Development of Flexible Antimicrobial Films Using Essential Oils as Active Agents

P. López,[†] C. Sánchez,*,[†,‡] R. Batlle,[†] and C. Nerín[†]

Department of Analytical Chemistry, Aragón Institute of Engineering Research, i³A, CPS-University of Zaragoza, María de Luna St. 3, E-50018 Zaragoza, Spain, and ARTIBAL S.A., Department of I+D+i, Cañada Real Street 12, E-22600 Sabiñánigo, Spain

The antimicrobial activity in the vapor-phase of laboratory-made flexible films of polypropylene (PP) and polyethylene/ethylene vinyl alcohol copolymer (PE/EVOH) incorporating essential oil of cinnamon (*Cinnamomum zeylanicum*), oregano (*Origanum vulgare*), clove (*Syzygium aromaticum*), or cinnamon fortified with cinnamaldehyde was evaluated against a wide range of microorganisms: the Gram-negative bacteria *Escherichia coli*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, and *Salmonella choleraesuis*; the Gram-positive bacteria *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Enterococcus faecalis*; the molds *Penicillium islandicum*, *Penicillium roqueforti*, *Penicillium nalgiovense*, *Eurotium repens*, and *Aspergillus flavus* and the yeasts *Candida albicans*, *Debaryomyces hansenii*, and *Zigosaccharomyces rouxii*. Films with a nominal concentration of 4% (w/w) of fortified cinnamon or oregano essential oil completely inhibited the growth of the fungi; higher concentrations were required to inhibit the Gram-positive bacteria (8 and 10%, respectively), and higher concentrations still were necessary to inhibit the Gram-negative bacteria. PP films were more effective than PE/EVOH films. The atmospheres generated by the antimicrobial films inside Petri dishes were quantitatively analyzed using headspace-single drop microextraction (HS-SDME) in combination with gas chromatography–mass spectrometry (GC–MS). The analyses showed that the oregano-fortified PP films released higher levels of carvacrol and thymol, and the cinnamon-fortified PP films released higher levels of cinnamaldehyde, during the first 3–6 h of incubation, than the corresponding PE/EVOH films. Shelf-life tests were also performed, demonstrating that the antifungal activities of the films persisted for more than two months after their manufacture. In addition, migration tests (overall and specific) were performed, using both aqueous and fatty simulants, to ensure that the films meet EU regulations regarding food contact materials. Following contact with the tested films, the substances that had migrated into the aqueous simulants were recovered by direct immersion-single drop extraction (DI-SDME) and then analyzed by GC–MS. The fatty stimulant (isooctane) was directly injected into the chromatographic system.

KEYWORDS: Antimicrobial packaging; essential oil; single-drop microextraction; migration; polypropylene; polyethylene/ethylene vinyl alcohol copolymer

INTRODUCTION

Foods, being perishable products, have very short shelf lives. During production, processing, distribution, and storage, food may deteriorate due to both chemical reactions and microbiological proliferation. Traditional, passive food packaging only provides barrier and protective functions, and traditional food preservation techniques or the use of refrigeration alone cannot ensure the quality and safety of all foods. Therefore, various alternative preservation techniques, such as modified atmosphere packaging (MAP), the use of pulsed light, electric, or magnetic fields, high pressure, irradiation, or a wide range of food-grade chemicals, are being researched and developed (*1–4*). Another alternative with promising potential is active antimicrobial packaging, in which antimicrobial agents are added directly to the packaging material (*5*).

Antimicrobial packaging is attracting increasing attention from the food and packaging industry due partly to increasing consumer demands for minimally processed, preservative-free products. The use of preservative food-packaging films offers several advantages compared to the direct addition of preservatives to food products since the preservative agents are applied

* Author to whom correspondence should be addressed (telephone 34-974483221; fax 34-974481190; e-mail: cristinasanchez@artibal.com).
† CPS-University of Zaragoza.
‡ ARTIBAL S.A.

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to the packaging material in such a way that only low levels of the preservatives come into contact with the food. Either of two approaches can be used to give packaging materials antimicrobial activities (*6*). In preservative-releasing or migrating approaches, preservatives are introduced into their bulk mass, or applied to their surfaces, which subsequently migrate into the food or the headspace surrounding the food. In nonmigrating approaches, compounds are applied to the packaging surfaces that inhibit target microorganisms when they come into contact with them.

Some antimicrobial films have already been evaluated for food-packaging applications, such as low-density polyethylene (LDPE) with incorporated imazalil (*7*), potassium sorbate (*8*), nisin (*9*), or hexamethylenetetramine (*10*), PE coated with an antimicrobial peptide (*11*), and nisin-incorporated cross-linked hydroxypropylmethyl-cellulose (HPMC) (*12*). Nevertheless, although the antimicrobial properties of natural extracts are widely known (*13, 14*) and they have been used as antimicrobial preservatives for centuries, there have been very few publications regarding their incorporation in active films (*15–19*).

The terms active packaging and intelligent packaging were first introduced by Regulation 2004/1935/EC of the European Parliament and Council (*20*). This regulation stipulates that every substance that is incorporated into food from packaging must meet criteria stated in Directive 89/107/EC concerning food additives. Directive 89/107/EC classifies additives in various categories including (inter alia) flavorings, colorants, preservatives, and heat stabilizers, depending on their effects on food. (*21*). Natural extracts, such as essential oils (EOs) and their constituents, are categorized as flavorings by the European Decision 2002/113/EC (*22*). In addition, EOs and their constituents are categorized as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration. Therefore, the incorporation of EOs in plastic films to avoid microbial food spoilage is considered an attractive option by packaging manufacturers and demanding consumers.

The aims of the present work were to develop and test antimicrobial films consisting of either polypropylene (PP) or polyethylene/ethylene vinyl alcohol copolymer (PE/EVOH), with various incorporated concentrations of EOs, whose efficiency has already been evaluated in previous studies (*23, 24*). The antimicrobial action is exerted by the controlled release of the active agents into the headspace enclosed by the packaging. The antimicrobial activity of various combinations of EOs and plastic matrices, and the atmosphere they generated was evaluated. Shelf-life tests and migration tests were also performed with the most effective antimicrobial films to assess their suitability as commercial food contact materials.

MATERIAL AND METHODS

Antimicrobial Films. The antimicrobial films were produced in the laboratory by incorporating known concentrations (w/w) of EOs in films of either PP or PE/EVOH, both of which are suitable for use as food packaging, via an innovative process protected by European Patent EP1657181 (*25*) held by the Spanish company ARTIBAL S.A. Briefly, the active package is prepared by applying to the packaging material or format a coating or active liquid containing the natural active EO.

The PP film was a 20-μm-thick, trilayer, coextruded bioriented PP film, obtained from Poligal, S.A. (Narón, Spain), consisting of external layers (5%) of polypropylene-3% ethylene copolymer from BP (Lillo, Belgium) with silica at parts per million levels as an antiblocking agent, and an inner layer (95%) of isotactic polypropylene homopolymer containing erucamide and quaternary amines at parts per million levels from Repsol (Tarragona, Spain). The PE/EVOH film was a 50-μm-thick polyethylene/ethylene vinyl alcohol copolymer (5 μm EVOH),

high barrier material (Bariflex) manufactured by Industria Termoplastica Pavese SpA (Milano, Italy).

Antimicrobial films were produced 24 h before the assays in which they were used began.

Essential Oils. The EOs were supplied by ARTIBAL (Sabiñánigo, Spain). Oils from the following plant species were tested in this work: *Cinnamomum zeylanicum* (cinnamon, Chemical Abstract Service, CAS, number: 8015-91-6), *Syzygium aromaticum* (clove, CAS 8000-34-6), and *Origanum vulgaris* (oregano, CAS 8007-11-2). Cinnamon EO fortified with cinnamaldehyde, which has been shown to have excellent antimicrobial properties (*23*), was also tested.

Microbial Cultures. The following food-borne microbial strains were selected for use in the assays because of their relevance in the food industry: the Gram-positive bacteria *Staphylococcus aureus* (American Culture Collection, ATCC 29213), *Bacillus cereus* (Colección Española de Cultivos Tipo, CECT 495), *Enterococcus faecalis* (ATCC 29212), and *Listeria monocytogenes* (ATCC 7644); the Gram-negative bacteria *Escherichia coli* (ATCC 29252), *Yersinia enterocolitica* (CECT 4315), *Salmonella choleraesuis* (CECT 4000), and *Pseudomonas aeruginosa* (ATCC 27853); the yeasts *Candida albicans* (ATCC 64550), *Debaryomyces hansenii* (CECT 10353), and *Zygasaccharomyces rouxii* (CECT 11928); and the molds *Penicillium islandicum* (CECT 2762NT), *Aspergillus flavus* (CECT 2687), *Penicillium nalgiovense* (Culture Collection of Fungi, IBT 12105), *Penicillium roqueforti* (IBT 21319), and *Eurotium repens* (IBT 1800). The CECT strains were obtained from the Department of Microbiology (University of Valencia, Spain), the ATCC strains were purchased by LGC Promochem (Barcelona, Spain), and the IBT strains were obtained from the Technical University of Denmark (Lyngby, Denmark).

The strains were stored at -18 °C in sterilized skimmed milk and subcultured as follows. Gram-positive bacteria were subcultured on Mueller-Hinton agar at 30 °C for 48 h, except for *B. cereus*, which was subcultured on Mueller-Hinton blood agar. Gram-negative bacteria were grown in Mueller-Hinton agar at 30 °C for 24 h. *C. albicans* and the molds were subcultured on Sabouroaud cloramphenicol agar at either 30 °C for 48 h (*C. albicans*) or at 36.5 °C for seven days (*P. islandicum* and *A. flavus*). The other two yeasts (*D. hansenii* and *Z. rouxii*) were subcultured on maltose extract agar at 30 °C for 3 days.

Chemicals. The following chemicals were used as standards in the analyses of the atmospheres generated by the antimicrobial films: α-pinene (98%, CAS 80-56-8), camphene (95%, CAS 79-92-5), β-pinene (99%, CAS 1872-67-3), *p*-cymene (99%, CAS 99-87-6), (+)-limonene (97%, CAS 5989-27-5), 1,8-cineole (99%, CAS 470-82-6), linalool (2,6-dimethylocta-2,7-dien-6-ol, 97%, CAS 78-70-6), camphor (96%, CAS 76-22-2), (−)-borneol (1,7,7-trimethyl-(1*S*)-endo-bicyclo[2.2.1]heptan-2-ol, 98%, CAS 464-45-9], estragol (1-methoxy-4-(2-propenyl)benzene, 98%, CAS 140-67-0), *trans*-cinnamaldehyde (99%, CAS 14371-10-9), eugenol (2-methoxy-4-(2-propenyl)-phenol, 99%, CAS 97-53-0) and α-humulene (98%, CAS 6753-98-6) supplied by Aldrich (Sigma-Aldrich Química S.A, Madrid, Spain); thymol (5-methyl-2-(1-methylethyl)-phenol, >99.5%, CAS 89-83-8) and β-caryophyllene (99%, CAS 87-44-5) by Sigma (Sigma-Aldrich Química S.A, Madrid, Spain) and α-terpinolene (1-methyl-4-(1-methylethylidene)cyclohexene, >97%, CAS 586-62-9), carvacrol (5-isopropyl-2-methyl phenol, >97%, CAS 499-75-2), verbenone (4,6,6-trimethylbicyclo[3,1,1]hept-3-en-2-one, CAS 18309-32-5, >97%), and *p*-xylene (CAS 106-42-3, >98%,) by Fluka (Sigma-Aldrich Química S.A, Madrid, Spain).

Antimicrobial Activity Tests. A series of test cultures (one for each film to be tested) of each of the test microorganisms was prepared by inoculating plates of the appropriate solidified agar medium (see above) in Petri dishes with 100 μL of a physiological saline solution containing between 10^5 and 10^8 colony forming units (CFU) per milliliter of the organism. Then, each antimicrobial film to be tested was placed over the tops of one of the series of Petri dishes, with no direct contact between it and the microorganisms. Each Petri dish and film ensemble were then sealed with a nylon cable tie (Coferdroza, Zaragoza, Spain) and incubated under the conditions previously described. After the incubation period, the number of colonies that had formed on each plate was counted. All tests were performed in triplicate. Controls without plastic films and blanks with PP or PE/EVOH films without active compounds were included.

Clearly, the experimental setup was not hermetic. Therefore, the antimicrobial performance of the films was evaluated under unfavorable conditions, in which losses of the more volatile antimicrobial compounds were inevitable.

Headspace-Single Drop Microextraction (HS-SDME). The atmosphere generated by each of the antimicrobial films was sampled using headspace-single drop microextraction (HS-SDME), after rinsing the syringe used for this purpose with acetone followed by ethanol and *p*-xylene, 10–15 times each, to avoid formation of air bubbles and carryover of compounds. Then, 5 μL of *p*-xylene containing 6.7 μg/g of verbenone, used as the injection standard, was drawn into the syringe (Hamilton 85RN (26S/51 mm/needle type 2; Hamilton Bonaduz AG, Bonaduz, Switzerland) and suspended over the Petri dish using a metal stand. The needle was introduced to a given depth, and the plunger was depressed to expel 2.5 μL of the solvent. The drop formed was discarded into the headspace, and the plunger was then fully depressed to generate a 2.5 μL droplet of solvent on the needle tip. When the extraction time (5 min) was complete, the drop was retracted, the syringe was taken out of the vial, and then the solvent and sampled compounds were injected into the GC. The partition coefficient after 5 min extraction, K_5, calculated in a previous work (*23*), was used to quantify the concentrations of the samples compounds in the headspace. All analyses were carried out in triplicate.

Shelf-Life of the Antimicrobial Films. Batches of PP and PE/EVOH films incorporating 4% (w/w) of either fortified-cinnamon EO or oregano EO were made and stored at room temperature. Their antimicrobial activities against a yeast (*C. albicans*) and a mold (*A. flavus* or *P. nalgiovense*) were then checked every week, following the procedure described above. Tests were carried out in triplicate, and the total duration of the experiment was 76 days.

Overall Migration Tests. Tests were performed using the following aqueous food simulants: water, 3% aqueous acetic acid solution (v/v), and 10% aqueous ethanol solution (v/v). These substances were designated simulants A, B and C, respectively. In addition, isooctane was used as a fatty food stimulant because it could be directly injected into the analytical system and was designated as simulant D.

Double-sided, total immersion migration tests (both sides of films immersed into the liquid stimulant) were performed as follows. For each combination of plastic and simulant, a 1 dm^2 sample of the plastic and 15 g of the simulant were placed in each of five 20-mL glass vials, which were incubated for 10 days in a thermostatic oven set at 40 °C according to Directive 97/48/CE (*26*). These conditions were more aggressive than those expected for the intended applications to examine the films' migration parameters in a "worst case scenario" for food packaging. Immediately after each test, the polymer was removed, the simulants were evaporated to dryness, and their solid residues were gravimetrically determined. Five independent replicates were analyzed for each plastic sample and each simulant. The tested plastics were PP and PE/EVOH (blanks), PP-Ca4 and PE/EVOH-Ca4 (4% (w/w) of cinnamon EO), PP-C4 and PE/EVOH-C4 (4% (w/w) of clove EO), PP-Or4 and PE/EVOH-Or4 (4% (w/w) of oregano EO), and PP-enrCa4 and PE/EVOH-enrCa4 (4% (w/w) of fortified-cinnamon EO).

Specific Migration Tests. One-sided specific migration tests (only one side of film is in contact with stimulant) were performed with the four simulants used in the overall migration tests, but only the most active films: PP-Or4, PE/EVOH-Or4, PP-enrCa4, and PE/EVOH-enrCa4. Both sides of the films were analysed to evaluate the amount of the active compounds that diffused through film thickness.

In these tests, a 250-mL glass container was filled with 41.7 g of simulant, and the film to be tested was placed over its mouth. After the sample was closed and checked so that no liquid was leaking, the flask was upturned to bring the film into contact with the simulant. The area of surface exposed to the simulant was 28.3 cm^2, so the stimulant contact area ratio was similar to the ratio stipulated by European Regulation 97/48/CE [1 kg^{-6} dm^2; (*26*)]. Immediately after the test, the polymer was removed from each flask, and the simulant was weighed. Portions of simulant D (isooctane) were directly injected into the analytical system [gas chromatography–mass spectrometry (GC–MS)] after addition of the internal standard (verbenone), while analytes in the aqueous extracts (simulants A, B, and C) were extracted

Table 1. Optimized Extraction Conditions from Aqueous Simulants by DI-SDME

variables	simulant A	simulant B	simulant C
vial volume (mL)	4	4	20
% filling	90	50	50
drop volume (μL)	2.5	2.5	2.5
extraction time (min)	10	10	10
extraction temperature (°C)	30	30	60
saturation (%NaCl)	0	0	0
agitation (rpm)	100	400	100

by direct immersion-single drop microextraction (DI-SDME), as described below, prior to GC–MS analysis. All analyses were carried out in triplicate.

Direct Immersion-Single Drop Microextraction (DI-SDME). A volume of 5 μL of *p*-xylene containing 6.7 μg/g of verbenone, used as the injection standard, was drawn into the syringe (Hamilton 85RN (26S/51 mm/needle type 2; Hamilton Bonaduz AG, Bonaduz, Switzerland)) and suspended into a vial containing the aqueous simulant. The needle was submerged to a given depth, and the plunger was depressed to expel 2.5 μL of the solvent. The drop formed was discarded into the simulant, and the plunger was then fully depressed to generate a 2.5 μL droplet of solvent on the needle tip. When the extraction time was complete, the drop was retracted, the syringe was taken out of the vial, and then the solvent and sampled analytes were injected into the GC. The extraction conditions, optimized in a previous work (*27*), are shown in **Table 1**.

Gas Chromatography–Mass Spectrometric (GC–MS) Analysis. GC–MS analyses were performed using a Hewlett-Packard 6890 gas chromatograph (Wilmington, DE, USA) equipped with a 5973-mass selective detector and an A HP-5 MS (60 m × 0.25 mm, 0.25 μm film thickness) capillary column.

The temperature program for the gas chromatography was as follows: initial temperature, 75 °C, followed by gradients of 10 °C/min to 190 °C, then 20 °C/min to 280 °C, which was held for 5 min. The injector temperature was 270 °C, injection was in splitless mode (splitless time, 18 s), and the temperature of the transfer line was 280 °C. The carrier gas was helium (99.999% purity, 1.0 mL/min) supplied by Carburos Metálicos (Barcelona, Spain). Analytes sampled from the aqueous simulants (A, B, and C) were quantitatively analyzed in scan mode, from 45 to 250 m/z at a rate of 6.61 uma s^{-1}. Both atmosphere samples and simulant D samples were first screened in scan mode, and the detected compounds of interest were then quantified by selected ion monitoring (SIM) analysis, once their characteristic masses had been selected from their full spectra.

RESULTS AND DISCUSSION

Figure 1 shows the antimicrobial activity of the different combinations of plastic film and EOs against several bacteria and fungi in tests using a nominal concentration of the active agent incorporated in the films of 4% (w/w), according to other studies carried out with an antioxidant film (*15, 18*).

Yeasts, followed by molds, were the most sensitive microorganism, in accordance with previous studies showing fungi to be the most susceptible microorganisms to the vapors of EOs (*23, 24*). They were totally inhibited when they were exposed to the atmospheres generated by the films with 4% of oregano or fortified-cinnamon EO (1 and 4%). Yeasts were also inhibited by films with 4% of clove EO. In tests with other combinations of film and fungi the numbers of viable cells were reduced (e.g., films with 1% of oregano or 4% of cinnamon EOs and *D. hansenii* or *Z. rouxii*) or delayed the growth phase of the mold (e.g., white colonies of *A. flavus* incubated in the atmosphere generated by PP with 4% of clove EO, in contrast to brown-greenish colonies of the species, which grew both in the control dishes and in the presence of the PP film). However, only the films with 4% fortified-cinnamon oil showed antibacte-
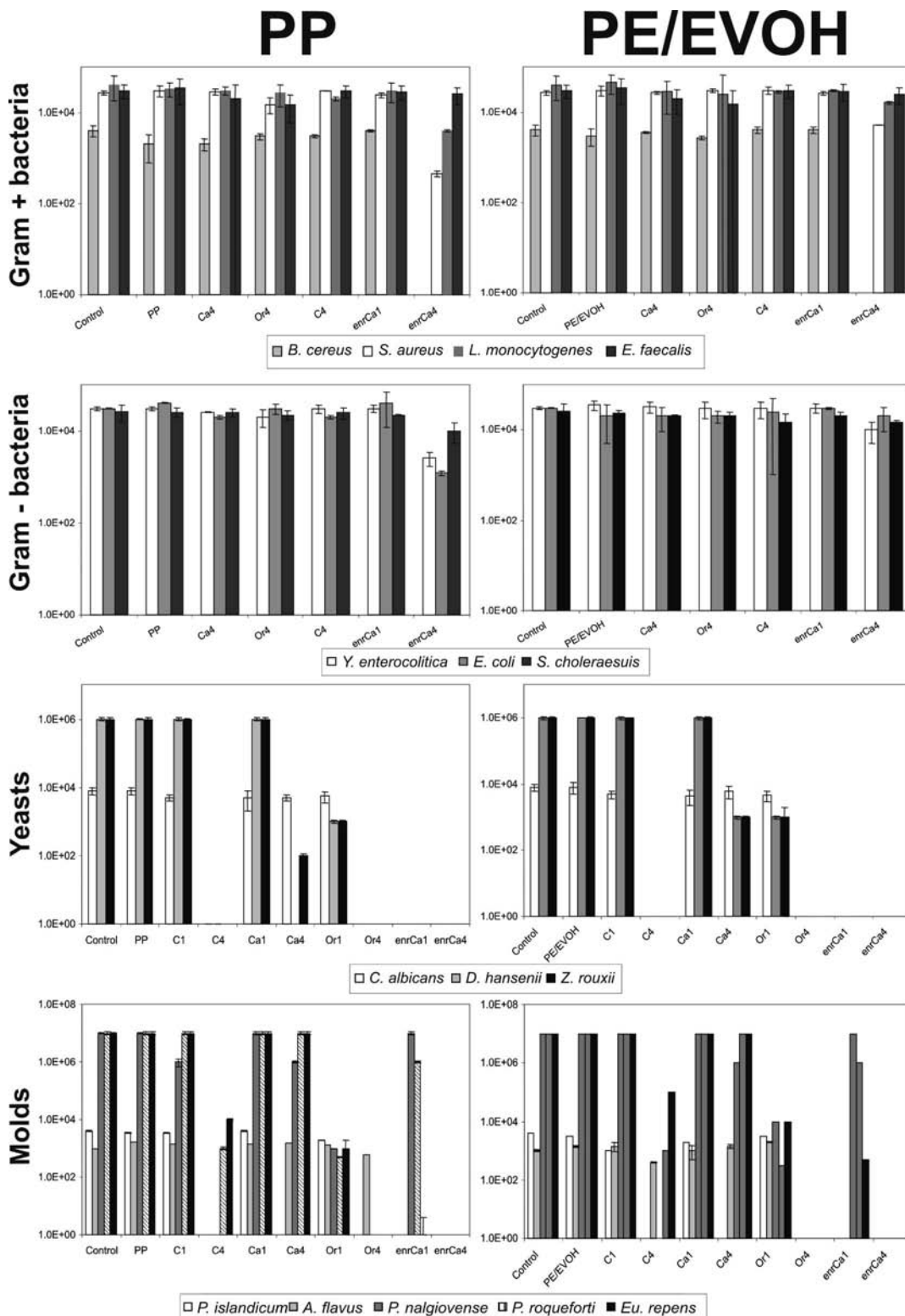
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Figure 1. Antimicrobial activity of films made of PP or PE/EVOH with incorporated cinnamon EO (Ca), clove EO (C), oregano EO (Or), or fortified-cinnamon EO (enrCa) at nominal concentrations of 1 or 4% (w/w). Y-axis: colony forming units (CFU)/mL.

rial activity. These films completely inhibited *B. cereus* and reduced the number of CFUs of *S. aureus* and *L. monocytogenes*. No effect was observed against Gram-negative bacteria. Overall, PP films were found to be more effective than PE/EVOH films, as illustrated in **Figure 1**.

The antifungal properties of the films with 4% (w/w) of oregano and fortified-cinnamon EO have been proven. However, this concentration is not high enough to inhibit the growth of

the bacteria. Thus, new films with higher contents of the extract were prepared in the laboratory.

As shown in **Figure 2**, films with fortified-cinnamon EO exhibited stronger antibacterial activity than those with oregano. Total inhibition of Gram-positive bacteria was achieved with 8% (w/w) of fortified-cinnamon EO and with 10% of oregano EO. Gram-negative bacteria, as expected, were more resistant, and 10% of fortified-cinnamon EO was required to inhibit them,
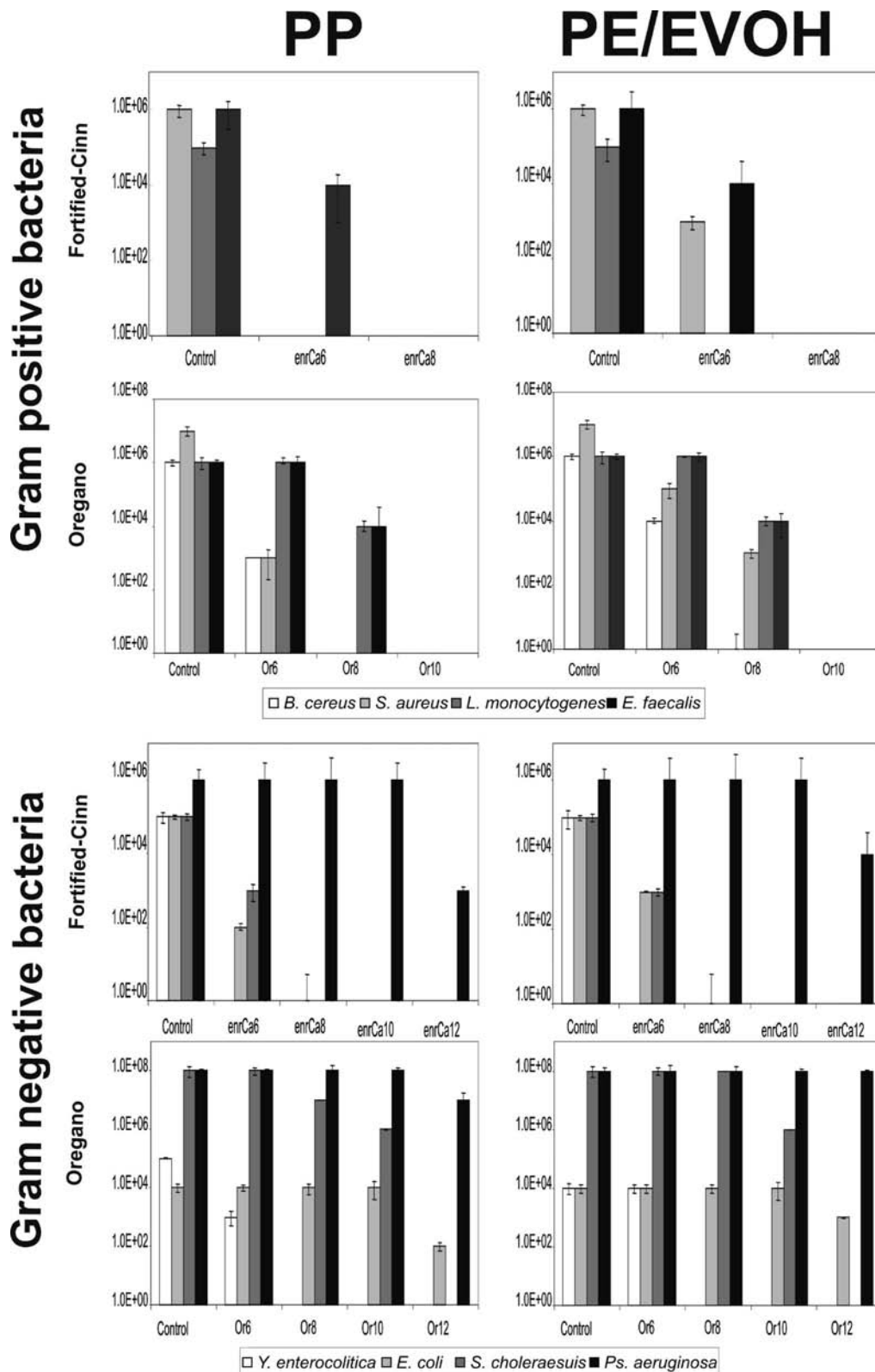
PP

PE/EVOH

Figure 2. Antimicrobial activity against bacteria of films made of PP or PE/EVOH with incorporated oregano EO (Or) or fortified-cinnamon EO (enrCa) at nominal concentrations ranging from 6 or 12% (w/w). Y-axis: colony forming unit (CFU)/mL.

although *Ps. aeruginosa* appeared to be unaffected even at this concentration, in accordance with its known high tolerance of EOs (*28*). Nevertheless, films with a 12% (w/w) of nominal concentration decreased the number of *Ps. aeruginosa* colonies and reduced their green coloration. Films with oregano, on the other hand, did not completely inhibit *E. coli*. Once again, PP films were more effective than PE/EVOH films.

Active films release volatile compounds to the headspace they enclose (in these tests, the Petri dishes). Thus, analysis of this atmosphere is a crucial step to correlate the release of volatile compounds and their effectiveness, so the atmospheres the films generated were sampled using HS-SDME then analyzed by GC–MS. **Figure 3** and **Table 2** show the results obtained when the atmospheres generated by films with incorporated fortified-cinnamon EO or oregano EO were sampled. As shown in **Figure**
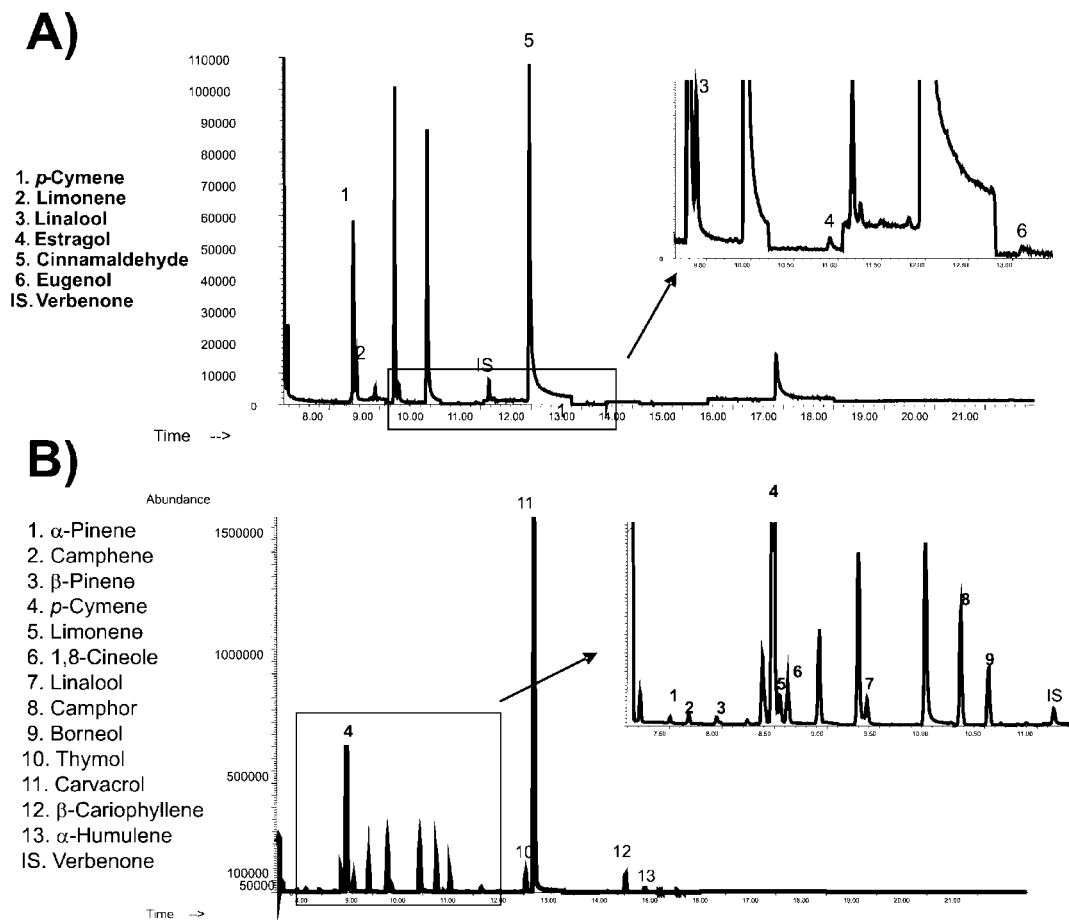
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Figure 3. GC–MS chromatograms of atmospheres generated by the antimicrobial films after three hours incubation sampled by HS-SDME. (**A**) PP with 6% (w/w) of fortified-cinnamon EO. (**B**) PE/EVOH with 12% (w/w) of oregano EO.

Table 2. Concentration of the Atmospheres Generated by the Antimicrobial Films After Three Hours Incubation Sampled by HS-SDME: (**A**) PP with 6% (w/w) of Fortified-Cinnamon EO and (**B**) PE/EVOH with 12% (w/w) of Oregano EO

compound	C (μg/L)
(A)	
p-cymene	57.5 ± 2.0
limonene	12.8 ± 3.7
1,8-cineol	36.2 ± 0.2
linalool	9.68 ± 0.34
camphor	24.3 ± 6.2
borneol	19.0 ± 4.4
thymol	37.8 ± 9.5
carvacrol	920 ± 21
β-caryophyllene	24.5 ± 4.1
α-humulene	5.52 ± 0.89
(B)	
p-cymene	13.2 ± 0.9
limonene	8.38 ± 1.45
linalool	2.61 ± 0.30
estragol	0.84 ± 0.10
cinnamaldehyde	257 ± 20
eugenol	49.5 ± 8.9

3a, fortified-cinnamon EO films released cinnamaldehyde (the major constituent of fortified-cinnamon EO; 80–95%), *p*-cymene, limonene, linalool, and traces of eugenol and estragol. Atmospheres generated by oregano EO films (**Figure 3b**) contained *p*-cymene, limonene, 1,8-cineole, camphor, borneol, thymol, carvacrol, β-caryophyllene, and α-humulene. Detectable traces of α-pinene, camphene, β-pinene, and estragol were also released by the films with the highest contents of oregano EO

(10–12%) during the first three hours of incubation. Carvacrol was the major constituent (80–90%), followed by thymol, borneol, and camphor, whose concentrations were very similar.

Cinnamaldehyde, carvacrol, and thymol are known to have excellent antimicrobial properties (*14, 29, 30*) and have been demonstrated to be responsible for the inhibition provided by fortified-cinnamon (cinnamaldehyde) and oregano EO (carvacrol and thymol), respectively (*23*).

The concentrations of these three compounds in the sampled atmospheres as functions of time, plastic material, and nominal incorporated concentration are shown in **Figure 4**. The amounts of cinnamaldehyde, carvacrol, and thymol releases to the Petri dish headspace during the first hours of the tests were clearly correlated with the concentrations of the EOs. The concentration of cinnamaldehyde released from fortified-cinnamon EO films increases to a maximum after 3 h of incubation and then decreases to a level that remained almost constant during the rest of the experiment. Carvacrol and thymol followed the same trend, except that the concentrations of these phenolic compounds peaked after 3–12 h of incubation, depending on the nominal concentration of oregano in the plastic film. It should be noted that the experimental system composed of the Petri dish, the plastic film, and the nylon cable tie never reaches a steady equilibrium because the system is not hermetically closed, and neither PP nor PE/EVOH is a barrier material to compounds in the vapor phase, so they are likely to diffuse through the films.

Although the antimicrobial mechanisms are still not fully understood, active compounds are thought to exert their effect during the lag phase (*29, 31*), which last several hours for
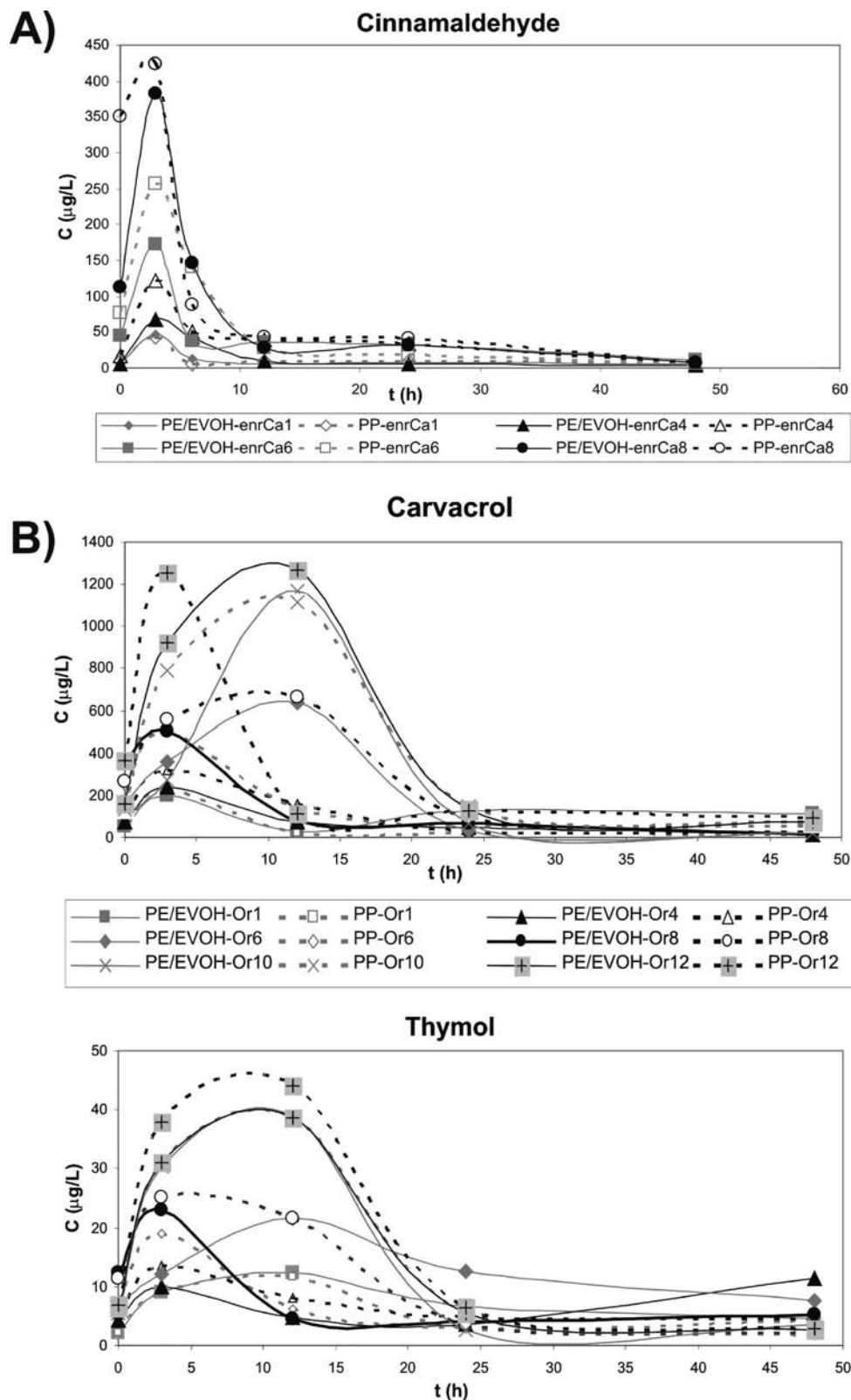
Figure 4. (**A**) Changes in cinnamaldehyde concentrations in the Petri dish headspaces as functions of time, nominal concentration of fortified-cinnamon EO (enrCa), and film material. (**B**) Changes in carvacrol and thymol concentrations in the Petri dish headspaces as functions of time, nominal concentration of oregano EO (Or), and film material.

bacteria. As can be seen in **Figure 4**, higher amounts of carvacrol, thymol, and cinnamaldehyde are liberated from PP than from PE/EVOH at the same nominal concentration of EO during the first six hours, which explains why PP films are more effective. These differences may be due to the difference of diffusion through the polymeric matrix. The diffusion coefficient

of organic species in polymer is mainly a function of molecular weight and polymer type (*32*). Since the range of molecular weight covered by active compounds is not very large, their diffusion coefficients are quite similar, and effective diffusion is mainly a function of polymer type and specifically of differences of polarity between polymeric materials (*33*).

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Table 3. Shelf-Life of Antifungal Films with 4% (w/w) of Fortified-Cinnamon (EnrCa) or Oregano (Or) EO[a]

| day | C. albicans | | | | | A. flavus | | | P. nalgiovense | | |
	control	PP-enrCa4	PE/EVOH-enrCa4	PP-Or4	PE/EVOH-Or4	control	PP-enrCa4	PE/EVOH-enrCa4	control	PP-Or4	PE/EVOH-Or4
1	1.0×10^4	n.g.	n.g.	n.g.	n.g.	2.0×10^3	n.g.	n.g.	1.0×10^7	n.g.	n.g.
8	1.0×10^4	n.g.	n.g.	n.g.	n.g.	4.0×10^3	n.g.	n.g.	1.0×10^6	n.g.	n.g.
15	1.0×10^4	n.g.	n.g.	n.g.	n.g.	1.0×10^4	n.g.	n.g.	1.0×10^6	n.g.	n.g.
22	2.0×10^4	n.g.	n.g.	n.g.	n.g.	1.0×10^4	n.g.	n.g.	1.0×10^6	n.g.	n.g.
29	1.1×10^4	n.g.	n.g.	n.g.	n.g.	1.0×10^4	n.g.	n.g.	1.0×10^7	n.g.	1.0×10^5
36	8.0×10^3	n.g.	n.g.	n.g.	n.g.	1.0×10^4	n.g.	n.g.	1.0×10^7	n.g.	1.0×10^5
42	1.0×10^4	n.g.	n.g.	n.g.	n.g.	1.0×10^4	n.g.	n.g.	1.0×10^8	1.0×10^2	1.0×10^5
49	1.5×10^4	n.g.	n.g.	n.g.	n.g.	1.0×10^5	n.g.	n.g.	1.0×10^8	1.0×10^2	1.0×10^5
69	1.0×10^4	n.g.	n.g.	n.g.	n.g.	1.0×10^5	n.g.	n.g.	1.0×10^8	1.0×10^2	1.0×10^5
76	1.0×10^4	n.g.	n.g.	n.g.	n.g.	1.0×10^5	n.g.	n.g.	1.0×10^8	1.0×10^2	1.0×10^5

[a] Results are given in CFU/mL. n.g., no growth was observed.

Table 4. Overall Migration (mg dm^{-2}) of the Antimicrobial Films Containing 4% (w/w) of Clove (C), Cinnamon (Ca), Oregano (Or), or Fortified-Cinnamon (EnrCa) Essential Oil

		blank	C4	Ca4	Or4	enrCa4
simulant A	PP	n.d.[a]	n.d.	1.24 ± 0.03	n.d.	0.02 ± 0.02
	PE/EVOH	n.d.	n.d.	n.d.	n.d.	n.d.
simulant B	PP	0.01 ± 0.02	n.d.	0.32 ± 0.05	n.d.	0.14 ± 0.10
	PE/EVOH	0.11 ± 0.10	n.d.	n.d.	n.d.	n.d.
simulant C	PP	n.d.	n.d.	n.d.	0.09 ± 0.08	n.d.
	PE/EVOH	0.05 ± 0.02	n.d.	0.02 ± 0.01	n.d.	n.d.
simulant D	PP	0.06 ± 0.03	0.24 ± 0.16	0.32 ± 0.19	0.13 ± 0.10	n.d.
	PE/EVOH	n.d.	0.84 ± 0.40	1.49 ± 1.08	0.41 ± 0.24	0.12 ± 0.04

[a] n.d., not detected.

Accordingly, the active compounds are more strongly retained in the PE/EVOH matrix, thus reducing diffusion through film thickness. Carvacrol, thymol, and cinnamaldehyde have polar functional groups, including hydroxyl and aldehyde groups, which could interact with the double bonds in the films, which are more reactive in polyethylene than in polypropylene, notably, in the alcohol vinyl layer of EVOH and the adhesives used to bind the layers of the PE/EVOH copolymer. It has been demonstrated that the amount of active compounds released from multilayer films is strongly influenced by the amount of free cross-linking agents they contain, which can react with the active compound, binding it to the polymeric matrix (*34*).

From an industrial perspective, the antimicrobial properties of the films should persist as long as possible since plastic manufacturers are rarely located near food packaging industries. Therefore, shelf-life tests were performed with the PP and PE/EOH antifungal films with nominal concentrations of 4% (w/w) of fortified-cinnamon or oregano EO. **Tables 3** and **4** show the results obtained.

Films containing oregano EO inhibited the growth of the yeast *C. albicans* for more than two months, but only PP films had a fungicidal effect. In tests with *P. nalgiovense*, small colonies appeared when they were incubated in the presence of these PP and PE/EVOH films after storage for 29 and 36 days, respectively.

The results described above show that the new PP and PE/EVOH antimicrobial films with incorporated EOs have promising potential for preserving foodstuffs that are frequently spoiled by fungi and remain effective for more than two months. In addition, they retarded bacterial growth. However, before they can be used in contact with foodstuffs, they have to be shown to have no harmful effects on human health; thus, migration tests (overall and specific) were performed.

Results obtained in the overall migration testing show (**Table 5**) that in all cases the values were far lower than the limit of

10 mg dm^{-2} stipulated in Directive 2002/72/EC (*35*). Migration values were highest for simulant D, the fatty food stimulant. As far as the other simulants were concerned, detectable amounts of migrating compounds were only detected in the tests with the PP films containing cinnamon, oregano, or fortified-cinnamon EO, in accordance with the finding that the EO constituents were less strongly retained by the polymeric matrix of these films than those of PE/EVOH films in the atmosphere analyses.

The specific migration tests validated the use of the antimicrobial films with oregano and fortified-cinnamon EOs in contact with foodstuff (**Tables 5** and **6**). The only problem is migration of eugenol to simulants A and B ($\approx$10 mg kg^{-1}) that exceeded its specific migration limit (0.2 mg kg^{-1}). Nevertheless, this chemical is not an isolated compound directly added to the packaging but a constituent of an EO (cinnamon), whose use as a flavoring is allowed, unless it alters the organoleptic or health properties of the foodstuff (*20, 22*).

cis(*Z*)- and *trans*(*Z*)-Cinnamaldehyde, eugenol, tributyl citrate, and traces of linalool migrated from fortified-cinnamon films (see **Figure 5A**), and thymol, carvacrol, tributyl citrate, and traces of 1,8-cineol, α-terpineol, 1-terpinen-4-ol, eugenol, borneol, and camphor migrated from films with oregano to the aqueous simulants. In addition to these substances, detectable amounts of a number of other more lipophilic terpenes (including *p*-cymene and limonene from films with fortified-cinnamon and α-pinene, camphene, β-caryophyllene, and α-humulene from films with oregano) migrated to the fatty food stimulant, isooctane.

Migration tests were performed with both sides of the films to study the diffusion of the molecules of active compounds and the simulants through the films. Diffusion depends on the nature of the migrant (size and solubility), as well as on the properties of the simulant and the polymeric matrix. Whatever side of PE/EVOH was in contact with the three aqueous simulants, migration values of molecules with a polar functional

Table 5. Specific Migration Values (μg g^{-1}) of Films Containing 4% (w/w) of Fortified-Cinnamon EO[a]

compound	simulant A (water)				simulant B (3% acetic acid)			
	AF1	rAF1	AF2	rAF2	AF1	rAF1	AF2	rAF2
linalool	0.03 ± 0.00	0.02 ± 0.00	0.04 ± 0.01	0.03 ± 0.01				
estragol								
(E)-cinnamaldehyde	0.03 ± 0.00	0.02 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
(Z)-cinnamaldehyde	5.14 ± 1.24	2.79 ± 0.84	5.58 ± 1.07	4.38 ± 1.14	3.32 ± 0.20	2.32 ± 0.44	3.30 ± 0.34	3.20 ± 0.14
eugenol	0.16 ± 0.04	0.07 ± 0.02	0.15 ± 0.08	0.10 ± 0.02	0.17 ± 0.01	0.08 ± 0.02	0.13 ± 0.02	0.09 ± 0.03
tributyl citrate	0.60 ± 0.04		1.36 ± 0.56		0.83 ± 0.10	0.19 ± 0.13	0.38 ± 0.09	0.04 ± 0.01

compound	simulant C (10% ethanol)				simulant D (isooctane)			
	rAF1	AF2	rAF2	AF1	rAF1	AF2	rAF2	AF1
p-cymene					0.01 ± 0.01		0.01 ± 0.00	
limonene					0.01 ± 0.01		0.001 ± 0.00	
1,8-cineol								
linalool	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
estragol	0.02 ± 0.04	0.01 ± 0.00						
(E)-cinnamaldehyde	0.03 ± 0.00	0.03 ± 0.00	0.05 ± 0.01	0.03 ± 0.00				
(Z)-cinnamaldehyde	4.22 ± 1.53	3.03 ± 1.08	6.13 ± 1.2	2.04 ± 1.0	3.45 ± 0.34	1.96 ± 0.63	3.03 ± 0.15	
eugenol	0.06 ± 0.01	0.08 ± 0.04	0.10 ± 0.02	0.04 ± 0.01	0.07 ± 0.04		0.02 ± 0.01	
tributyl citrate	0.20 ± 0.06	0.07 ± 0.02	0.35 ± 0.09	0.04 ± 0.02	3.91 ± 0.38	5.69 ± 0.72	3.74 ± 0.27	0.11 ± 0.06

[a] AF1: PP; AF2: PE/EVOH; r-side with less active compound exposed to the simulant.

Table 6. Specific Migration Values (μg g^{-1}) of Films Containing 4% (w/w) of Oregano EO[a]

compound	simulant A (water)				simulant B (3% acetic acid)			
	AF1	rAF1	AF2	rAF2	AF1	rAF1	AF2	rAF2
1,8-cineol	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.05 ± 0.01	0.02 ± 0.00	0.06 ± 0.01	0.05 ± 0.00
linalool	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00				
camphor	0.09 ± 0.00	0.02 ± 0.01	0.08 ± 0.03	0.04 ± 0.02	0.13 ± 0.03	0.012 ± 0.00	0.14 ± 0.02	0.08 ± 0.00
borneol	0.03 ± 0.00		0.03 ± 0.00	0.02 ± 0.01	0.06 ± 0.02		0.03 ± 0.02	0.05 ± 0.00
thymol	0.14 ± 0.00	0.07 ± 0.03	0.05 ± 0.01	0.04 ± 0.01	0.15 ± 0.04	0.03 ± 0.01	0.10 ± 0..01	0.09 ± 0.00
carvacrol	2.89 ± 0.11	1.43 ± 0.23	0.86 ± 0.30	0.78 ± 0.23	3.58 ± 0.73	0.82 ± 0.24	2.61 ± 0.31	2.28 ± 0.15
eugenol	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
tributyl citrate	0.73 ± 0.11		1.45 ± 0.039		0.57 ± 0.20	0.08 ± 0.02	0.68 ± 0.11	0.16 ± 0.00

compound	simulant C (10% ethanol)				simulant D (isooctane)			
	rAF1	AF2	rAF2	AF1	rAF1	AF2	rAF2	AF1
α-pinene					0.01 ± 0.00			
camphene					0.01 ± 0.00			
p-cymene					0.02 ± 0.01	0.01 ± 0.00	0.04 ± 0.02	
limonene					0.01 ± 0.00			
1,8-cineol					0.05 ± 0.01	0.06 ± 0.01	0.09 ± 0.02	0.05 ± 0.00
linalool	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
camphor	0.05 ± 0.01	0.01 ± 0.00	0.07 ± 0.03	0.06 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.18 ± 0.01	0.04 ± 0.00
borneol			0.01 ± 0.01		0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	
thymol	0.06 ± 0.01	0.02 ± 0.01	0.04 ± 0.02	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.02	0.12 ± 0.03	
carvacrol	2.82 ± 0.44	1.09 ± 0.2	3.10 ± 0.60	3.42 ± 0.70	2.92 ± 0.05	3.39 ± 0.26	5.17 ± 0.23	0.39 ± 0.14
eugenol	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.07 ± 0.02			
b-caryophyllene					0.07 ± 0.02	0.05 ± 0.01	0.08 ± 0.02	0.01 ± 0.00
a-humulene					0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	
tributyl citrate	0.30 ± 0.03	0.03 ± 0.01	0.40 ± 0.02	0.12 ± 0.01	5.96 ± 0.31	4.69 ± 0.25	4.65 ± 0.09	0.45 ± 0.11

[a] AF1: PP; AF2: PE/EVOH; r-side with less active compound exposed to the simulant.

group, such as linalool, thymol, cinnamaldehyde, carvacrol, or eugenol, were similar. EVOH properties are highly deteriorated in the presence of water. It is thought that water molecules intercept the hydroxyl groups from inter- and intramolecular hydrogen bonding. As consequence of this plasticization effect, the fractional free volume of the polymer for permeants to travel across polymer packages increases. Small molecules are solubilized and transported through the films and reach the simulant (*36*). Nevertheless, the cinnamaldehyde concentration in simulant C decreases without direct contact. This could have been due to reactions between the ethanol and the aldehyde, forming an acetal, while the molecule diffused through PE/EVOH.

Larger molecules, for example, tributyl citrate, diffuse slowly, and consequently their migration values depended on the contact side. However, simulant D is capable of extracting the analytes of interest from PP, whereas EVOH acts as a barrier and prevents them from diffusing through it to the layer in contact with isooctane (*37*).

The results of this study demonstrate the potential utility of PP or PE/EVOH films with incorporated oregano or fortified-cinnamon EOs at concentrations of 4% (w/w) as antifungal packaging materials. They maintain their antimicrobial properties for more than two months, and their use in contact with foodstuffs has been demonstrated not to be harmful to consum-
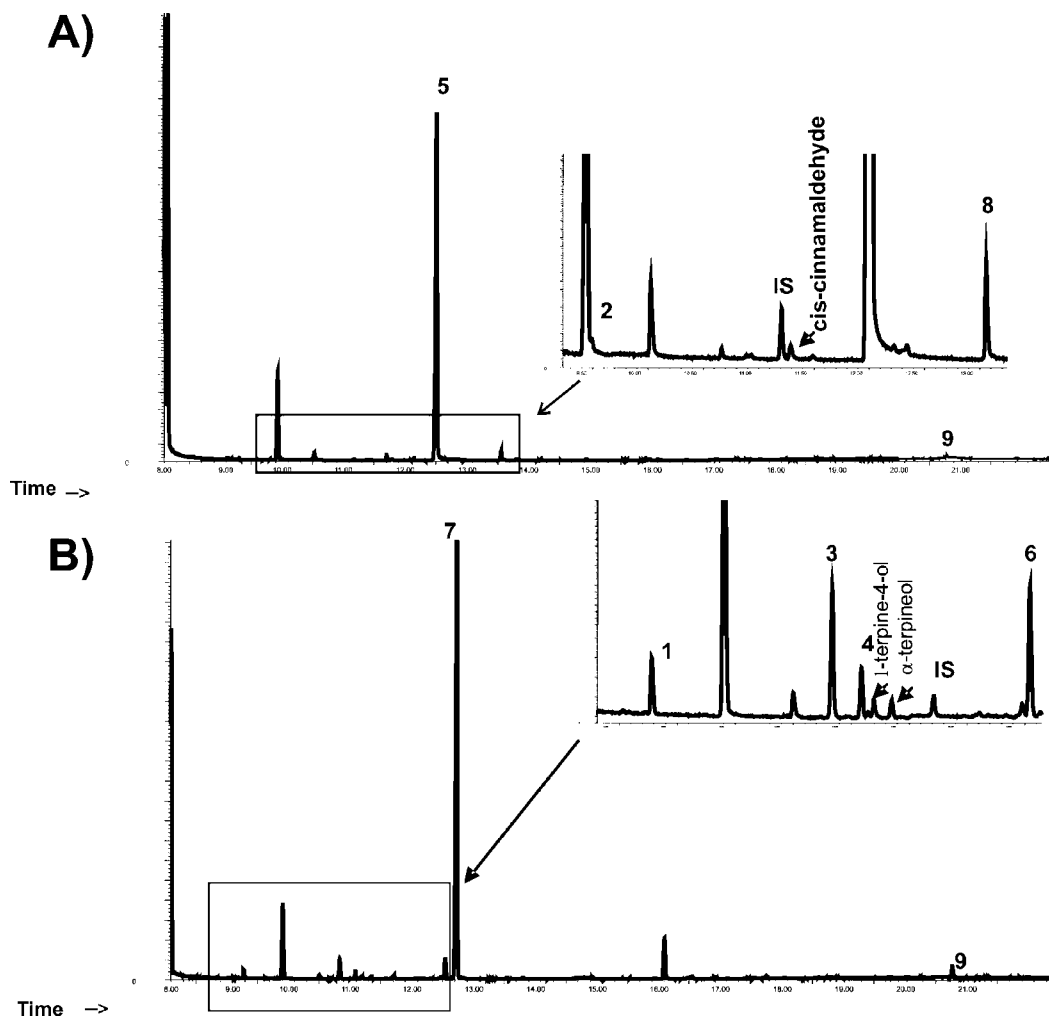
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Figure 5. GC–MS chromatograms of DI-SDME samples from the specific-migration-tests. (**A**) Migration from PP with 4% (w/w) of fortified-cinnamon EO to simulant C. (**B**) Migration from PE/EVOH with 4% (w/w) of oregano EO to simulant B. (1 = 1,8-cineol; 2 = linalool; 3 = camphor; 4 = borneol; 5 = *trans*-cinnamaldehyde; 6 = thymol; 7 = carvacrol; 8 = eugenol; 9 = tributyl citrate; IS = verbenone).

ers' health. Main drawback could be the organoleptical alteration of the packaged food due to the chemicals released by the active package. According to Valero and Giner (*29*), levels of 2 μL cinnamaldehyde/100 mL carrot broth, which is around 2 μg/g, were organoleptically acceptable. Only when the concentration rose up to 6 μg/g was the effect on taste and smell negative. Sensory evaluation of oregano EO added to bologna suggested that concentrations lower than 45 ppm, which could mean concentrations of carvacrol lower than 20 μg/g, would be acceptable to consumers (*38*). Although a further investigation is currently under way to evaluate the organoleptical effect of active films on consumers' acceptance, the comparison of literature data with the results obtained from specific migration tests is promising. The final result will depend on how the constituents of the food matrix ad/absorb the antimicrobial agents.

However, food matrices are more complex than culture media, so some reactions between active compounds and food constituents are likely to occur. These reactions could reduce the films' antimicrobial effectiveness in some cases, for example, if EOs containing cinnamaldehyde are used in conjunction with alcoholic foodstuffs. For these reasons, customized or ad hoc solutions should be applied to specific foodstuffs, depending on their natural microbiota, composition, and capacity to absorb candidate antimicrobial agents. As a preliminary selection, a combination of different bakery

products with the cinnamon package and meat items with oregano is being evaluated.

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