Loss of AM additives from antimicrobial films during storage

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Abstract

Films based on linear low-density polyethylene (LLDPE) and low-density polyethylene (LDPE) containing linalool or methylchavicol were prepared by extrusion film blowing. Film rolls of LLDPE containing linalool or methylchavicol were stored at ambient temperature for 1 year. Samples of these films were then evaluated for the amount of linalool or methylchavicol retained and for their antimicrobial (AM) activity by the agar disc diffusion assay. In addition, film rolls of LDPE-EVA (LDPE-ethylene vinyl acetate) containing linalool or methylchavicol were stored at 25 and 35°C. Samples of these films were periodically collected to quantify the amount of linalool or methylchavicol retained as a function of time. For the AM LLDPE films, a decrease in additive retention was observed but there was no statistically significant difference in their AM activity against *E. coli* at the beginning and after 1 year of storage. For the AM LDPE-EVA films, the amount of additive in the film decreased with time and the additive retention in all films tended to deviate from the theoretical first-order decay. These findings suggest that an amount of linalool or methylchavicol that is sufficient to maintain AM activity remained in the polymeric matrix after the storage period. This study confirms the potential use of polymeric films containing basil constituents as AM films for enhancing quality and safety as well as the extension of the shelf life of foods.

Keywords: Active packaging; Antimicrobial film; Antimicrobial activity; Linalool; Methylchavicol; Long-term storage; Accelerated storage

1. Introduction

During the past decade, there has been an increasing interest in developing antimicrobial (AM) packaging materials to prevent microbial growth on food surfaces

during storage, by a slow release of AM additives onto the food surface. The AM additives that have been mentioned include acid anhydrides, amines, bacteriocin, enzymes, fungicides, metal ions, organic acids and their salts, paraben and plant extracts (Suppakul et al., 2003a; Appendini and Hotchkiss, 2002; Quintavalla and Vicini, 2002; Vermeiren et al., 2002;; Kerry et al., 2006; Coma, 2008). Dainelli et al. (2008) reviewed the progress in the area of AM packaging technology and reported that this rapidly emerging technology is expected to grow in the next decade. There is a growing interest in the incorporation of natural AM additives into packaging films to be used as "AM packaging" for the purpose of improving food quality and safety as well as extending shelf life (Becerril et al., 2007; López et al., 2007; Rodríguez et al., 2007; Gutiérrez et al., 2009).

Essential oils are well-known inhibitors of microorganisms (Burt, 2004; López er al., 2005; Di Pasqua et al., 2007; Goñi et al., 2009; Gutiérrez et al., 2010). Basil (*Ocimum basilicum* L.) is one of the oldest identified spices and its essential oils have been used extensively for many years in food flavoring and perfumery. Numerous investigations on basil essential oils have been reported including taxonomy, chemistry and AM activity (Kalemba and Kunicka, 2003). Suppakul et al. (2003b) reviewed the topic of basil essential oils with regards to their chemical composition, their effect on microorganisms, and their possible future use in food preservation or as an AM additive in packaging materials. When focusing on natural plant extracts, basil extract is one of the promising potential AM additives due to its AM activity against a broad spectrum of Gram-positive and Gram-negative bacteria, and yeasts as well as moulds. The principal constituents of basil, namely linalool and methylchavicol, exhibit AM activity against many microorganisms (Suppakul et al., 2003b). These compounds possess "GRAS" status (Suppakul et al, 2003a). They are stable at relatively high temperatures and may therefore

have the potential to be incorporated into polymers and used in AM packaging applications. In studies by Suppakul et al. (2006) and Suppakul et al. (2008), linalool or methylchavicol was incorporated into polyethylene-based films. The barrier, optical, physico-chemical and thermal properties and the antimicrobial efficacy of the films were investigated. The storage temperature may affect the additive retention in the AM films and therefore their AM activity. However, no published information could be found in the scientific literature in regard to the loss of AM additives and their retained AM activity during film storage. The present study was aimed at determining the effect of time and temperature, at either long-term or accelerated storage conditions, on the retention of basil components that had been impregnated into polyethylene-based films.

2. Materials and methods

2.1. 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).

2.2. Antimicrobial 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.

2.3. Chemicals

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.

2.4. Media

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

2.5. Microorganism

The microorganism used in this research was *Escherichia coli* (FSA 1301), obtained from the Culture Collection of Food Science Australia, Werribee, Victoria, Australia.

2.6. Preparation of AM LLDPE films

Linear low-density polyethylene (LLDPE) films of 45-50 μm in thickness, with and without linalool or methylchavicol, were prepared from LLDPE pellets. Additive-free LLDPE pellets were ground and the powder was doped in linalool or methylchavicol dissolved in isooctane. This AM agent-impregnated powder was used as the master batch. The master batch powder containing linalool or methylchavicol was mixed with virgin 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). Films without linalool or methylchavicol were used as controls and were prepared under similar conditions to the films containing the active agents.

2.7. Additive quantification in AM films

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. Isooctane was used since it was anticipated that an end-use of the films would be for the packaging of hard cheeses that contain predominantly non-polar substances such as fats, etc. The extraction efficiency was checked by periodically analyzing the extract until no further

change in the concentration of the AM agent was observed after a period of 18 h extraction. 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, then kept at this temperature for 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.

2.8. Antimicrobial activity of LLDPE films in solid media

The films were tested for their inhibition against the selected microorganism *Escherichia coli* (Gram-negative bacteria) by using an agar disc diffusion method (Acar and Goldstein, 1986; Parish and Davidson, 1993).

The microorganism 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 *Escherichia coli*. Cell densities were estimated from standard curves and confirmed by the "pour plate" method on plate count agar for bacteria (Swanson et al., 1992).

Each film sample was cut into a circle of 5 mm in diameter and sterilized 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 of the 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.

2.9. Long-term storage of AM LLDPE films

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 antimicrobial activity in solid media, as described in the previous section.

For determining the effect of the worst-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 (Acar and Goldstein, 1986; Parish and Davidson, 1993). The reason for this is that loss of active agents over time is expected to be greater from the exposed outside and side regions of the roll than from the inside and center regions.

2.10. Preparation of AM LDPE-EVA films

The LDPE-EVA films of 45-50 μm in thickness, with and without linalool or methylchavicol, were prepared from LDPE pellets. A pre-blended master batch of an ethylene vinyl acetate (EVA) copolymer powder containing linalool or methylchavicol was mixed with virgin LDPE pellets and manufactured into films using the same extruder mentioned above. The purpose of using this copolymer was to enhance the solubility and/or partial anchoring of the AM additives in the polymer matrix. Films without linalool or methylchavicol were used as controls and were prepared under similar conditions as the films containing the active agents.

2.11. Accelerated storage of AM LDPE-EVA films

The LDPE-EVA AM films were used in this work for the study of ambient and accelerated storage conditions. The rolls that comprised approximately 100 m of film

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, as described in the previous section.

2.12. 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. Results and discussion

During the preparation of the LDPE-based AM films, the additive could be properly incorporated in the polymer melt, leading to a film with a uniformly dispersed AM agent. This result was observed and confirmed by scanning electron microscopy (Suppakul et al., 2006). This finding is consistent with the study by Hong et al. (2000) on AM films in which clove extract had been incorporated.

Suppakul et al. (2006) reported that the transparency of the LDPE-based AM films decreased slightly compared to the control LDPE film. Methylchavicol had a larger effect on the transparency than linalool. The transparency of the AM films in the present study was in the acceptable range for transparent films and no difficulty in commercialization of these films is envisioned.

The temperature profile of 90-95 °C previously used by Han and Floros (1997) for the production of their films could not be used in the present study since the current polymers have higher melting temperatures (the vast majority of commercial polyethylene grades have a melting temperature above 100 °C and therefore it is not clear how the above mentioned temperature range could be applied). Nonetheless, the

199 temperature profile of 160-190 °C, previously used by Ha et al. (2001), was used for
200 manufacturing of the LDPE-based AM films by extrusion film blowing and resulted in a
201 high loss of the AM agent. Lower manufacturing temperatures are preferable in order to
202 minimize loss of the active agent by evaporation. The limitations of the single screw
203 extruder available for our experiments further affected the expected results.

204 In the first experiment, LLDPE was used. However, because of the higher melting
205 temperature of this polymer compared to LDPE, higher processing temperatures (about
206 190˚C) had to be applied resulting in a much greater loss of the AM agents during
207 processing. Thus, the residual AM concentration in the polymer was about 0.05 g/100 g
208 only. At a later stage, a blend of LDPE and EVA was used to prepare films at around
209 160˚C. This combination increased the retention of the residual active agent in the
210 extruded films to 0.34 g/100 g (initial concentration was 1.0 g/100 g in the blend; See
211 Table 1 and Table 2). The increased AM agent concentration in this polymer mixture is
212 attributed to the lower processing temperature and to the interaction between the AM
213 agents and the copolymer enabling the "anchoring and solubilizing" of the AM molecules
214 within the polymeric matrix (Suppakul et al., 2008).

215 Linalool may be oxidized in the presence of air at normal elevated temperatures.
216 Several different oxidation products of linalool include 7-hydroperoxy-3,7-dimethyl-octa-
217 1,5-diene-3-ol and 6-hydroperoxy-3,7-dimethyl-octa-1,7-diene-3-ol together with
218 secondary product of 8-hydroperoxy-3,7-dimethyl-octa-1,6-diene-3-ol (Bäcktorp et al.,
219 2006). Oxidation products of methyl chavicol include 4-methoxybenzaldehyde and 4-
220 methoxy benzene acetaldehyde (Bouvier-Brown et al., 2008). However, at the relatively
221 mild extrusion conditions used in the present study the presence of such degradation
222 products is expected to be minor.

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224

225 *3.1. Residual concentration and AM activity after long-term storage*

226 All prepared LLDPE films containing linalool or methylchavicol showed a
227 positive AM activity against *E. coli* in the agar disc diffusion test (Table 1). The size of
228 the zone was taken as a quantitative measure of AM activity. It should be noted, however,
229 that the size of the zone of inhibition might be limited since the AM agents have to
230 diffuse from the polymer through the agar. Colonies of *E. coli* could not be viewed in the
231 circular region directly below the film samples containing the constituents of basil;
232 however such colonies were formed in the control plates containing the film without an
233 AM agent. Consequently, when there is direct contact between the AM film and the agar,
234 an inhibition halo (clear zone) is formed under and around the disc because of diffusion
235 of the AM from the film to the medium in which the microorganism is growing.

236 A concern exists about a possible depletion, by diffusion into the environment, of
237 AM additives, especially volatile compounds, during long-term storage. During long-term
238 storage at ambient conditions for 1 year, the films were subjected to the temperature cycle
239 between day and night. The residual concentration and AM activity of linalool-LLDPE
240 and methylchavicol-LLDPE films are shown in Table 1. The additive retentions of
241 linalool and methylchavicol in the films were 66.1% and 52.8% respectively of the
242 original values. Fig. 1 shows a clear zone with symmetrical characteristics of LLDPE AM
243 film against *E. coli* after 1 year of storage. The additive retention was still high enough to
244 exhibit anitmicrobial activity. This might be due to the low initial concentration of the
245 additives and therefore a low driving force for diffusion as well as the temperature cycle
246 that slowed down the diffusion rate at night. Although a decrease in additive retention
247 was observed, there was no difference in AM activity of the films between the beginning
248 and after 1 year of storage. Clearly, this finding suggests that linalool or methylchavicol

AM films exhibited inhibitory effects against *E. coli*, a Gram-negative bacterium, even at low residual concentrations.

Colonies of *E. coli* could not be viewed in the clear zone around the film samples containing the constituents of basil, whereas 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 extruded film sample 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 are slightly soluble in the water held by the agar due to the presence of some hydrophobic substances (Suppakul et al., 2003b). According to Elgayyar et al. (2001), the present results show that LLDPE AM films possess "moderately inhibitory" characteristics against *E. coli*. Linalool showed a higher level of inhibition than methylchavicol in the AM films prepared by extrusion, despite the fact that methylchavicol possesses a greater extent of AM activity than linalool. The reason may stem from the faster diffusion of linalool and its greater solubility in water (and subsequently a more pronounced presence in the aqueous-based agar media), compared to methylchavicol (Suppakul et al., 2003b). Linalool and methylchavicol are known to possess a broad spectrum of AM activity against a variety of microorganism such as *Aeromonas hydrophila*, *Bacillus cereus*, *E. coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Aspergillus* sp., and *Penicillium* sp. (Suppakul et al., 2003b).

Other AM additives, including Ag-zirconium (An et al., 1998), clove extract (Hong et al, 2000), lacticin and nisin (An et al., 2000; Pranoto et al., 2005), garlic oil (Pranoto et al., 2005), potassium sorbate (Pranoto et al., 2005) and rosemary oil (Seydim and Sarikus, 2006) failed to retard the growth of *E. coli*, even at much higher concentrations. The reason might be that Gram-negative bacteria are generally more

274 resistant to the growth inhibition and killing effects of various antibiotics and AM agents
275 (Salton, 1994) due to the strong hydrophilicity of their surface that acts as a strong
276 permeability barrier (Nikaido and Vaara, 1985). The surface also possesses divalent
277 cations that stabilize the lipopolysaccharide association within the membrane and may
278 prevent active compounds from reaching the cytoplasmic membrane (Russel, 1991).

279 Due to the low water solubility of lipophilic molecules, emulsifiers such as Tween
280 20 (polyoxyehtylene-2-sorbitan monolaurate), Tween 80 (polysorbate 80) and Triton
281 X100, or solvents like ethanol, are often used to enhance the solubility of hydrophobic
282 compounds in both solid and liquid media. These lipophilic molecules may become
283 soluble within the micelles formed by non-ionic surfactants such as Tween 20 and Tween
284 80, and thereby be partitioned out from the aqueous phase of the suspension (Schmolka,
285 1973). Kazmi and Mitchell (1978) claimed that AM agents solubilised within micelles do
286 not contribute to the AM activity, as they do not come into direct contact with the target
287 microorganisms.

288 *3.2. Effect of temperature on additive retention after accelerated storage*

289 The depletion of the specific additives was used to determine the reaction rate
290 constants associated with the AM films. The reduction in additive concentration of the
291 LDPE-EVA AM films during storage at 25 and 35 °C is presented in Fig. 2. A first-order
292 decay for the additive retention was initially supposed (Eq. 1):

293 $$\ln(C) = - kt \qquad (1)$$

294 where C is the additive retention and k is a rate constant. Equation 1 is the only kinetic
295 model that is required to apply this approach to accelerated shelf life testing (ASLT) and
296 the extrapolation process (to a limited extent), after evaluation the value of k from the
297 initial rate, is clearly very simple (Mizrahi, 2000). The end of the film shelf life (t_s) is
298 therefore:

$$t_s = \ln(C)/-k \quad (2)$$

The theoretical plots of ln(C) versus time for a first order decay are shown in Fig. 3. 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 (Mizrahi, 2000). 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_∞) versus time, as a first-order decay with an offset, described by (Eq. 3) have been used as depicted in Fig. 4:

$$\ln(C - C_\infty) = - kt \quad (3)$$

where C_∞ is the additive retention observed after an "infinite" time. From this figure, the infinite concentration, rate constant (k) and half-life ($\theta_{1/2}$), as described in Eq. 4, can be obtained and are shown in Table 2:

$$\theta_{1/2} = \ln(2)/-k \quad (4)$$

where $\theta_{1/2}$ is the time required for the additive retention to decrease to half of its initial value.

The calculated levels of bound additive in linalool-LDPE-EVA films at 25 and 35 °C were found to be 0.051, and 0.036 % w/w, respectively. For methylchavicol-LDPE-EVA films the levels at 25 and 35 °C were 0.045 and 0.029% w/w respectively. These results for LDPE-EVA AM films stored at 35 °C were close to the actual concentrations of the AM agents remaining in the LLDPE AM films stored at ambient conditions for 1 year. Thus, it might be concluded that AM films containing basil extracts retain their inhibitory action against *E. coli* even when stored at 35 °C for a long period. The rate constant of linalool-LDPE-EVA and methylchavicol-LDPE-EVA films at 25°C were 9.0 $\times 10^4$ and 10.6 $\times 10^4$ h^{-1} respectively and at 35 °C they were 10.8 $\times 10^4$ and 12.5 $\times 10^4$ h^{-1}. The estimated half-life, $\theta_{1/2}$, can be calculated in accordance with Eq. 4 (Labuza, 1982; Man and Jones, 1994). The values ranged between 27 and 32 days at 25 °C and 4-5 days

at 35 °C. The sensitivity of the deterioration of linalool-LDPE-EVA and methylchavicol-LDPE-EVA films can be calculated from the rate constant and expressed in terms of the parameter Q_{10} (Eq. 5), which is the ratio between the rate constants at two temperatures differing by 10 degrees (Labuza, 1982; Man and Jones, 1994):

$$Q_{10} = k_{T+10}/k_T \quad (5)$$

where k_T is the rate constant measured at the absolute temperature, T, and k_{T+10} is rate constant measured at the absolute temperature $T + 10$. The value of Q_{10} may also be expressed as in Eq. 6 (Mizrahi, 2000):

$$Q_{10} = \exp\{10E_a/[RT(T + 10)]\} \quad (6)$$

where E_a is the activation energy, R is the ideal gas constant and T is the absolute temperature.

From plots of $\ln(C - C_\infty)$ versus time (assuming a first-order decay with an offset in the additive retention in the LDPE-EVA AM films) at 25 and 35 °C, the Q_{10} of linalool and methylchavicol were found to be 1.19 and 1.17, respectively. The temperature dependence of the additive retention is well described by an Arrhenius relation (Eq. 6) with activation energies of 13.3 kJ mol^{-1} and 12.0 kJ mol^{-1} for linalool and methylchavicol, respectively. Methylchavicol showed 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. 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 depletion of the AM additives depends on the initial concentration of the additive in the film, the storage conditions and the nature of the

additive and polymer. Therefore, in commercial film production, it is possible in principle to define the requested half-life of the AM film and calculate from this value, the initial concentration that should be used.

4. Conclusion

The natural AM components of basil (linalool and methylchavicol) can be successfully incorporated into either LLDPE or LDPE-EVA polymer and retain their inhibitory effect against the growth of *E. coli* in a model (i.e. solid medium) system. Film storage studies suggested 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 storage temperature of 35 °C.

Acknowledgements

On behalf of the Royal Thai Government, author Suppakul would like to express his thank and appreciation to the Australian Agency for International Development (AusAID) for providing financial support. The authors would like to thank Ms. Alison Caruana-Smith, Ms. Irawati Prasatya, Mr. Juan Carrasset, Mr. Phillip Bovis, Mr. Joe Pelle, Mr. Dale Tomlinson and Mr. Ku Heng Sai for their assistance during the conducting of the experiments. The authors would like to gratefully thank Dow Chemical (Australia) Ltd. and Qenos Pty. Ltd. for generously donating raw materials.

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