Influence of pH and temperature variations on vapor phase action of an antifungal food packaging against five mold strains

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A R T I C L E   I N F O

Article history:
Received 9 March 2014
Received in revised form 3 June 2014
Accepted 10 June 2014
Available online 17 June 2014

Keywords:
Antifungal
Cinnamon
Mold
Vapor phase
Active packaging

A B S T R A C T

An antifungal active food packaging containing cinnamon essential oil has been evaluated under different pH and temperature. Cinnamon essential oil was previously chosen among oregano, clove and their major components cinnamaldehyde, carvacrol and eugenol respectively. Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC) were determined by macrodilution method against two strains of Aspergillus flavus as well as Aspergillus niger, Penicillium roqueforti and Penicillium expansum. Despite pH and temperature modified the growth rate of the molds, these variables did not affect the MIC and MFC values. The active food packaging, consisting of Polypropylene films coated with a formula containing 2%–6% cinnamon were produced and evaluated. The antifungal activity was studied against two A flavus, P roqueforti and P. expansum at different pH and temperature. The results highlighted the growth delay caused by the PP 2% in all cases. Total inhibition was obtained with higher amount of cinnamon. This fungicidal action was maintained beyond the 30 days of the experiments. Long-term properties were confirmed after 3 months. The fridge temperature did not hamper the antifungal action of the cinnamon volatile compounds. Finally, all these results demonstrated the high-performance of the antifungal packaging, what guarantee an efficient and durable antifungal protection.

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1. Introduction

Since the last decade, active packaging has outcome a reliable alternative not only as a food conservative method, but also as a shelf life extension. Essential oils are natural substances categorized as GRAS by the Food and Drug Administration (FDA) and most of them are derived from plants. They have shown recognized antibacterial and antifungal properties achieved both in direct contact and in vapor phase. The possibility of achieving an antimicrobial action by the release of the volatile compounds has increased the interest of including them into the packaging (Appendini & Hotchkiss, 2002; Becerril, Gómez-Lus, Goni, López, & Nerín, 2007; Nielsen & Rios, 2000; Rodriguez, Batlle, & Nerín, 2007; Rodríguez, Nerín, & Batlle, 2008) Many examples can be found in the bibliography concerning different substrates, active agents and microorganisms target (da Cruz Cabral, Fernández Pinto, & Patriarca, 2013; Matan et al., 2006; Nielsen & Rios, 2000; Nostro et al., 2012; Ojagh, Rezaei, Razavi, & Hosseini, 2010; Souza, Goto, Mainardi, Coelho, & Tadini, 2013). Due to the good results obtained, their application towards different food products has also increased (Coma, 2008; Cristina, Annalisa, Amalia, Francesco, & Del Nobile, 2013; Gutiérrez, Sánchez, Battle, & Nerín, 2009; Guynot, Sanchis, Ramos, & Marín, 2003; Matan, 2012; Montero-Prado, Rodriguez-Lafuente, & Nerín, 2011; Rodríguez-Lafuente, Nerín, & Battle, 2010; Skandamis & Nychas, 2002) in order to inhibit the growth, or inactivate pathogenic or spoilage microorganisms (Davidson, Critzer, & Taylor, 2013). However, developing a new antimicrobial active packaging is a delicate task where different variables must be studied. One critical point is the choice of the material used, because the nature of the substrate may affect the freedom of the active agent incorporated. Hence, due to a minor porosity, plastic films such as PET (Polyethylene terephthalate), PP (Polypropylene) and PE/EVOH (polyethylene/ethylene vinyl alcohol copolymer) have revealed a faster release of the active compounds than paper (Manso, Cacho-Nerin, Becerril, & Nerín, 2013). Moreover, another important aspect is the choice of the active agent. For that, the kind of food product has to be considered, because of three fundamental reasons: Firstly, the food properties cause a
prevalence of some microbial species. Secondly, the organoleptic properties of the packaged food should not be affected by the volatile compounds released by the active agents. This is a critical point of the essential oils, although in some cases this issue can be used as an advantage. Finally, variables such as temperature and pH of the product may affect the antimicrobial activity, what would condition the use of the active agent.

The first two reasons have been discussed by different authors in several papers but it is the first time that the influence of pH and temperature variation is considered for antifungal packaging in vapor phase. The aim of this work was to deeply study an antifungal food packaging here presented constitutes a clear advantage due to the action of the vapor phase of cinnamon. Also, the activity demonstrated at pH 3, pH 5.6 and at three temperatures (6 °C, 25 °C and 37 °C) opens a large number of possibilities concerning different food products.

2. Materials and methods

2.1. Strain and culture media

A. flavus CECT 2940 (A. flavus 2949), A. flavus CECT 2687 (A. flavus 2687), Aspergillus niger CECT 2088 (A. niger), P. roqueforti CECT 2905 (P. roqueforti) and P. expansum CECT 2278 (P. expansum), all from the Spanish collection CECT (University of Valencia) (Spain) were used as reference strains. PDA (Potato Dextrose Agar), MEA (Malt Extract Agar N’1) and agar—agar as solid media and PDB (Potato Dextrose Broth), MEB (Malt Broth) and YEB (Yeast Extract Broth) as liquid media were all supplied by Scharlau (Spain).

2.2. Essential oils and antimicrobial films

As active agents, the essential oils of cinnamon (CIN) (Cinnamomum zeylanicum CAS 8015-91-6) from the bark fortified with cinnamaldehyde, orégano (OR) (Origanum Vulgare CAS 8007-11-2) and clove (CLO) (Syzygium aromaticum CAS 8000-34-6), as well as their respective major compounds trans-cinnamaldehyde 99% (CINAM) (CAS 14371-10-9), carvacrol > 97% (CARV) (CAS 499-75-2) and eugenol 99% (EUG) (CAS 97-53-0) were used. The essential oils were supplied by Argolide (Barcelona, Spain) while the major compounds were provided by Sigma (Sigma—Aldrich Química S.A.) (Madrid, Spain). The composition of the cinnamon from the vapor released from bark and oréarn was previously analyzed (López, Sánchez, Battile, & Nerín, 2007b) as well as from the clove (López, Sánchez, Battile, & Nerín, 2005). Active PP (with 2% CIN, 4% CIN and 6% CIN) and blank PP (PP 0%) were supplied by Artibal (Sabiñánigo, Spain). It consisted of 30 μm thick layer coated with an organic base formulation containing the essential oil at different concentrations, according to the EU patent (Garcés & Nerín, 2004). The grammage of the coating was 2.5 g/m2.

2.3. Screening of the six active agents activity

The MIC (Minimal Inhibitory Concentration) and MFC (Minimal Fungicidal Concentration) of six compounds (CIN, CINAM, OR, CARV, CLO, and EUG) were established for the molds A. flavus CECT 2949 and P. roqueforti. Spores were harvested from a 7 days old PDA culture with a sterile swab (Scharlau), adjusted in sterile NaCl 0.9% (Panrec) until 10⁶ CFU/ml by using the hemocytometer (Assistant) and confirmed later by plate counting. MIC was determined by macrodilution method in YEB following the same procedure as used in a previous work (Manso et al., 2013). In summary, serial EOs dilutions in ethanol were added to a YEB tube containing 10⁶ CFU/ml reaching a final range of 3.2 mg/mL–0.05 mg/mL EO concentration. Controls with 20 μL of ethanol were added to the test. Samples were incubated for 48 h at 25 °C under continuous shaking and MIC was determined by visual method as the lowest concentration where non-growth was observed. Tubes were kept at 25 °C for a total of 5 days to assure that there were not changes versus time. After that, 100 μL of these inhibitory concentrations were seeded on PDA for P. roqueforti and on MEA for A. flavus 2949. After 5 days of incubation period at 25 °C, minimal fungicidal concentration (MFC) was determined as the lowest concentration where non-growth was observed. This test was carried out at least three times by duplicate each time.

2.4. Influence of pH and temperature on the CIN and OR essential oils activity

After the first screening, the influence of pH and temperature on the CIN and OR activity was studied. In this case, five strains were evaluated: A. flavus 2949, A. flavus 2687, A. niger, P. roqueforti and P. expansum. The inhibitory concentrations were evaluated under the same conditions of pH and temperature as explained in Section 2.5. However, in this case, the pH of YEB was modified before autoclaving. MIC was determined as in Section 2.3 but incubating the 6 °C samples for 7 days. Besides, in order to assure the final values, tubes were kept in the incubator beyond the MIC determination, that is, 5 days for the 25 °C and 37 °C samples, and 15 days for the 6 °C samples. Previously, MFC was determined in all cases after 5 days at 25 °C, seeding A. niger, P. roqueforti and P. expansum on PDA and A. flavus 2949 and A. flavus 2687 on MEA. As before, this test was carried out at least three times by duplicate.

2.5. Diameter of fungal colony at different pH and temperature

The pH of the different culture media was adjusted to pH 3, pH 5 and pH 7. For that, known volumes of PDB and MEB were prepared and the amount of NaOH (0.1 M) or HCl (0.1 M) needed to modify the pH was calculated. After adding the agar–agar, they were autoclaved (121 °C for 15 °C) and the correspondent amount of the acidic or alkaline solution was added. An aliquot was extracted from the media and the three pH values were checked. Finally, 15 mL of each were directly poured onto the Petri dishes.

Once the plates were solidified, 10 μL of 10⁶ CFU/mL were inoculated in the center of PDA (in the case of A. niger, P. roqueforti and P. expansum) and MEA Petri dishes (A. flavus 2949 and A. flavus 2687) at the three pH tested. Finally, the plates were incubated at 25 °C and 37 °C for the genera Aspergillus and 25 °C and 6 °C for the Penicillium. For each colony, the mean diameter was calculated by measuring both directions at right angles to each other with a digital caliper every 24 h, considering at least 4 replicates from each strain, pH and temperature. The distance (diameter in mm) was plotted versus the time (days) needed to reach the end of the Petri dish, which varies depending on the strain growth. Also, two plates from each different pH were kept in the incubator at the three temperatures tested (25 °C, 37 °C and 6 °C) without fungal inoculation, and the final pH was measured at the end of the experiment in order to assure the pH stability.
2.6. Active PP films under different pH and temperature: vapor phase

This experiment was designed in two different steps. Firstly, the effect of temperature on the CIN active film vapor phase activity was studied. For that, 100 μl of 10^5 CFU/ml from the five strains were seeded into PDA and MEA plates depending on the strain as in Section 2.6. Then, the active PP films coated with the active formula at 2%–6% of CIN essential oil concentration were placed over the inoculated plates without being in contact with the agar. The film was attached with a nylon cable replacing the normal lid of the Petri dish (López, Sánchez, Batlle, & Nerín, 2007a) in order to maintain the vapor phase released by the film in the Petri dish. Controls were performed in the same way with inactive PP (0% CIN). Plates were incubated at two possible temperatures for each strain (25 °C and 37 °C for the genera Aspergillus and 25 °C and 6 °C for Penicillium). The films that caused a total mold inhibition were evaluated to determine if the action was fungistatic or fungicidal. For that, three plugs from the inhibited plate were placed in a new Petri dish containing MEA or PDA culture medium. Fungicidal was confirmed in the case of non-re-growth, otherwise it was fungistatic.

The aim of the second step of this experiment was to evaluate the influence of the two variables, pH and temperature. In this case only the strains A. flavus 2949 and P. roqueforti were evaluated, using MEA and PDA respectively at pH 3. The pH was modified as described in Section 2.5. Then, after inoculating 100 μl of 10^6 CFU/ml mold suspension, active and control films were applied as in the first step and plates were incubated at two temperatures mentioned for each strain. The whole experiment was performed by triplicate.

3. Results and discussion

3.1. Screening of the six active agents activity

A. flavus 2949 and P. roqueforti were chosen for the initial screening of the six compounds. As a dilution assay, broth dilution method was chosen because it provides more accurate results than agar diffusion assay (David, Steenson, & Davidson, 2013; Suppakul, Miltz, Sonneveld, & Bigger, 2003). The MIC (Minimum Inhibitory Concentration) and MFC (Minimum Fungicidal Concentration) values are shown in Table 1. Concentrations (mg/ml), expressed as a range, are referred to replicates where both results were found. Minor variations in external variables such as the initial fungal suspension and slight differences in time and temperature incubation may incline the results towards one or the upper MIC or MFC value. Despite only differences higher than one dilution order were considered as relevant, some aspects can be mentioned.

As we can see, cinnamon, oregano and clove gave similar results as their major components cinnamaldehyde, carvacrol and eugenol respectively. This finding demonstrates the strong dependence of these major compounds on the antifungal efficiency of the essential oils and matches with the results from the vapor phase composition mentioned in Section 2.2. Data reported by López et al. about the vapor phase composition of cinnamon, oregano and clove have been used in the present work because the same essential oils were used. This is particularly interesting because of the known heterogeneity in the composition of these natural substances.

López et al. (2007b) attributed the oregano and cinnamon vapor phase activities mainly to the presence of carvacrol and cinnamaldehyde respectively (López et al., 2007b). A stronger antifungal activity for cinnamon from the bark than cinnamon from the leaf was found in another work, due to a higher amount of cinnamaldehyde in the first case (Pawar & Thaker, 2007). Also, eugenol has been identified as the major compound in the vapor phase composition of clove (López et al., 2005).

Among the two strains, MFC concentrations were slightly higher in P. roqueforti, finding also a greater difference between their MIC and MFC values, being more accused in the case of clove and eugenol.

Concerning the different substances, the activity obtained decreased in the following order: cinnamon > cinnamaldehyde > carvacrol = oregano > eugenol > clove. Hence, cinnamon and cinnamaldehyde were the most active agents, with the lowest MIC of 0.1 mg/ml for cinnamon in both strains, and 0.1–0.2 mg/ml for cinnamaldehyde in A. flavus and P. roqueforti respectively. Cinnamon obtained the same MFC as cinnamaldehyde in A. flavus and the lowest fungicidal concentration in P. roqueforti.

Oregano and carvacrol gave the intermediate activity. Despite the same MIC and MFC values were found for both compounds in A. flavus and P. roqueforti, carvacrol appeared as slightly more active. Thus, a minor mold development was observed in the tube samples incubated at the carvacrol sub-inhibitory concentrations compared to oregano.

Finally, clove and eugenol were the less active substances, needing the highest MIC and MFC concentrations, highlighting the fungicidal value for P. roqueforti of 3.2 mg/ml. Eugenol was slightly more active than clove, reporting the same or minor concentrations than clove. Besides, eugenol showed the same behavior as carvacrol at sub-inhibitory concentrations.

Due to the lack of a standard method to determine the activity of the essential oils, only the broth dilution method has been considered to compare the present results. Similar MIC for cinnamon (Koceski et al., 2013), oregano (Mitchell, Stamford, de Souza, Lima, & Carmo, 2010) and clove (Omidbeygi, Barzegar, Hamidi, & Naghdibadi, 2007) has been found.

3.2. Influence of pH and temperature on the CIN and OR essential oils activity

Due to their lower MIC and MFC values compared to clove essential oil (Table 1), cinnamon and oregano were chosen to evaluate the influence of pH and temperature in the essential oil activity. MIC and MFC concentrations were determined at three pH and two possible temperature incubation depending on the strain as explained in Section 2.4.

As can be seen in Table 2, cinnamon resulted as in the previous experiment (Table 1) more active than oregano, giving in general lower inhibitory concentrations. As can be appreciated, MIC and MFC values obtained at different pH and temperature were very similar but with a tendency to decrease as the temperature increases.

Table 1

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<tr>
<th></th>
<th>CIN</th>
<th>CINAM</th>
<th>OR</th>
<th>CARV</th>
<th>CLOVE</th>
<th>EUG</th>
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<tbody>
<tr>
<td>MIC</td>
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<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.4</td>
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<tr>
<td>MFC</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.4</td>
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A. flavus 2949

<table>
<thead>
<tr>
<th></th>
<th>CIN</th>
<th>CINAM</th>
<th>OR</th>
<th>CARV</th>
<th>CLOVE</th>
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<tr>
<td>MIC</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>MFC</td>
<td>0.1</td>
<td>0.4</td>
<td>0.1</td>
<td>0.8–1.6</td>
<td>0.4</td>
<td>0.8</td>
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closed, being the maximal difference of only one dilution order. This result indicates that these variables did not affect the essential oils activity in a relevant manner. The only exception was found for \( P.\ roqueforti \) at 6 °C, when the MFC concentrations were slightly lower at pH 7.

Despite it is known that the modification of the incubation conditions affects the mold growth, the results discussed above demonstrated that these variations do not affect the essential oils activity tested, where the same MIC and MFC values were maintained. The same conclusion has been already published by other authors with different natural substances (López-Malo, Alzamora, & Palou, 2002; López-Malo, Barreto-Valdivieso, Palou, & Martín, 2007; Suhr & Nielsen, 2003). Also, essential oils present a clear advantage comparing to other antifungal synthetic substances as potassium sorbate and sodium benzoate, which are more active on the acidic pH due to the prevalence of the undissociated form (Davidson et al., 2013; López-Malo et al., 2002; López-Malo et al., 2007).

### 3.3. Diameter of the fungal colony at different pH and temperature

As explained in Section 3.2, cinnamon and oregano essential oils did not modify their MIC and MFC values at different pH and temperature conditions. However, it does not mean that these variables do not affect the mold growth. In fact, despite the mold growing at sub-inhibitory concentration was not measured, some variation on the mold quantification is expected when modifying these variables. In order to analyze their influence on the mold growth without essential oils, the five strains were cultured in solid media at the pH and temperature values employed in Section 3.2. The colony diameter of the five molds was measured every 24 h, and the distance (mm) was plotted versus time.

The results are shown in Table 3. The growth rate (GR) was taken from the slope of each linear regression equation. The correlation \((R^2)\) was obtained within the normal values for this experiment, showing in all cases a coefficient higher than 0.95. The growth rate of the different options was analyzed by statistical analyses (SPSS 15.0 for Windows). In order to diagnose possible differences, one-way Anova was performed applying Anova or Welch test depending on the variance’s homogeneity (Levene test). Post-hoc multiple comparisons tests (Bonferroni or T2 Tamhane) were used in order to identify the origin of the difference. Significant differences were considered at the \( p < 0.05 \) level.

As expected, the pH 3 caused in all cases a slower GR (Table 3). Both strains of \( A.\ flaus \) resulted influenced by the pH diminution while the variation of temperature did not affect significantly (significant differences at \( P < 0.05 \) level). However, \( A.\ niger \) showed the opposite behavior where only significant differences were found when the temperature was modified. In fact, this was the only strain where the GR increased when the temperature rose from 25 °C to 37 °C. Obviously the two species of \( Penicillium \) showed a clear influence of the temperature, decreasing strongly the GR when incubating the plates at 6 °C. Besides, \( P.\ expansum \) showed significant differences between pH 3 and pH 7. These results are in agreement with other authors. López-Malo, Alzamora, and Palou (2005) found a clear diminution on the GR of \( A.\ flaus \) when decreasing pH and water activity (aw), with a slight modification on germination time (López-Malo et al., 2005). Holmqvist, Walker, and Stahr (1983) reached at the same conclusion, with a significant \( Aspergillus \) reduction at pH 3 (Holmquist et al., 1983). Also, Plaza, Usall, Teixido, and Vinas (2003), demonstrated that decreasing temperature from 25 °C until 10 °C and 4 °C in \( Penicillium\ digitatum \) and \( Penicillium\ italicum \), caused an increase of the Lag phase with a diminution of the germination rates (Plaza et al., 2003).

### 3.4. Active cinnamon PP films under different pH and temperature: vapor phase

The three CIN percentages applied onto the PP films provided antifungal activity, even at the lowest 2% CIN concentration. Despite the total inhibition was achieved only in few samples, a clear reduction of growth was obtained in all cases compared to the controls. The growth rate (GR) of the five different strains under different pH and incubation temperature. The GR values (in mm/day) are presented as the mean (±Standard Deviation) and correlation coefficient values \((R^2)\) of at least 4 independent replicates.
controls (PP 0%), that is why we referred PP 2% CIN as the sub-inhibitory concentration. The results are shown in Fig. 1. As will be discussed in detail, this activity was strongly influenced by the change of temperature and pH. Active PP films with 4% and 6% CIN reported a remarkable fungicidal activity, due to the total inhibition in all cases, considering different strains, temperatures and pH evaluated. Besides, the mold was not able to re-growth after placing plugs in new fresh dishes. Hence, PP 4% and PP 6% were named as fungicidal concentrations.

Due to the qualitative results of this experiment, data concerning the growth of the controls and PP 2% were converted into a scale in order to quantify the activity. Four points were established, that is: total inhibition (0), initial growth (1), medium growth (2), strong growth (3) and complete growth (4). Supplementary data Fig. 2 shows an example of the scale applied, in the case of a different growth degree obtained by an active material. A similar scale has been used to relate fungal active packaging development (Manso et al., 2013; Wallstrom, Stromberg, & Karlsson, 2005).

However, as we can see in Fig. 1, the scale has been employed to convert the data into percentage of growth, so that the three replicates form each sample were considered. Hence, the points obtained for each replicate were summed up, and the final sum for each sample (three replicates per sample) was related to the control (PP 0%). The complete growth of the controls means a 100% of mold growth, that is, 4 points for each replicate which sum up the 12 points in total for the control sample. This qualitative scale allowed us to measure the active packaging activity, but it is not capable of distinguishing minor differences. However, in all cases the growth obtained was carefully evaluated every day, in order to detect minor differences, even if they were not quantifiable. As mentioned, the PP 4% CIN and PP 6% CIN films caused a total inhibition in all cases and for this reason these data are not represented in Fig. 1. Hence, only the control (PP 0%) and PP 2% CIN are discussed.

Mold growth was affected when the temperature was modified. As can be observed, both Aspergillus strains were completely inhibited by the PP 2% CIN at 37 °C (Fig. 1a,b). At 25 °C A. flavus 2949

Fig. 1. Development of A. flavus 2949 (a,e), A. flavus 2687 (b), P. roqueforti (c,f) and P. expansum (d) in time, under the influence of active PP with 2% cinnamon essential oil. Vertical axis has been converted applying a 5 point scale described in the text and expressed as percentage of growth considering the three replicates of each option. All the strains were evaluated at two possible temperatures (25 °C and 37 °C for the genus Aspergillus) and (25 °C and 6 °C for the genera Penicillium). Besides, A. flavus 2949 and P. roqueforti were also evaluated at pH 3 (figures “e” and “f” respectively).
experienced a total growth in two of the three replicates (66% of growth), meanwhile in *A. flavus* 2687 only one replicate showed a partial growth, which started after 20 days of incubation (20.83% of growth). The total inhibition achieved at 37 °C by PP 2% CIN in both *Aspergillus* may be due to a minor rate sporulation in both strains at this temperature.

As expected, the fridge temperature caused a strong slowdown and minor sporulation rate in both *Penicillium*. As mentioned before, this fact could explain the high activity resulted by the PP 2% CIN at 6 °C, where only one plate of *P. expansum* growth partially, whereas a total inhibition was found for *P. roqueforti*.

Concerning the pH, *A. flavus* 2949 and *P. roqueforti* were evaluated at pH 5.6 and pH 3. As can be appreciated, at 25 °C controls of both strains grew two days later when the pH was decreased to pH 3. In the case of 37 °C and 6 °C a higher difference was found between controls at pH 5.6 and pH 3. Thus, comparing both pH, a slowdown of 6 days and 5 days was found for *A. flavus* 2949 at 37 °C (Fig. 1a,e) and *P. roqueforti* at 6 °C (Fig. 1d,f) respectively. As can be seen, controls of both strains showed a similar behavior under these variables. However, this slowdown influenced more the PP 2% CIN activity in the case of *P. roqueforti*, reducing at 25 °C the growth from 33.33% at pH 5.6 (Fig. 1d) towards a complete inhibition at pH 3 (Fig. 1f).

The results evidenced that the active packaging activity can be enhanced when negative conditions for the mold growth take place, causing a clear growth delay at sub-inhibitory concentrations (PP 2% CIN). Besides, the active materials at sub-inhibitory and fungicidal concentrations were able to reduce or inhibit the mold growth under all incubation conditions. This is an important outcome that highlights that volatile compounds from cinnamon can be released from the active film towards the culture medium even at fridge temperature. This fact is very important, as most of the perishable food are kept under refrigeration, and in these conditions this active packaging is also efficient as antifungal.

Furthermore, the active films that caused a total inhibition during the experiment still showed antifungal activity after three months, confirming the durability of the antifungal action, as it was previously demonstrated by a PET (Polyethylene Terephthalate) antifungal packaging containing cinnamon (Manso et al., 2013). In the same line, other authors have found the same antimicrobial activity versus bacteria after 1 year of storage of the packaging materials (Suppakul, Sonneveld, Bigger, & Miltz, 2011).

Besides, PP 2% CIN demonstrated a clear reduction of an important mycotoxin, aflatoxin B1, whereas PP 4% CIN reduced completely the mycotoxin content (unpublished results).

The good results obtained are due to the homogeneous distribution of the volatile compounds of the EO over the whole Petri dish, reducing the time needed to reach the equilibrium (Becerril et al., 2007). The authors Gutiérrez, Batlle, Sánchez, and Nerín (2010) demonstrated in a previous work, that the antimicrobial action of the active packaging was more related to the kinetic release of the compounds from the packaging than to the final active concentration in the agar. However, the release of the active compounds depends on the porosity and thickness of the substrate as well as on the formula used to incorporate the active agent on the polymer, explaining the strong differences found in antimicrobial packaging approaches with paper and plastic films (Manso et al., 2013; Rodríguez et al., 2008). This means that each packaging material require a previous optimization of the concentration of the active agent to get the maximum antifungal activity.

Finally, the sensory perception of the same PP cinnamon packaging used in this work was previously analyzed, resulting compatible with the most frequent aromas used in food (Gutiérrez, Escudero, Batlle, & Nerín, 2009). Of course if the concentration of cinnamon increases the sensory perception could be compromised.

### 4. Conclusions

The present work demonstrates the potential of the cinnamon essential oil as an antifungal agent against several strains, needing lower inhibitory and fungicidal concentrations than the rest of the compounds tested.

As expected, the variations in external conditions as pH and temperature incubation may modify the growth rate of the molds evaluated, showing significant differences in the colony diameter in some cases (*P < 0.05*). However, the Minimal Inhibitory Concentrations and Minimal Fungicidal Concentrations of the six compounds tested (cinnamon, cinnamaldehyde, oregano, carvacrol, clove and eugenol) determined by broth dilution method, maintained the same values in all the conditions of incubation showing a non-influence of pH and temperature. This performance is very important, as means that the active material can work under refrigeration conditions and in any type of food in the wide range of pH studied.

The antifungal packaging consisting of PP (Polypropylene) films containing cinnamon essential oil resulted in a high-performance antifungal system. The same sub-inhibitory concentration (PP 2% CIN) was established for the four strains incubated at different pH and temperature. In general, a retard in mold growth and sporulation was achieved under this concentration. A clear slowdown was obtained for the *Penicillium* controls (PP 0%) and active (PP 2% CIN) incubated at 6 °C compared to those at 25 °C, playing this fact an important role on the antifungal protection. Slight differences between pH 5.6 and pH 3 were also obtained and discussed. Higher amount of cinnamon incorporated into the PP films (PP 4% CIN and PP 6% CIN) produced a total inhibition in all cases.

Besides the antifungal properties, in a recent work the same active materials have demonstrated an antimycotoxigenic action caused by the cinnamon essential oil in vapor phase. Hence, the fungicidal concentrations (PP 4% and 6% CIN) caused a total mold reduction accompanied by a complete inhibition of the aflatoxin B1, meanwhile the sub-inhibitory concentration (PP 2% CIN) resulted in a reduction of both mold growth and mycotoxin content (Manso, Pezo, Gómez-Lus, & Nerín, 2014).

To sum up, the results propose an efficient antifungal packaging containing cinnamon essential oil. In the case of total inhibition, the potent antifungal activity was maintained for at least 3 months. Besides, data presented confirmed that the volatile compounds of the cinnamon essential oil can be released from the packaging independently on the temperature incubation. This fact, together with the long-term properties achieved, guaranty an efficient antifungal protection of packaged food in all cases, opening the possibilities of these packaging materials towards a wide range of food products.

### Acknowledgments

Financial support was generously provided by the Spanish Ministry of Science and Innovation (AGL2008-04363 and AGL2012-37886) and by Fondo Europeo de Desarrollo Regional (FEDER) funds.

### Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2014.06.014.

### References


