solubility of diffusing molecules in films, on the nature of adhesive forces at interfaces (Brydson 2000), and on molecular mobility (Vojdani and Torres 1990; Myint et al. 1996). The fact that the relationship between diffusion and temperature is described by an Arrhenius equation in this study, suggests that the influence of temperature is thermodynamic in nature, essentially regulated by the proportion of energy provided to the activation energy (Daniels and Alberty 1972).

4.7 Storage of AM films

4.7.1 Residual concentration and AM activity after long-term storage

A concern exists about a possible depletion, by diffusion into the environment, of AM additives, especially volatile compounds, during long-term storage. During long-term storage at ambient conditions for 1 year, the films were subjected to the temperature cycle between day and night times. The residual concentration and AM activity of linalool-LLDPE and methylchavicol-LLDPE films are shown in **Table 4.13**. The additive retention of linalool and methylchavicol in the films were 66.07% and 52.83% respectively. **Figure 4.22** shows a clear zone with symmetric characteristic of AM LLDPE film against *E. coli* after 1 year of storage. The additive retention was still comparatively high. This might be due to the low initial concentration of the additives and therefore a low driving force for diffusion as well as the temperature cycle that slowed down the diffusion rate at night. Although a decrease in additive retention had been observed, there was no difference in AM activity of the films between the beginning and after 1 year of storage. Obviously, this finding suggests that linalool or methylchavicol AM films exhibited inhibitory effects against *E. coli*, a Gram-negative bacterium, even at low residual concentrations.

4.7.2 Effect of temperature on additive retention after accelerated storage

The depletion of the specific additives was used to determine the reaction rate constant of AM films. The reduction in additive concentration of the AM

Table 4.13 AM activity of LLDPE films against *E. coli* as observed by agar disc diffusion assay after long-term storage

Treatment	Target Conc./ %w/w	Actual Conc./ %w/w	Zone of Inhibition/ mm[1]
At the beginning			
LLDPE	-	-	-[2]
linalool-LLDPE	1.00	0.0559	11.27±1.89[3]b
methylchavicol-LLDPE	1.00	0.0529	8.76±0.44a[4]
At 1 year of storage			
linalool-LLDPE	1.00	0.0371	11.08±0.11b
methylchavicol-LLDPE	1.00	0.0281	8.15±0.12a

[1] no antimicrobial activity against *Staphylococcus aureus* and *Saccharomyces cerevisiae*

[2] -, no reaction

[3] Values for zone of inhibition are represented as mean±SEM

[4] The treatment with same letter within row is not statistically significant difference ($p > 0.05$)

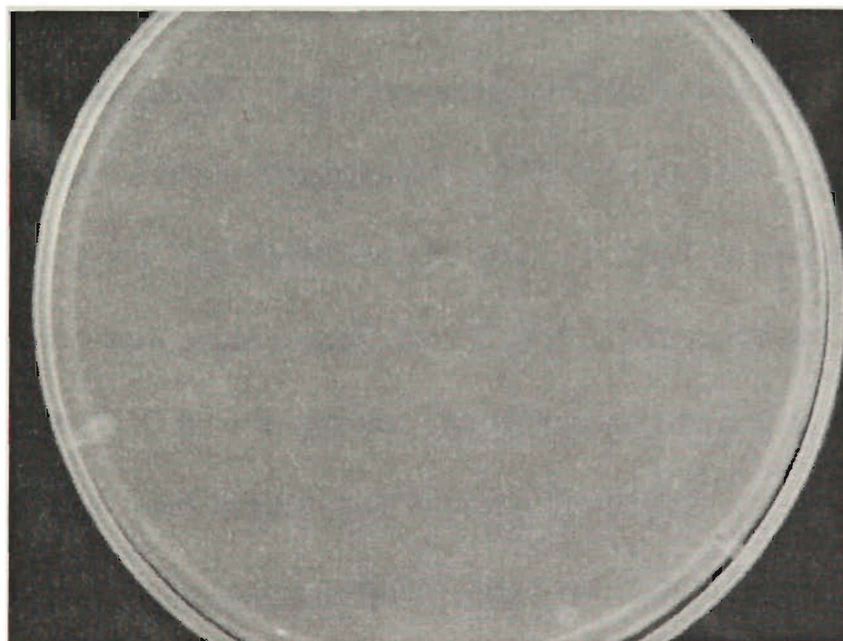


Figure 4.22 Growth inhibition of *E. coli* by LLDPE film containing linalool or methylchavicol after 1 year of storage

LDPE-EVA films during storage at 25 and 35 °C is represented in **Figure 4.23**. A first order decay for additive retention was initially supposed. The theoretical plots of ln(C) versus time for a first order decay are shown in **Figure 4.24**. It was found that additive retention of all film samples tend to deviate from a theoretical first order decay plot of ln(C) versus time. This might result from the fact that a certain amount of linalool or methylchavicol is bound within the polymeric matrix. Hence, plots of $\ln(C-C_{\infty})$ versus time as first order decay with an offset have been employed as depicted in **Figure 4.25**. From these figures, the infinite additive retention (C_{∞}), rate constant (k) and half-life can be obtained and are shown in **Table 4.14**.

The calculated levels of bound additive in linalool-LDPE-EVA films at 25 and 35 °C were found to be 0.0514, and 0.0360% w/w, respectively. For methylchavicol-LDPE-EVA films the levels at 25 and 35 °C were 0.0453 and 0.0285% w/w respectively. These results for AM LDPE-EVA films stored at 35 °C were close to the actual concentration of AM LLDPE films stored at ambient conditions for 1 year (**see Section 4.7.1**). Thus, it might be assumed that AM films containing basil extracts retain their inhibitory action against *E. coli* even when stored at 35 °C for a long term. The determined rate constant of linalool-LDPE-EVA and methylchavicol-LDPE-EVA films at 25 were 9.0×10^{4} and 10.6×10^{4} h^{-1}, respectively, and at 35 °C were 10.8×10^{4} and 12.5×10^{4} h^{-1}. The estimated half-life, $\theta_{1/2}$, can be calculated by dividing ln(2) by the rate constant (Labuza 1982; Man and Jones 1994). The values ranged between 27 and 32 days at 25 °C and 4-5 days at 35 °C. From the rate constant, the sensitivity of the

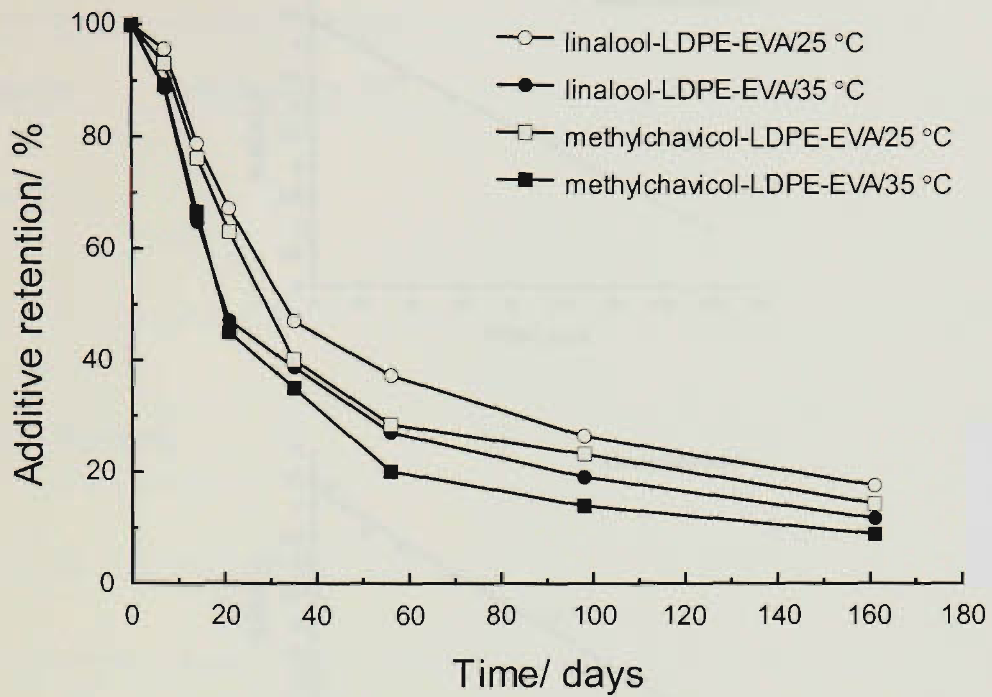


Figure 4.23 Decrease in additive retention of AM LDPE-EVA during storage at 25 and 35 °C

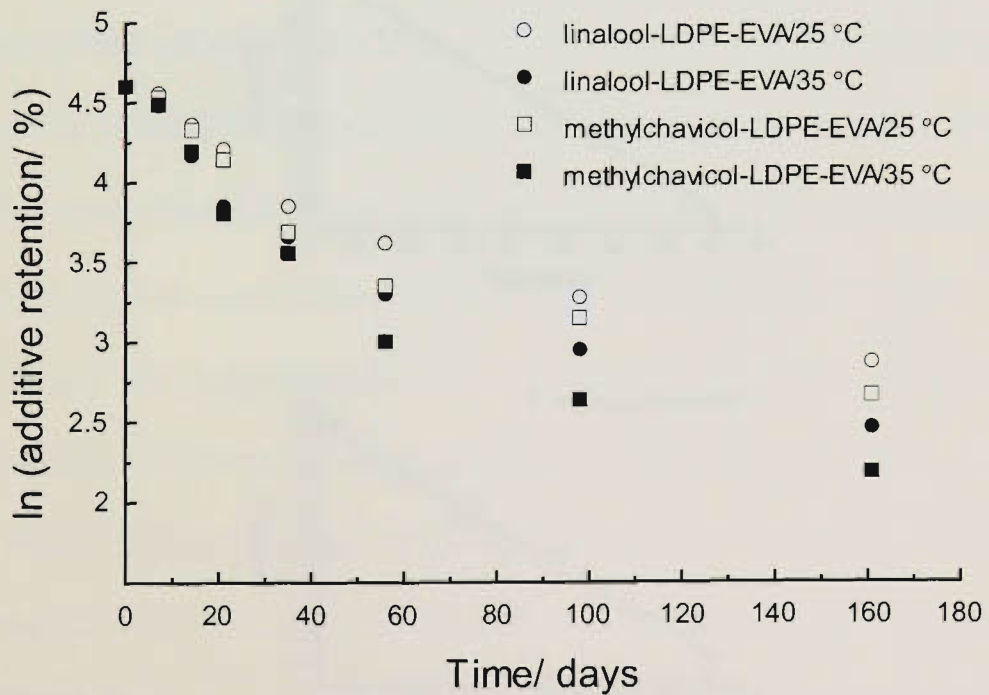


Figure 4.24 Plot of ln(C) versus time as first order decay in additive retention of AM LDPE-EVA films during storage at 25 and 35 °C

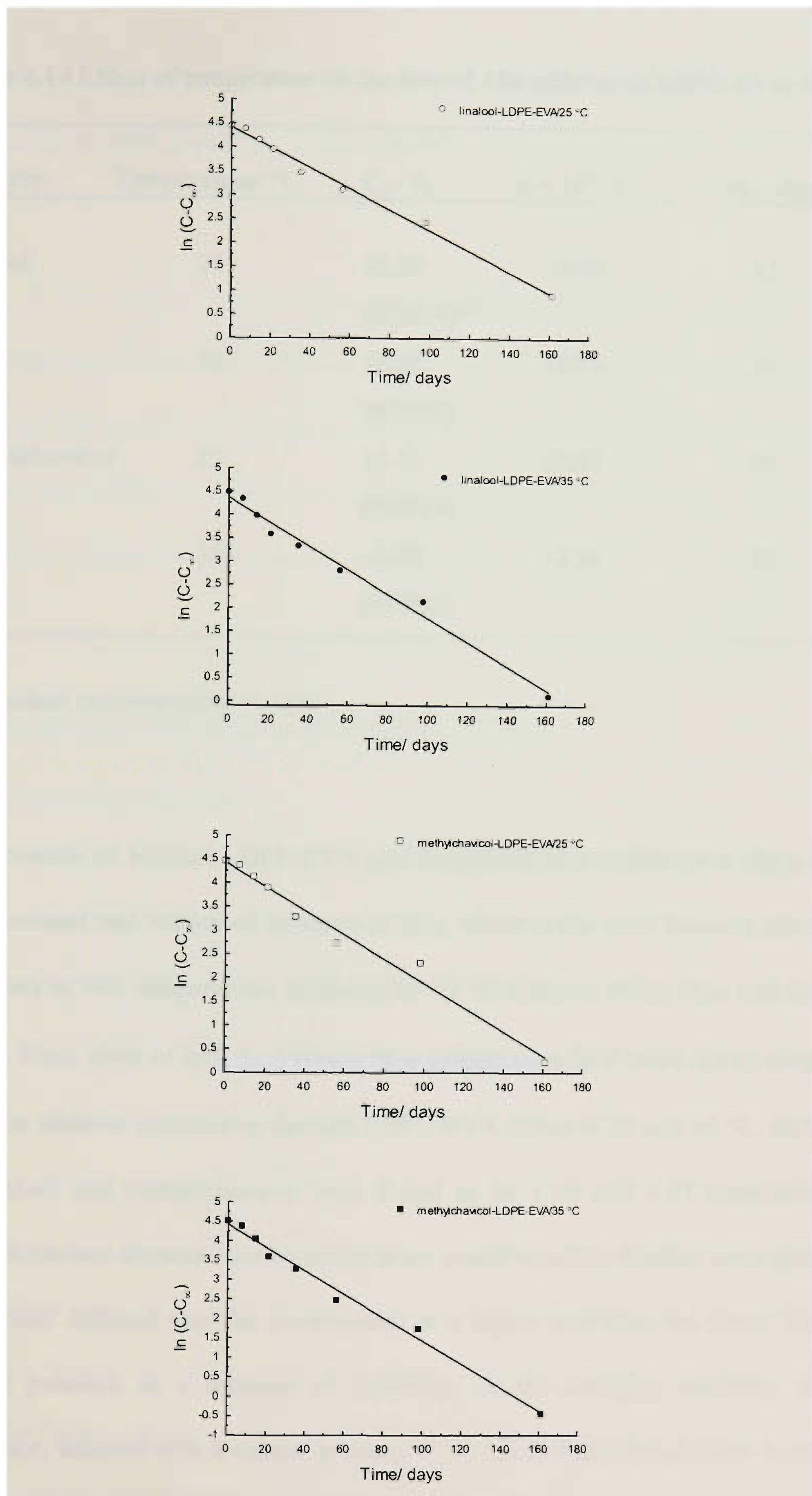


Figure 4.25 Plot of ln(C-C_∞) versus time as first order decay with offset in additive retention of AM-LDPE-EVA films during storage at 25 and 35 °C

Table 4.14 Effect of temperature on the loss of AM additive in LDPE-EVA films

Additive	Temperature/ °C	C_∞/ %	$k \times 10^4$/ h^{-1}	$\theta_{1/2}$/ day
linalool	25	15.20 (0.0514)[1]	9.04	32
	35	10.64 (0.0360)	10.79	27
methylchavicol	25	13.15 (0.0453)	10.62	27
	35	8.28 (0.0285)	12.50	23

[1] Residual concentration/ % w/w

deterioration of linalool-LDPE-EVA and methylchavicol-LDPE-EVA films can be calculated and expressed in terms of Q_{10}, which is the ratio between the rate constants at two temperatures differing by 10 °C (Labuza 1982; Man and Jones 1994). From plots of $\ln(C-C_\infty)$ versus time (assuming a first order decay with an offset in additive retention in the AM LDPE-EVA films) at 25 and 35 °C, the Q_{10} of linalool and methylchavicol were found to be 1.19 and 1.17 respectively. Methylchavicol showed a lower temperature sensitivity than linalool even though the former diffused into the environment at a higher rate than the latter. Using vapour pressure as a measure of volatility, or the escaping tendency of a substance, linalool with a vapour pressure of 21 Pa at room temperature is more volatile than methylchavicol with a vapour pressure of 12 Pa (**see Appendix B**).

The results suggest that linalool, with the higher volatility, had stronger molecular interaction with the polymer matrix than methylchavicol.

It is worthy to note that the deterioration of the AM additives depends on the initial concentration of additive in the film, the storage conditions and the nature of the additive and polymer. Therefore, in commercial film production, it is possible to define the requested half-life of the AM film and calculate from this the initial concentration that should be used.

4.8 Application of AM films on Cheddar cheese

4.8.1 Microbial growth

Changes in total aerobic mesophilic bacterial counts in Cheddar cheese samples wrapped with the additive-free LDPE, the linalool-LDPE-EVA and the methylchavicol-LDPE-EVA films during storage under refrigeration are presented in **Figure 4.26**. Total aerobic mesophilic plate counts in control samples gradually increased from 4.18 to 4.78 log(cfu g^{-1}) at the end of the storage period (21 days). Samples wrapped with linalool-LDPE-EVA and methylchavicol-LDPE-EVA films showed a significant reduction in bacterial populations by 2.14 and 1.90 log units, respectively, during the first 2 days. Then bacterial growth in the samples increased progressively up to 4.35 and 4.29 log(cfu g^{-1}). This pattern of bacterial growth observed in treated films was similar to the change in total aerobic mesophilic bacterial counts in ground beef samples irradiated at 1 kGy and coated with protein-based film during storage at 4 °C for 10 days, studied by Ouattara et al. (2002).

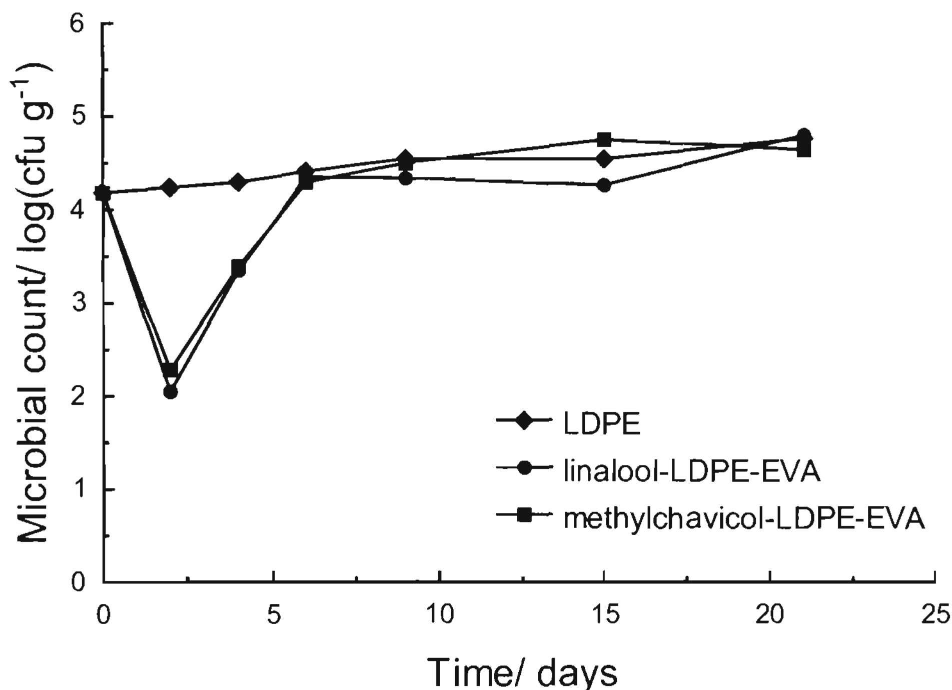


Figure 4.26 Inhibition of total aerobic mesophilic bacteria on Cheddar cheese with AM-LDPE-EVA films refrigerated at 4 °C

In the present study, Cheddar cheeses packaged in LDPE-EVA film with 0.34% w/w linalool or methylchavicol showed significantly lower growth of total aerobic mesophilic bacteria for the period of 15 and 9 days, respectively, compared to the control (**Figure 4.26**). On the basis of 10^7 cfu g^{-1} as an acceptable microbial criterion for quality limit, unfortunately there were insufficient data to evaluate the shelf life of Cheddar cheese wrapped with AM films compared to the control.

There were no significant differences between AM LDPE-EVA films and additive-free LDPE film in coliform bacteria count, and yeast and mould count through the storage period. Both counts were less than 0.5 cfu g^{-1}. There was also

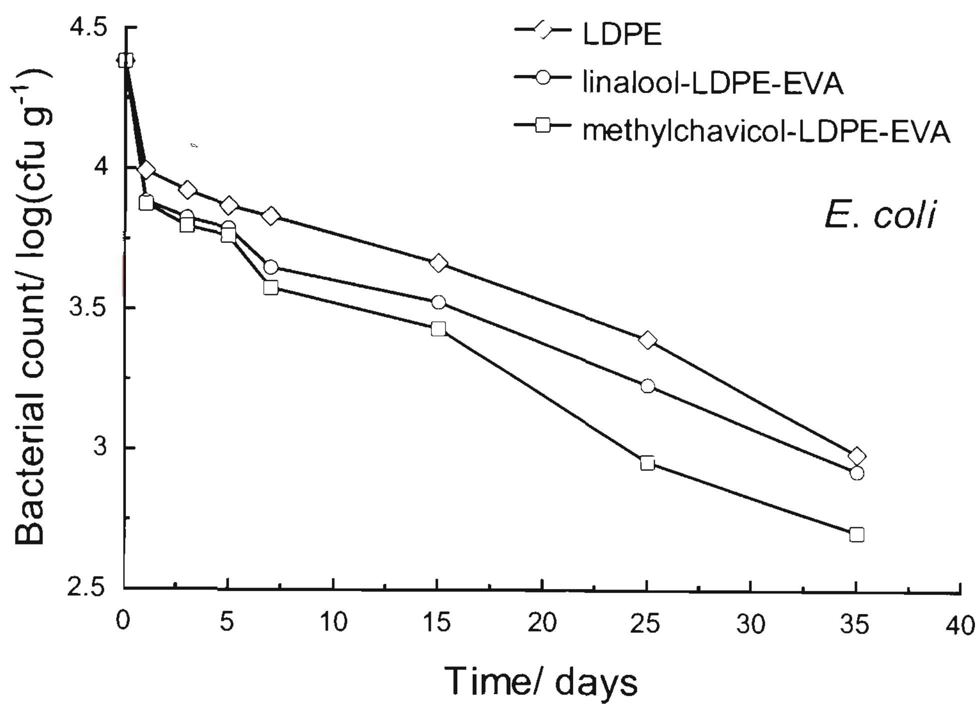
no visual colony of mould on cheese surfaces at the end of the storage. Mould growth was visible a week later in the sample wrapped with LDPE film. The delay on cheese wrapped with this film suggested that a longer period was required for mould to access oxygen and to develop mycelium reaching out beyond the surface area covered by the LDPE film. No visual colony of mould was observed up to 2 months when LDPE-EVA film containing 0.34% w/w linalool or methylchavicol was used. Weng and Hotchkiss (1992) reported that no growth of *Aspergillus toxicarius* and *Penicillium* sp. was visually observed up to 10 days when LDPE film containing 1000 mg kg^{-1} Imazalil, an antimycotic agent, was used to wrap Cheddar cheese.

The results of this study show that LDPE-EVA film containing linalool or methylchavicol has an inhibitory effect for microbial growth in natural microbial-contaminated Cheddar cheese. Nevertheless, the complexity of the microbial flora (Beresford et al. 2001) in Cheddar cheese makes it difficult to explain completely the phenomenon of the slowed microbial growth caused by the linalool-LDPE-EVA and methylchavicol-LDPE-EVA films. Ha et al. (2001) found in their study that grapefruit seed extract-coated film showed AM activity against *B. cereus*, *B. subtilis*, *E. coli*, *L. mesenteroides*, *M. flavus*, *S. aureus*, and *S. cerevisiae* on the agar disc diffusion assay. However, the level of grapefruit seed extract addition (0.5 and 1.0% w/w) did not produce any discernible difference in microbial growth on packaged ground beef.

No evidence of chemical interaction between polymers and linalool or methylchavicol has been found in the literature. However, it could be hypothesised that impregnation of active compounds in a polymeric matrix would lower their diffusion and bring to a higher concentration of the compounds at the surfaces of foods for a longer period, as commended by Ouattara et al. (2001). It was reported that incorporation of hydrophobic compounds into hydrophilic polymers cause structural modifications of the polymer matrix, leading to an increase in network tortuosity (Redl et al. 1996; Callegarin et al. 1997), impeding the transport of molecules through the network (Papadokostaki et al. 1997), and reducing water uptake (Vazquez et al. 1997). Further research is ongoing to investigate the AM effects of these agents in Cheddar cheese with food spoilage and/or pathogenic culture inoculation.

4.8.2 Bacterial growth

Escherichia coli. The numbers of *E. coli* on Cheddar cheese wrapped with LDPE, linalool-LDPE-EVA, and methylchavicol-LDPE-EVA films decreased by a factor of 24, 28 and 47, respectively, after 35 days of storage at 4 °C (**Figure 4.27**). Storing the samples at 12 °C was done in order to mimic temperature abuse situations that might occur in the food distribution chain (Cutter 1999; Siragusa et al. 1999). A similar pattern of inhibition of *E. coli* on Cheddar cheese subjected to temperature abuse was found and is shown in **Figure 4.28**. LDPE, linalool-LDPE-EVA and methylchavicol-LDPE-EVA films reduced the *E. coli* populations by a factor of 14, 27 and 34, respectively, after 15 days.



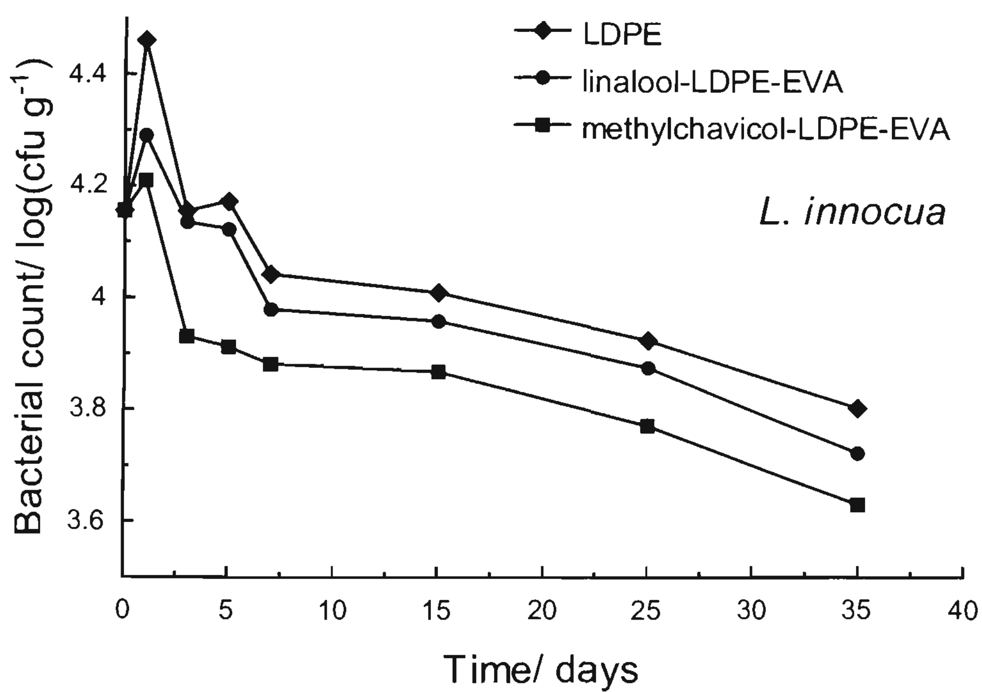
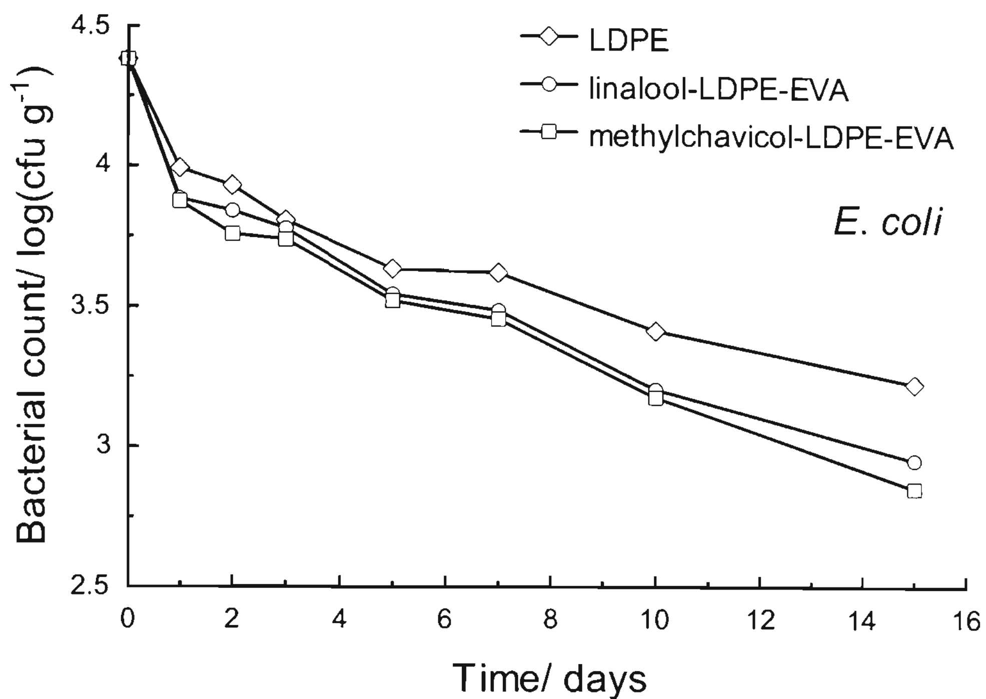


Figure 4.27 Inhibition of *E. coli* and *L. innocua* on Cheddar cheese with AM-LDPE-EVA films refrigerated at 4 °C



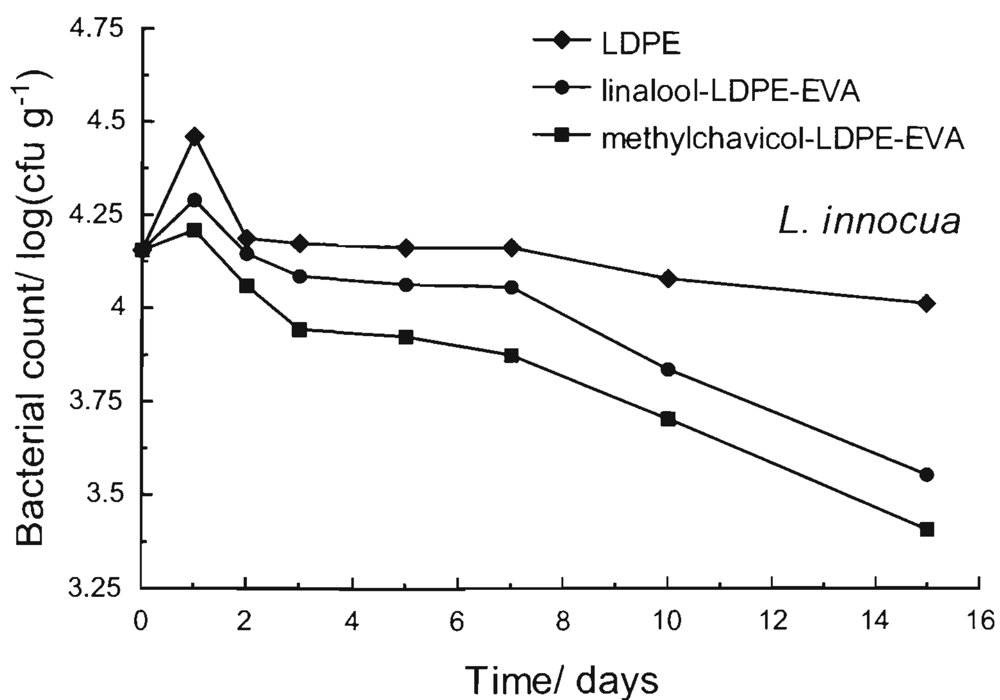


Figure 4.28 Inhibition of *E. coli* and *L. innocua* on Cheddar cheese with AM-LDPE-EVA films subjected to temperature abuse condition of 12 °C

Listeria innocua. Films containing linalool or methylchavicol reduced *L. innocua* populations by a factor of 2.7 and 3.4, respectively after 35 days of storage at 4 °C (**Figure 4.27**). In control samples, the *Listeria* population was also reduced by a factor of 2.2 after 35 days at refrigerated storage. The inhibition of *L. innocua* on Cheddar cheese subjected to temperature abuse is depicted in **Figure 4.28**. The values of *L. innocua* on Cheddar cheese packed with linalool-LDPE-EVA and methylchavicol-LDPE-EVA films were reduced by a factor of 4.0 and 5.6, respectively after 15 days of temperature abuse simulation. For the additive-free LDPE film, the *Listeria* population initially remained constant and decreased slightly (by a factor of 1.4) during the following 7 days of temperature abuse condition. Regardless of storage temperatures and sample treatments, the growth of *Listeria* on Cheddar cheese during the first day may have occurred due to the availability of nutrients leached from the cheese into the moisture and due to the presence of oxygen on the surface of the product as a result of previous exposure to the relatively high moisture and oxygen environment (Holliday et al. 2003).

In the control, populations of *E. coli* also decreased in Cheddar cheese samples throughout the end of the storage period. The decrease was rapid in samples stored at the temperature abuse conditions, compared to 4 °C. This is consistent with the study by Holliday et al. (2003) of the viability of *E. coli* O157: H7 in butter, "yellow fat spread" and margarine stored at 4.4 and 21 °C and also consistent with the work of Cagri et al (2002) on survival of *E. coli* O157: H7 in Bologna and fermented summer sausage stored at 4 °C. As observed

with *E. coli*, the population of *L. innocua* was reduced faster in cheese samples stored at 4 °C compared to the abuse conditions at which the population of *L. innocua* remained almost constant. Holliday et al. (2003) also showed that the population of *L. monocytogenes* was continually decreased in light salted butter and "yellow fat spread" stored at 4.4 °C, whilst it almost remained unchanged in sweet, cream-whipped salted butter stored at 21 °C until 7 days. This effect might be explained by the results of Dawson et al (2002) who claimed that the growth rate of *L. monocytogenes* is expected to be slower at 4 °C than at elevated temperatures.

Limjaroen et al. (2002) reported that 1.5 and 3.0% w/w sorbic acid containing polyvinylidene chloride (Saran F-310) films decreased *L. monocytogenes* populations by a factor of 6.3 and 21.4, respectively on Cheddar cheese after 35 days of storage at 4 °C. Cutter (1999) investigated the effectiveness of Triclosan-incorporated plastic (TIP) against bacteria on beef surfaces. During 5 days of storage of vacuum-packaged beef wrapped with TIP containing 1,500 ppm of Triclosan under simulated temperature abuse conditions, a slight decrease in the number of *E. coli* was observed. However, after an additional storage up to 14 days at this condition, the number of *E. coli* increased.

It seems that the agar disc diffusion assay on packaging films with typical test strains of microorganisms provides limited information on the effectiveness of the film in actual food packaging situations. Actual packaging trials need to be performed in order to evaluate the efficacy of the AM packaging film (Ha et al. 2001). In the previous study (**Section 4.4.2**), there was no AM activity against *L.*

innocua shown in the agar disc diffusion test. A later study was therefore carried out to challenge the efficacy of these AM films against *L. innocua* in Cheddar cheese. It was interesting to learn that linalool-LDPE-EVA and methylchavicol-LDPE-EVA films showed significantly inhibitory effects against *L. innocua* innocuated on Cheddar cheese.

In addition, the inhibition of *E. coli* and *L. innocua* on Cheddar cheese using linalool-LDPE-EVA and methylchavicol-LDPE-EVA films stored at the temperature abuse conditions exhibited promising results. It is known that in general, an increase in temperature from a refrigerated level (4 °C) to a temperature of 12 °C enhances or expedites the growth of microorganisms (Montville 1997). In spite of that, these films showed a reduction in the growth of *E. coli* and a decrease in the growth of *L. innocua*. This result could be explained by the fact that the higher the temperature, the higher release of the active agent from the film into food surfaces. Thus, there is a merit in these kinds of natural plant extracts over conventional AM agents like sorbic acid, K-sorbate or Triclosan. AM films containing basil extract yielded encouraging protection against *E. coli* and *L. innocua* in Cheddar cheese, especially at temperature abuse conditions. It should be noted, however, that for this maximum inhibition to occur, there is a need for direct contact between the food and the AM packaging material. It is believed that AM films containing basil extracts would contribute to the shelf life extension of Cheddar cheese and probably other foods.

4.8.3 Sensory evaluation

The sensory evaluation results are summarised in **Tables 4.15** and **4.16**. The panelists did not perceive a difference in flavour between Cheddar cheese wrapped in linalool-LDDPE-EVA film and LDPE film (control) throughout the storage period of 6 weeks at 4 °C.

A significant difference in flavour, at the 99% confidence level, was found after 1 week between Cheddar cheese wrapped in methylchavicol-LDPE-EVA film and LDPE film. After that period, the panelists found there to be no difference in flavour and 80% of the panelists had correct answers at 4 weeks of storage. This might be a result of correct guessing by the panelists with the probability of 1/3 in the triangle test. It was observed that the panelists spent less time for the sensory evaluation in the first week in order to indirectly detect methylchavicol in the cheese sample. After that week the panelists spent more time in the sensory evaluation and were unable to detect differences in the samples. The majority of panelists detected a methylchavicol flavour but could not detect a linalool flavour at 1 week of storage. This might be a result of the more distinct and pleasant flavour of mehtlychavicol compared to linalool (Wright 1999).

It can be summarised that the present study shows that taints in flavour from linalool or methylchavicol was not significantly detectable by the sensory evaluation panels. Low doses of linalool or methylchavicol can be used as AM

Table 4.15 Triangle test results of Cheddar cheese wrapped with linalool-LDPE-EVA film refrigerated at 4°C

Storage time/ weeks	Panelists	Correct response	Significance level
1	10	2	NS[1]
2	10	3	NS
3	10	3	NS
4	10	3	NS
6	10	5	NS

[1] NS = Non-significant difference

Table 4.16 Triangle test results of Cheddar cheese wrapped with methylchavicol-LDPE-EVA film refrigerated at 4°C

Storage time/ weeks	Panelists	Correct response	Significance level
1	10	8	99%
2	10	4	NS[1]
3	10	3	NS
4	10	8	99%
6	10	5	NS

[1] NS = Non-significant difference

additives in AM films to extend the shelf life of Cheddar cheese (which has its own unique flavour), without any detrimental effect on flavour. Ouattara et al. (2001) reported that low scores were obtained in sensory evaluation tests for odour and taste of pre-cooked shrimp (*Penaeus* sp.) coated with a protein-based solution containing 0.9 or 1.8% v/w of mixture of thyme oil and *trans*-cinnamaldehyde. They claimed that these low scores resulted from the intrinsic sensory attributes of thyme oil and *trans*-cinnamaldehyde. Mejlholm and Dalgaard (2002) claimed that 0.05% v/w oregano oil yielded a distinct, although pleasant, flavour to cod fillets, and the oil delayed spoilage reactions and extended the shelf life of the fish.

5 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The results of the present study highlight the promising potential and feasibility of LDPE-EVA films containing linalool or methylchavicol for use as AM packaging materials. The following are the conclusions, related to the research questions as presented in Chapter 1 that can be drawn from the present study:

1. The first research question relates to investigating the target microorganisms against which the film should be effective.

In the preliminary study with LLDPE and the study with LDPE-EVA films, all prepared films containing either linalool or methylchavicol presented an AM effect against *E. coli*, a Gram-negative bacterium, in the agar disc diffusion assay and in the liquid growth experiments.

2. Research question 2 is associated with exploring the AM effectiveness in terms of the retained AM activity.

The LLDPE films in the preliminary study showed effective inhibition of the growth of *E. coli*, although the residual amount of linalool or methylchavicol in the extruded films was found to be approximately 0.05% w/w (target concentration was 1.0% w/w in blend). With a relatively low concentration of 0.05% w/w linalool or methylchavicol in the extruded film, such formulations

might be not suitable for use in liquid food applications as demonstrated by the results obtained from the liquid medium model. However, the films showed significantly good results in the solid medium model (compared to other works which normally incorporate the AM agent at 1% w/w. Film storage studies showed that the AM additives continued to inhibit the growth of *E. coli* even after long-term storage (1 year). A certain concentration (above the minimum for bacteria growth inhibition) of linalool or methylchavicol was retained in the polymeric matrix even at the higher temperature of storage of 35 °C.

3. Research question 3 deals with understanding the effect of the AM additives on the properties of the film.

From the film characterisation experiments it became evident that incorporation of either linalool or methylchavicol into LDPE-EVA films may slightly change the barrier and optical properties of the (packaging) material. However, there was almost no difference in the thermal properties and polymer morphology of the AM films in comparison with the additive-free LDPE film.

4. Research question 4 relates to understanding the diffusion behaviour of the AM additives in the polymeric material.

Studies on the release of the AM additives showed the kinetics of releasing linalool and methylchavicol to present a non-Fickian behaviour at 4 °C. The kinetic data could be fitted better by a non-linear least-squares correlation of the time-response function using the Hill coefficient. The effect of temperature on

migration could be described well by an Arrhenius-type model based on the values calculated by using the time-response function with Hill coefficient.

5. Research question 5 deals with exemplifying to what extent the AM films maintain the microbiological quality and/or safety of food wrapped in that material.

In assessing the AM efficacy of LDPE-EVA films containing linalool or methylchavicol by wrapping samples of both naturally contaminated and inoculated Cheddar cheese, it was found that the films exhibited an inhibitory effect against microbial growth, and against the growth of *E. coli* and *L. innocua* on the surface of the cheese. As far as taint in flavour is concerned, the prepared films containing linalool or methylchavicol did not present a significant problem for packaging of Cheddar cheese.

In addition to the conclusions related to the research questions some further conclusions, related to the side-track studies, can be drawn.

6. Both linalool and methylchavicol could be encapsulated in β-CD by a precipitation method. The residual quantities of AM additives encapsulated in β-CD were, however, low.

7. The sensitivity of the optical density (OD) method for measuring microbial activity was enhanced as the wavelength at which the measurements

were made decreased. The minimum inoculum size of *E. coli* that could be detected reliably by OD measurements at 470 nm was about 5×10^5 cfu mL^{-1}.

5.2 Significance of the findings

Antimicrobial agents are often added directly to foods to control microbial growth and extend the shelf life of many non-sterile foods leading, in some cases, to over-use. Moreover, the negative consumer perception of certain food additives, such as chemical preservatives, has become an issue in recent years. Consequently, the integration of naturally derived components of basil as AM additives in polymeric packaging materials, presents an alternative means of food preservation as opposed to direct addition to the food. Such AM films offer potential economic savings by increasing productivity, and improved product safety *via* reducing the over-use of preservatives and/or the growth of pathogenic bacteria. This study provides a reliable basis and guide for the further scientific and technical development of such technology including expanding the potential use in other food applications and beyond, such as the medical sector. Since the commercial introduction of AM films, incorporating synthetic AM compounds, is restricted due to food contact issues (health and safety concerns/regulations), the development of this technology could have merit.

5.3 Recommendations for further research

This study has laid some foundations for the future manufacturing and application of natural AM packaging films by focusing on the use of the principal constituents of basil namely, linalool and methylchavicol. It has identified the

potential for use of these natural compounds in AM packaging applications for certain foods. However, there are a number of issues to be further studied to enable the development of the technology from its fundamental basis to a commercial application. In this respect it is recommended to:

1. Investigate the effect of incorporating two or more natural antimicrobial plant constituents (in various concentrations) on retention and inhibition efficacy.

2. Investigate the potential for applying other natural plant constituents (such as eugenol, thymol, and cinnamaldehyde) in this technology.

3. Research the options for optimising the structure of the AM film including the retention of the AM additive during manufacturing. In this respect it should be useful to explore:

- The use of alternative blends to further lower the temperature in the extrusion process. One alternative could be the use of ionomers in the blend with LDPE
- Material structures (e.g. barrier coating or layer) that will restrict the migration and release of the AM agent at the outer surface of the packaging.

4. Further investigate the effect of higher retention rates on the inhibition of microbial growth of common food spoilage bacteria and pathogens other then *E. coli*.

5. Further investigate the release mechanism in gaseous systems given the volatile nature of the basil extract.

6. To explore the applicability of basil extract AM films for the preservation of other types of food such as meat, poultry, seafood and high moisture bakery products.

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APPENDIX A-Typical Properties of Polymers

Table A.1 Typical properties of polymers used in this research[1]

Properties		Polymer			Test Method
		DOWLEX	ALKATHENE	ESCORENE	
Melt index/ g 10min^{-1}		1.0	2.5	2.0	ASTM D1238
Density/ g (cm^3)$^{-1}$		0.920	0.921	0.930	ASTM D1505
Haze/ %		NT[2]	5.5	3	ASTM D1003
Gloss		NT	70	85	ASTM D2457
Dart impact/ g		197	80	NT	ASTM D1709
Tensile strength/ MPa	MD	30	24	30	ASTM D882
	TD	23	17	21	
Tensile yield/ MPa	MD	8.5	8	6.9	ASTM D882
	TD	9	11	6.2	
Ultimate elongation/ %	MD	515	300	275	ASTM D882
	TD	620	810	600	
Tensile modulus[3]/ MPa	MD	178	170	100	ASTM D882
	TD	225	190	117	

[1] As per supplier specifications

[2] Not tested

[3] With 2% secant-DOWLEX and ALKATHENE

With 1% secant-ESCORENE

APPENDIX B-Description and Typical Properties of Linalool and Methylchavicol

Table A.2 Description and typical properties of linalool and methylchavicol[1]

Description and Properties	linalool	methylchavicol
CAS[1] number	78-70-6	140-67-0
Molecular formula	$C_{10}H_{18}O$	$C_{10}H_{12}O$
Synonym	linalol, linalyl alcohol	estragole, 4-allylanisole
Structural class	tertiary alcohol	phenyl ether
Molecular weight	154.25	148.21
Boiling point/ °C	198	216
Flash point/ °C	76	81
Density/ g $(cm^3)^{-1}$	0.863	0.965
Vapour pressure/ Pa	21	12
Solubility in water/ g $(100mL)^{-1}$	0.16	< 0.1

[1] As per supplier

[2] Chemical abstracts service

APPENDIX C-Standard Curves of Linalool and Methylchavicol

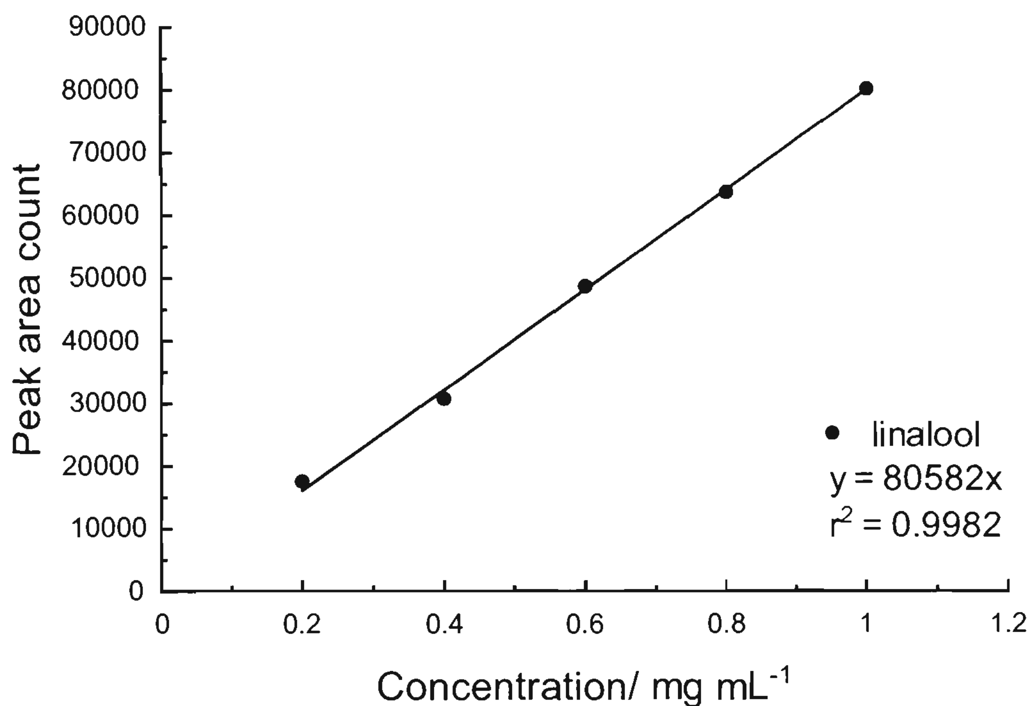

Figure A.1 Standard curve of linalool quantification by gas chromatography

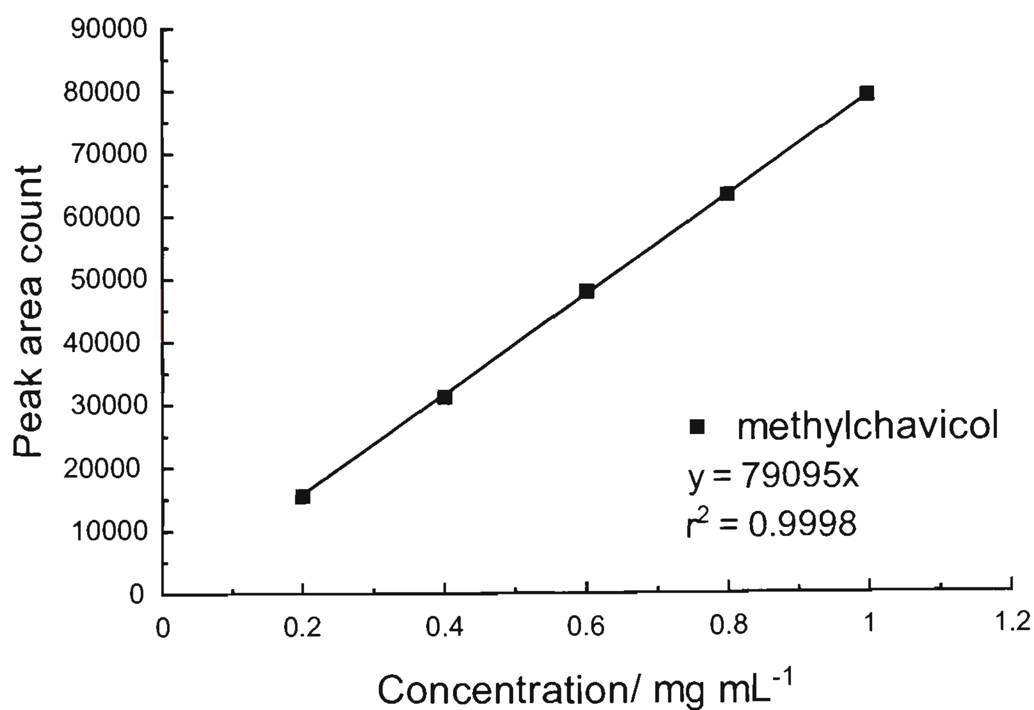

Figure A.2 Standard curve of methylchavicol quantification by gas chromatography

APPENDIX D-IR Spectra of Linalool and Methylchavicol

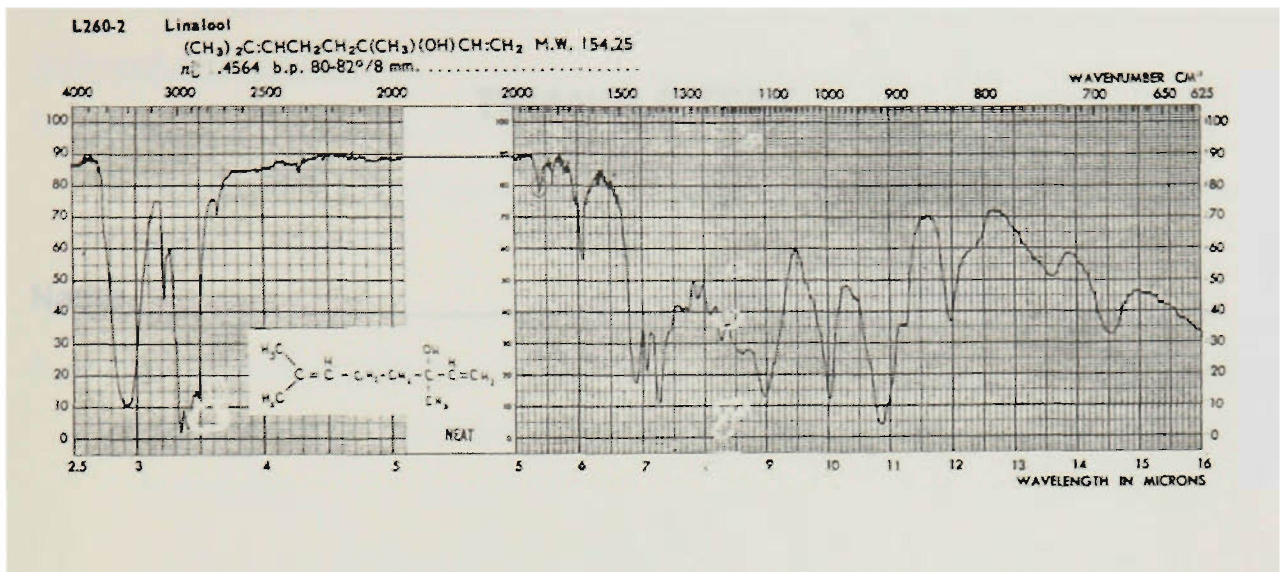

Figure A.3 IR spectrum of linalool (Pouchert 1981)

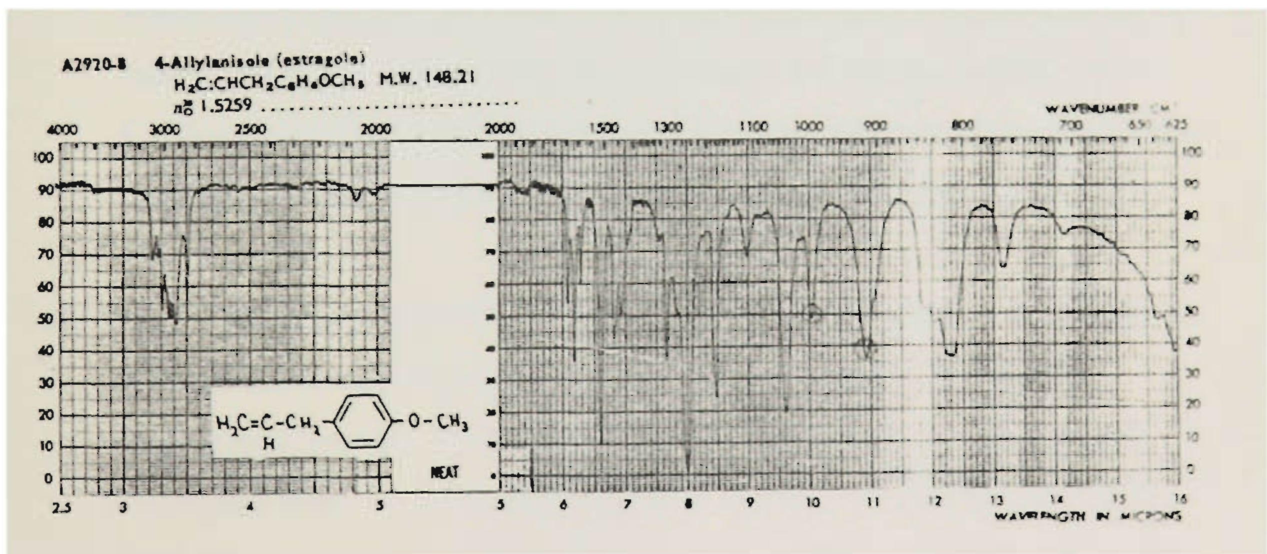

Figure A.4 IR spectrum of methylchavicol (Pouchert 1981)

APPENDIX E-Scorecard for the Triangle Test

TRIANGLE TEST

Name__________________________________**Code**__________**Date**_______________

In front of you are three coded Cheddar cheese samples, two are the same and one is different; starting from the left evaluate the samples and circle the code that is different from the other two. You may reevaluate the samples. You must make a choice. Thank you very much for your kindly participation.

APPENDIX F-List of Publications

Refereed Journal Papers:

Suppakul, P.; Miltz, J.; Sonneveld, K.; Bigger, S. W. Active packaging technologies with an emphasis on antimicrobial packaging and its applications. *J. Food Sci.* **2003**, *68*, 408-420.

Suppakul, P.; Miltz, J.; Sonneveld, K.; Bigger, S. W. Antimicrobial properties of basil and its possible application in food packaging. *J. Agric. Food Chem.* **2003**, *51*, 3197-3207.

Refereed Conference Presentations:

Suppakul, P.; Miltz, J.; Sonneveld, K.; Bigger, S. W. Preliminary study of antimicrobial films containing the principal constituents of basil. Oral Presentation, Session 22 on Antimicrobial Packaging. Proceedings of the 13th International Association of Packaging Research Institutes (IAPRI) World Conference on Packaging (WorldPak2002), Michigan State University, East Lansing, MI, U.S.A., June 23-28. CRC Press LLC, FL, 2002; pp 834-839.

Suppakul, P.; Miltz, J.; Sonneveld, K.; Bigger, S. W. Microencapsulation of linalool and methylchavicol in β-cyclodextrin. Oral Presentation, Session 2 on Packaging Materials. Proceedings of the 21st International Association of Packaging Research Institutes (IAPRI) Symposium on Packaging, ITENE Packaging, Transport and Logistics Research Institute, Valencia, Spain, May 18-21. ITENE, Valencia, 2003; pp 96-99.

Suppakul, P.; Miltz, J.; Sonneveld, K.; Bigger, S. W. Antimicrobial effect of polyethylene films containing the principal constituents of basil. Oral Presentation, Session 3 on Product-Packaging Interaction and Active Packaging. Proceedings of the 21st International Association of Packaging Research Institutes (IAPRI) Symposium on Packaging, ITENE Packaging, Transport and Logistics Research Institute, Valencia, Spain, May 18-21. ITENE, Valencia, 2003; pp 132-137.

Suppakul, P.; Miltz, J.; Sonneveld, K.; Bigger, S. W. Loss of antimicrobial additive from polyethylene films containing the principal constituents of basil. Poster Presentation. Proceedings of the 14th International Association of Packaging Research Institutes (IAPRI) World Conference on Packaging(WorldPak2004), Packforsk, Stockholm, Sweden, June 13-16, 2004: CD; The Abstracts Catalogue: p. 91

Suppakul, P.; Miltz, J.; Sonneveld, K.; Bigger, S. W. Inhibition of *Escherichia coli* and *Listeria innocua* on Cheddar cheese using antimicrobial films containing the principal constituents of basil. Oral Presentation, Session: Materials. Proceedings of the 14th International Association of Packaging Research Institutes (IAPRI) World Conference on Packaging (WorldPak2004), Packforsk, Stockholm, Sweden, June 13-16, 2004: CD; The Abstracts Catalogue: p. 43.

BIOGRAPHY

The author, Panuwat Suppakul, was born July 25, 1971 in Ranong, Thailand. He graduated with a Bachelor of Science (Agro-Industrial Product Development) with Second Class Honours from Kasetsart University (KU) in Bangkok, Thailand in 1993. Then he received a Postgraduate Studies Scholarship from the National Science and Technology Development Agency (NSTDA) to pursue a Masters degree in the same field. After graduation in 1996, he firstly joined the Department of Food Technology at Silpakorn University (SU) as a lecturer for a period of 6 months and subsequently joined the Department of Packaging Technology, Faculty of Agro-Industry at KU till the present day. He is a recipient of a Distinction Award for Thesis from the Graduate School, KU. He also received Royal Thai Government Scholarships for the undertaking postgraduate studies, initially for a Masters and later for a Doctoral degree, in the area of packaging materials.

Due to economic crisis in Thailand in 1997, the release of the scholarship was delayed. In conjunction with this Scholarship, in 1999 he was granted an Australian Development Scholarship (ADS) by the Australian Agency for International Development (AusAID) to undertake a Masters by Coursework degree at the School of Material Science and Engineering, The University of New South Wales (UNSW), Sydney, Australia. In 2000 he completed this degree with Distinction, following which he was offered an opportunity from AusAID for a scholarship upgrade to pursue his a Doctoral degree. He commenced his studies towards the degree of Doctor of Philosophy (Packaging Technology) at Victoria University (VU), Melbourne, Australia in the July 2000.