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Diminution of aflatoxin B1 production caused by an active packaging containing cinnamon essential oil

S. Manso [a], D. Pezo [a], R. Gómez-Lus [b], C. Nerín [a,*]

[a] Universidad de Zaragoza, Departamento de Química Analítica, Instituto de Investigación en Ingeniería de Aragón (I3A), María de Luna 3, 50018 Zaragoza, Spain

[b] Universidad de Zaragoza, Departamento de Microbiología Medicina Preventiva y Salud Pública, Instituto de Investigación de Ciencias Ambientales (IUCA), Domingo Miral S/N, 50009 Zaragoza, Spain

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ABSTRACT

The antifungal and antimycotoxigenic action of an active package containing cinnamon essential oil have been evaluated against the mold *Aspergillus flavus* on the aflatoxin B1 production. Two independent experiments were carried out, the first one with cinnamon on a paper diffusion disc placed in vapor phase and the second one with an active PP (Polypropylene) films containing the essential oil. The culture media, exposure time, closure of the Petri dish and cinnamon concentration were evaluated. The first experiment revealed an important reduction on mycotoxin, even when the mold grew, and the action remained for 15 days. The second experiment highlighted the importance of cinnamon concentration on the antimycotoxigenic action, achieving a strong reduction with the sub-inhibitory concentration (2% of cinnamon) and a complete reduction with fungicidal concentration (4% and 6% cinnamon). The UPLC system coupled to a fluorescence detector was optimized for analysis of aflatoxin B1.

1. Introduction

Essential oils are natural substances commonly present in plants, whose antimicrobial activity has been widely reported since the last decade (Bakkali, Averbeck, Averbeck, & Waomar, 2008; Burt, 2004; Kalemba & Kunicka, 2003; Seow, Yeo, Chung, & Yuk, 2014). They are categorized as GRAS (Generally Recognized as Safe) by the US Food and Drug Administration. Besides, the volatile compounds released from these substances confer them an additional property as protective agents, which could be included into a food package. Several applications can be found in the literature (Gutiérrez, Sánchez, Batlle, & Nerín, 2009; Matan et al., 2006; Rodriguez-Lafuente, Nerin, & Batlle, 2010; Suhr & Nielsen, 2005).

The positive results achieved in the active packaging field, has roused the interest of considering different antimicrobials and materials, like whey protein films (Fernández-Pan, Mendoza, & Maté, 2013), wheat gluten papers (Mascheroni, Guillard, Gastaldi, Gontard, & Chalier, 2011), cellulose films (Sanla-Ead, Jangchud, Chonhenchob, & Suppakul, 2012; Souza, Goto, Mainardi, Coelho, & Tadini, 2013) and gliadin whey protein films (Pau Balaguer, Lopez-Carballo, Catala, Gavara, & Hernandez-Munoz, 2013). However, polymeric antimicrobial materials are still in use (Hanusová et al., 2010; Junqueira-Gonçalves, Alarcon, & Niranjan, 2013; Nostro et al., 2012), and despite different testing methods are employed (Appendini & Hotchkiss, 2002), all of them are focused on protecting the product and avoiding microbial contamination.

Nowadays, concern about mold contamination on the food product has increased, mainly due to two reasons. The first one is the possible production of secondary metabolites, such as mycotoxins, which may be produced by some species of molds in certain conditions. The second one, the great economic loss as result of the decay and rottenness of some perishable products such as fruits, vegetables, bread and cereals. Mycotoxins are formed during the growth of molds on foods. Because of their physical and chemical resistance, a final product could contain mycotoxin if any moldy material is employed during the process (Filtenborg, Frisvad, & Thrane, 1996). Among them, aflatoxins are one of the most relevant families, being aflatoxin B1 classified as group 1-agent by IARC (International Agency for Research on Cancer), with significantly increased risk for hepatocellular carcinoma (HCC) in individuals exposed. Aflatoxin B1 can be produced by the following species: *Aspergillus flavus, Aspergillus arachidicola, Aspergillus parasiticus, Aspergillus nomius and Aspergillus minisclerotigenes* (Samson,

* Corresponding author. Tel.: +34 976 761873.
E-mail address: cnerin@unizar.es (C. Nerín).
URL: http://i3a.unizar.es/grupo/guia-17

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Houbraken, Thrane, Frisvad, & Andersen, 2010). Despite the temperate climate is more favorable for *Aspergillus* occurrence, nowadays, globalization involves an international trade, where any product can be found out of the proper climate, and thus, increasing the possibility of finding contaminated foods everywhere. In the last years essentials oils have been studied not only as mold growth inhibitors but also as antimycotoxigenic action with a large number of examples (El-Nagerabi, Al-Bahry, Elshafie, & AlHilali, 2012; Ferreira et al., 2013; Passone, Girardi, Ferrand, & Etcheverry, 2012; Rasooli et al., 2008; Rasooli & Owlia, 2005). Hence, a recent review compiles an exhaustive research concerning the antifungal and antimycotoxigenic action of these natural compounds (Cabral, Pinto, & Patriarca, 2013). However, only one example was found concerning the antimycotoxigenic action by the vapor phase of essential oils (Passone, Girardi, & Etcheverry, 2013).

The present work shows an antifungal and anti-mycotoxin packaging able to act in vapor phase against the mold *Aspergillus flavus* and therefore, the aflatoxin B1 production. The application of vapor phase permits to avoid a direct contact with the food product. Hence, the principal aim of this system is to reduce or inhibit completely the aflatoxin B1 production, using active packaging containing cinnamon at different concentration.

The experimental plan was divided into two different experiments, the first one carried out by a paper diffusion disc containing cinnamon essential oil in vapor phase and the second one with active polypropylene (PP) films containing cinnamon essential oil, acting also in vapor phase. Previously the sensory perception was studied, confirming the compatibility of the same PP films used in this work with most of food aromas (Gutiérrez et al., 2009). Besides, it is well known that accumulation of aflatoxins depends on the substrate, being some of them extremely favorable for the growth of aflatoxigenic fungi and therefore, the formation of aflatoxins (Rocha, Freire, Maia, Guedes, & Rondina, 2014). Because of that, five different culture media were employed in the two experiments, to study the antimycotoxigenic action of the vapor phase of cinnamon on different composition substrates. Moreover, the influence of exposure time of cinnamon essential and the seal of the Petri dish were evaluated as additional variables. Finally, the antifungal and antimycotoxigenic actions of both sub-inhibitory and fungicidal cinnamon concentrations in the PP (Polypropylene) active films were evaluated.

Aflatoxin B1 quantification was carried out by UPLC^R system, coupled to a fluorescence detector, without needing a previous derivatization due to the high sensitivity of this analytical technique. Besides, the chromatographic method was also optimized, achieving a specific separation of the mycotoxin from matrix effects.

2. Materials and methods

2.1. Microbial culture

A. flavus (A. flavus) CECT 2687 from the University of Valencia (Spain) was used as aflatoxin B1 (AFB1) producer strain. As solid media, Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Czapek Dox-Agar (CZP) and Agar–Agar were provided by Scharlab *(Scharlau)*. Yeast Extract Agar (YES) and Czapek Yeast Autolisiate Agar (CYA) were constituted following Samson et al. (2010) indications, with some variations for CYA: 50 g CZAPEK agar *(Scharlau)*, 5 g Yeast Extract *(Scharlau)*, 0.01 g $ZnSO_4$ *(Scharlau)*, 0.005 g $CuSO_4$ *(Scharlau)*, distilled water (1 L). Physiological solution (NaCl 0.9%) *(Panreac)* was employed as diluent.

2.2. Cinnamon essential oil (CIN EO)

Cinnamon essential oil (*cinnamomum zeylanicum*, CAS 8015-91-6) from the bark was used as active agent. It was fortified and supplied by Argolide (Spain). Usually CIN EO from the leaves contains eugenol and trans-cinnamaldheyde as major components. However, CIN fortified used in this work, presents trans-cinnamaldehyde as major compound (900 mg/g) and eugenol as minor compound (3.62 mg/g). Other components at trace level concentration were also present.

2.3. Reagents and aflatoxin extraction

Dichloromethane (DCM) (GC 99.8%, *Scharlau*), ethyl acetate (HPLC, 99.7% *Sygma-Aldrich*), methanol (MeOH) (99.9% LC-MS Fluka, *Sigma–Aldrich*), formic acid (98–100% *Scharlau*), acetonitrile (ACN) *(Scharlau)*, orthofosforic acid 85% (*PANREAC*, Barcelona – España) and water (milli-Q water 18.2 Ωm), were used as solvents. Aflatoxin B1 (AFB1) was provided as standard by Sigma–Aldrich (A6636, CAS 1162-65-8) in 10 mg powder. It was kept at 2–8 °C until use and reconstituted in methanol (99.9% LC-MS Fluka, *Sigma–Aldrich*) for further dilutions. For extraction process, a 6 mm core borer *(Scharlau)*, amber vials and PTFE 0.22 μm filter *(Scharlau)* (13 mm diameter) were employed before the UPLC analysis. Flow of N_2 (ALPHAGAZ1, 99,999% Global Purity, *Air Liquide* Zaragoza-Spain) was used to evaporate the extraction solvents.

2.4. Antifungal materials

For the antifungal characterization of the CIN over the mold *A. flavus*, 9 mm Whatman paper disc *(Scharlau)* and polypropylene (PP) films were used in two independent experiments. Active PP with 2%, 4% and 6% CIN (PP 2% CIN, PP 4% CIN, PP 6% CIN) and blank PP without CIN (PP 0%) were supplied by Artibal (Sabiñánigo, Spain), consisting of 30 μm thick layer coated with an organic base formula containing the essential oil at different concentrations, according to the EU patent (Garcés & Nerín, 2004). The grammage of the coating was 2.5 g/m^2. Besides, parafilm *(Scharlau)* and a digital caliper *(Comecta S.A.)* to measure the inhibition area in the first experiment were employed.

2.5. Chromatographic conditions

An acquity UPLC^R binary solvent manager system, coupled to a W474 fluorescence detector was used (*Waters*, Palo Alto, CA, USA). Excitation at 362 nm and emission at 425 nm were recorded. In order to better adapt the variability of the samples to the AFB1 determination, two different methods ("A" and "B") were applied. A C18 Phenomenex HPLC column (250 × 2 mm, Luna 3 μm) and a C18 UPLC BEH Acquity column (100 × 2.1 mm, 1.7 μm) were used for the first and second method respectively. The same precolumn (Van Guard™ BEH C18, 1.7 μm) was maintained in both cases. Detection Limit (LOD) and Quantification Limit (LOQ) were calculated for both of them.

For the method A, methanol was used as eluant A and milli-Q water as eluant B. The mobile phases consisted of (15:85) (MeOH:milli-Q water) both with 0.1% orthophosphoric acid. The flow rate was 0.3 mL/min and the gradient system consisted of: 0–0.67 min, 15% solvent A; 0.67–4 min, linear gradient from 15 to 35% A; 4–5 min, linear gradient from 35 to 90% A; 5–5.1 min, linear gradient from 90 to 15% A; 5.01–8 min, 15% A. Post-run time was 1 min. The column temperature was set to 30 °C. Injection volume was 10 μL.

Method B was developed in isocratic system at 0.3 mL/min with a combination of three different solvents as mobile phase (MeOH:ACN:milli-Q water) (45:15:40). The same column temperature (30 °C) and injection volume (10 μL) were employed.

In both experiments, samples were obtained in a wide range of AFB1 concentration. The concentration of AFB1 in the samples was calculated from the peak areas using the standard AFB1calibration

curve. With the aim of reducing at minimum level the possible error in the quantification, different lineal ranges from the calibration curve were applied according to the AFB1 concentration of the sample.

2.6. Diffusion disc experiment

2.6.1. Experimental plan

Fungal conidia were harvested after previous inoculation on PDA for 7 days at 25 °C, and transferred into a test tube with physiological saline solution (NaCl 0.9%). A final concentration of 10^6 sp/mL (spores/mL) was reached and confirmed by Neubauer chamber. From this suspension, 100 μL were seeded with a sterile Drigalsky spatel onto Petri dishes containing 15 mL of the corresponding culture media.

Samples were performed according to the experimental plan designed. Three different variables were considered: exposure time, closure of the Petri dish and culture media used. Within the closure of the dish, the influence of the cinnamon essential oil applied to active samples was also evaluated. For active plates, the inhibition area was measured everyday with a digital caliper. The experiment was carried out by triplicate.

2.6.2. Influence of the CIN exposure time

The whole experiment was performed four times as above described, forming four identical independent set of samples, but extracting the AFB1 at 4 days, 7 days, 10 days and 15 days respectively.

2.6.3. Influence of the closure of the Petri dish

Once the mold was inoculated in all plates, three different types of samples were performed. Disc active samples were performed by placing a 9 mm Whatman disc containing 10 uL of CIN in vapor phase, i.e. without any contact with the inoculated mold. After that, these active plates were closed with parafilm to avoid the rapid loss of the CIN as well as to get a better reproducibility within the replicates. As has been demonstrated, samples containing essential oils must be analyzed under sealed conditions to prevent the complete loss of essential oil (Inouye, Tsuruoka, Uchida, & Yamaguchi, 2001).

To evaluate the influence of the parafilm in the aflatoxin production, two different samples were performed: “control 1” and “control 2” samples. On one hand “control 1” samples are referred to those inoculated plates without cinnamon and closed with the normal lid without parafilm. On the other hand “control 2” samples were named to the inoculated plates without cinnamon, closing the Petri dish with the lid and finally, sealing the system with the parafilm. All plates were incubated at 25 °C.

2.6.4. Influence of the culture media

Five different culture media were used, that is, YES, CYA, MEA, PDA, and CZP. At the moment of the aflatoxin extraction, an additional sample was summed up, named as POOL and constituted by a homogeneous mixture of the five mentioned media. Each Petri dish was performed by triplicate.

2.7. PP experiment

2.7.1. Experimental plan

Plates were inoculated as in the previous experiment. Three variables were also evaluated: the closure of the Petri dish, the CIN EO concentration in the active PP films and the culture media used. The experiment was also carried out by triplicate.

2.7.2. Influence of the closure of the Petri dish

In this case, active PP films with 2%, 4% and 6% CIN were evaluated, adding also two different controls. “Control 1” was performed as described in the previous experiment. A PP film control named as “PP 0%” was added by closing the Petri dishes with blank PP without cinnamon. In all cases, films were attached to the Petri dish using a nylon cable tie (López, Sánchez, Batlle, & Nerín, 2007). All plates were incubated at 25 °C with agar at the bottom. Growth in the active plates (PP 2% CIN, PP 4% CIN and PP 6% CIN) was checked and quantified everyday.

2.7.3. Influence of CIN concentration

The PP experiment was performed at different CIN concentrations. Active films with PP 2% CIN as sub-inhibitory concentration and PP 4% CIN and PP 6% CIN as fungicidal concentrations were evaluated. For these last concentrations, only YES and CYA media were evaluated, having also 3 replicates for each medium and each CIN concentration.

2.8. Aflatoxin B1

2.8.1. Collecting sample from the agar

Plugs were taken with a 6 mm core borer on the agar surface, placed into a first amber vial and weighted. In the case of controls (control 1, control 2 and PP 0%), plugs were taken randomly from the whole surface of the dish due to the homogenous growth. However, a different nuance was applied for the active plates. In the disc diffusion experiment plugs in the active plates were taken on the retard area. However, in the PP experiment the mold under PP 2% CIN did not experiment the same growth degree in the three replicates of each culture media. Because of that, two different areas were defined in each PP 2% CIN plate, the “growth area” (with mold growth) and the “clean area” (agar without visible mold). An extraction scale from 0 to 8 points was built in order to correlate the AFB1 extracted with the fungal growth obtained. The scale was built as follows: Eight plugs were taken when the visual growth showed the total plate covered by the mold (100% of growth area), combining the number of plugs from both areas in relation to the fungal development, in such a way that the total number of plugs from each plate was always 8. Hence, 7 plugs from the growth area and 1 plug from the clean area were taken when the mold occupied more than 75% of the plate and so on; 6 plugs, 5 plugs, 4 plugs, 3 plugs, 2 plugs and 1 plug from the growth area were taken when the mold covered 75%, more than 50%, 50%, more than 25%, 25% and less than 25% respectively. In the case of total mold inhibition, the 8 plugs were taken from the clean area.

2.8.2. Aflatoxin B1 extraction

Once plugs were placed into the first amber vial, the next steps were the same for both experiments, following the procedure (O’Brien et al., 2006) with slights differences. First, 1.8 mL of solvent mixture ((Ethyl Acetate: DCM: MeOH) *(3:2:1)* + 1% Formic acid) were added to each vial, and the vial was left closed during the night at room temperature and kept away from light. Next day, the vials were ultrasonicated for 45 min (without heat), and for each sample, the extracted fraction was transferred into a second amber vial, and the solvent evaporated under N_2 flow at 30 °C to dryness. Then, the residue was re-dissolved with 1.2 mL of MeOH, ultrasonicated for 15 min, filtered by 0.22 μm PTFE and placed into 2 mL amber vials for chromatographic analysis.

3. Results and discussion

3.1. Chromatographic separation and analytical characteristics

The disc experiment was the first step of the experimental plan. Thus, the samples were prepared and analyzed by method A. However, in the second experiment an interference coming from

the culture media was observed at similar retention time as that of AFB1 under fungicidal concentrations (PP 4% CIN and PP 6% CIN). In the first case (diffusion disc experiment) samples were correctly analyzed and quantified by method A, as none influence of the unknown peak (near to baseline noise) was observed compared to the high AFB1 concentration in the samples.

However, fungicidal concentrations in PP experiment revealed the need of an improved method (B) for those samples with a very low AFB1 content. For this reason, plugs from CZP Petri dishes without inoculation (blank samples) were spiked before UPLC injection, with three concentrations of the AFB1 standard: 25, 50 and 250 ng/g. Moreover, two additional standard AFB1 concentrations (50 and 500 ng/g) were added without CZP culture media. Three different vials were prepared and processed following the extraction step mentioned for the rest of samples of this experiment (see Section 2.8.2). However, 0.4 mL instead of 1.2 mL of MeOH were used to dissolve the final sample, in order to enhance the signal and facilitate the optimization of the method. A complete separation of the mycotoxin was achieved at 4.6 min, finding the interference between 1.8 and 2.8 min. The total separation was also confirmed, with a good correlation ($R^2 = 0.9987$) between the areas of AFB1 peak extracted from the five samples. Because PP experiment was designed in a second step, this method B was applied to all samples of the PP experiment.

As Detection Limit (LOD) and Quantification Limit (LOQ), 0.85 ng/g and 2.84 ng/g were achieved for method A, and 1.03 ng/g and 3.45 ng/g respectively for method B. Also, a high accuracy and linearity (0.999 in almost all samples) was achieved.

3.2. Diffusion disc experiment

The vapor phase of CIN creates three different zones or areas in the same plate (Manso, Cacho-Nerin, Becerril, & Nerin, 2013). Once the vapor phase of the essential oil is absorbed by the agar, a progressive diffusion takes place, and the cinnamon concentration is reduced from the central point towards the periphery of the dish. The inhibition area is situated under the active disc and presents the highest CIN concentration absorbed by the culture media that prevents the mold to develop. Then, the essential oil is diffused creating the retard area, that corresponds to the sub-inhibitory concentration, where the mold is able to grow but with a notable decrease of sporulation. Finally, in the outline of the dish, the CIN concentration is very low, permitting the mold to develop in an apparent normal way.

The principal aim of this work was to study the production or inhibition of AFB1 on the mold exposed to the vapor phase of the CIN. For this reason, samples were collected from the retard area, where the mold was developed and a strong influence of the essential oil was evidenced.

The results of AFB1 concentrations in the samples from the disc diffusion experiment are shown in Suplementary Table 1. The concentration (μg/g) is expressed as average of three independent replicates. A low standard deviation was obtained between replicates, indicating the good reproducibility achieved. In order to clarify the data, concentrations from control 2 and active samples have been converted to % of AFB1 reduction, both referred to control 1 (Fig. 1).

3.2.1. Influence of the CIN and the closure of the Petri dish

Control 1 samples contained in all cases the highest mycotoxin concentration, in the range of 11.34–39.63 μg/g. This was expected, considering that in these samples the mold is able to grow under favorable conditions. Due to the use of parafilm in the active samples, control 2 and active samples were compared in order to reveal the effect of the CIN concentration on AFB1 production. For this purpose, statistical analyses were carried out by SPSS software (SPSS 15.0 for Windows). For each individual set (4 days, 7 days, 10 days and 15 days), an independent analysis was made for each culture media. One way ANOVA (Analysis Of Variance) was used to determine the statistical differences between control 2 and active samples. First, depending on the variance's homogeneity (Levene test), Anova or Welch test were applied to detect possible differences between AFB1 concentrations in control 2 and active samples. After that, the origin of the differences was identified by Post-Hoc multiple comparisons tests (Tukey or T3 Dunnet, depending on the previous Levene conclusion). Significant differences were determined at $P < 0.05$ level.

As can be seen in Fig. 1, AFB1 concentration was significantly reduced ($P < 0.05$) in all the active samples containing CIN, with only three exceptions. On one hand, at 4 days YES and CYA active samples produced 0.68 μg/g and 1.81 μg/g of AFB1 respectively, and 0.71 μg/g and 2 μg/g in the control 2 samples. Moreover, at 7 days YES experimented more reduction in control 2 (0.36 μg/g) than in the active samples (2.09 μg/g). To explain this finding control 1 (control without parafilm) and control 2 (control with parafilm) were also compared applying the same statistical methods as above mentioned. A significant difference was obtained in all cases ($P < 0.05$), that is, in the four independent sets and for all media. Besides, microbiological changes were also evident when the control plate was closed with parafilm (control 2). In general, a decrease of sporulation and a slight retard in growth were observed comparing those to the control 1 samples. This fact may be explained by a possible reduction of oxygen in the plate when sealing with parafilm.

3.2.2. Influence of culture media and CIN exposure time

Despite the parafilm influenced the growth in all the culture media, different intensity was observed between them, being YES and CYA the most affected substrates. Among all the media tested, YES is the most enriched medium, provoking a rapid and strong growth on *A. flavus* and a high and green sporulation all over the whole plate from the fourth day. This explains why control 1 samples made in YES media contained the highest level of AFB1 in most of the cases, with maximum of 39.63 μg/g at 15 days. However, when parafilm is used to close the Petri dish (control 2 samples) the mold grows heterogeneously over the plate, resulting in a white mass of mycelia with small areas of irregular sporulation. The size of those sporulation areas became wider in the last sets of 10 days and 15 days of incubation, which explains why from the 10th day, a significant reduction ($P < 0.05$) was obtained in YES active samples comparing to YES control 2 samples. CYA experimented similar behavior than YES, but the parafilm effect was not as strong as in YES. When closing the CYA plates with parafilm, minor changes on mold growth was observed compared to YES culture medium. Besides, as mentioned above, CYA experimented a significant reduction ($P < 0.05$) in all cases except for the first set of 4 days.

Concerning PDA and MEA, only a decrease of sporulation with minor color intensity was observed, evidencing a lower effect of parafilm on mold growth. Microbiological observations revealed a concordance between growth affectation and AFB1 concentration. The fact that parafilm itself reduced the sporulation suggests that the headspace of the Petri dish could be affected, as parafilm closure is more efficient seal than only the Petri dish lid. The results indicate that parafilm caused more diminution on AFB1 concentration compared to the active samples in the three cases above mentioned.

However, the comparisons between active and control 2 samples demonstrated a significant reduction on AFB1 content,

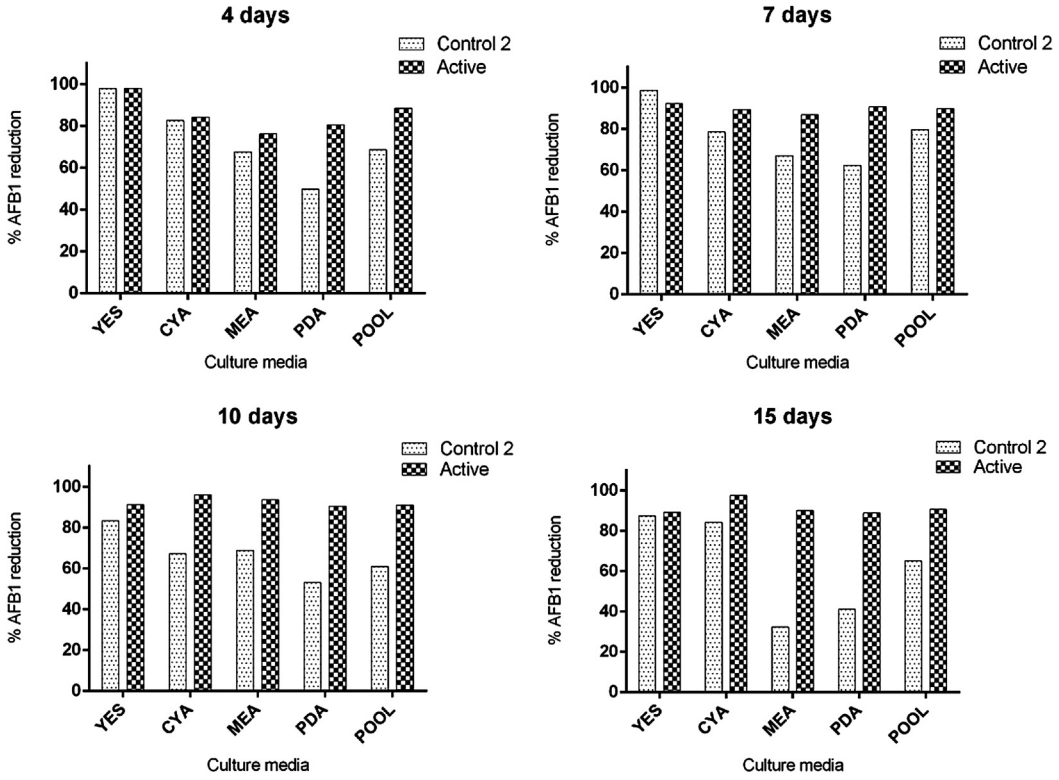

Fig. 1. Disc experiment. Percentage of reduction of AFB1 concentration in the "control 2" samples and "active" samples in relation to the "control 1" samples. The antimycotoxigenic action is manifest after 4 days, 7 days, 10 days and 15 days of incubation time.

highlighting the positive action achieved by the volatile compounds released from CIN.

Among the media tested in this work, CZP appeared as the least appropriate for AFB1 production, highlighting the importance of the substrate composition on AFB1 production. All samples extracted from CZP were prepared and analyzed, but only a few control samples gave a low signal, being the majority below the LOQ. For this reason, data from CZP have been excluded in this experiment and are not presented in Suplementary Table 2 and Fig. 1. Finally, POOL samples are the result of a homogeneous extraction from the five culture media, which explains the absence of microbiological observations in those cases.

3.3. PP experiment

The results of AFB1 concentrations contained in the samples from the PP experiment are shown in Suplementary Table 2. The aflatoxin concentration (μg/g) is expressed as average of three independent replicates. A low standard deviation was obtained between replicates, indicating the good reproducibility achieved. As in the first experiment, AFB1 concentrations have been converted to percentage of AFB1 reduction, referring as in the first experiment, the concentrations from PP 0% and PP 2 %–6% CIN to the control 1 samples (Fig. 2). In this case, the increment of the number of plugs (from 20 plugs to 24 plugs) was enough to obtain low but quantifiable concentrations (>LOQ) for all CZP control samples.

3.3.1. Influence of the closure of the Petri dish

The highest AFB1 concentrations corresponded again to control 1 samples. Similar statistical analyses as in the disc experiment were performed. One way-Anova was done for each culture media between the AFB1 concentrations from three types of samples: control 1, PP 0% and PP 2% CIN.

Control blank film without CIN (PP 0%) also caused a significant diminution ($P < 0.05$) in AFB1 compared to control 1 sample. However, this fact was not as strong as the parafilm effect, that is, the mycotoxin was not reduced in the same degree. Moreover, in this case the macroscopic growth in PP 0% and control 1 sample was very similar. The mold experimented the same lag phase and the sporulation achieved the whole surface of the Petri dish at 4th day of incubation time in both cases. Despite a slight minor intensity in the sporulation was observed in PP 0% plates, no differences on color were perceived. The microbiological observations of controls

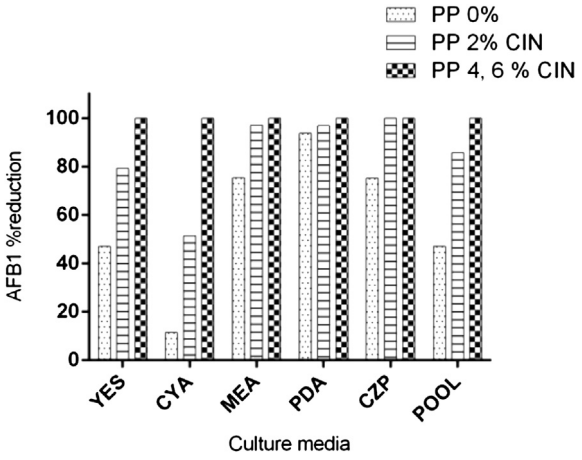

Fig. 2. PP (Polypropylene) experiment. Bars indicate the percentage of reduction of AFB1 concentration in PP 0% and active samples (PP 2% CIN, PP 4% CIN and PP 6% CIN) in relation to the control 1 samples. A clear reduction was obtained in the case of the PP 2% CIN meanwhile the PP 4% CIN and PP 6% CIN reached the complete reduction of the mycotoxin (100%).

were equal for all media tested. However, in contrast to the first experiment, in this case PDA and MEA experimented more reduction in AFB1 content when PP 0% was applied (Fig. 2). The following AFB1 concentrations were found in control 1 samples: 38.24 μg/g (YES), 13.29 μg/g (CYA), 24.96 μg/g (MEA), 17.72 μg/g (PDA), 0.18 μg/g (CZP) and 19.76 μg/g (POOL). In the case of PP 0% samples, AFB1 concentrations obtained were: 20.26 μg/g, 11.78 μg/g (CYA), 6.13 μg/g (MEA), 1.12 μg/g (PDA), 0.04 μg/g (CZP) and 10.47 μg/g (POOL). Hence, similarly to parafilm, the closure by the blank PP film seems to alter the atmosphere in the plate, affecting the mold sporulation intensity and the further mycotoxin production, but in a much lower degree than parafilm did.

3.3.2. *Influence of the CIN concentration*

On one hand, the mold growth was clearly affected by PP 2% CIN compared to the control samples, that is why it was referred as "sub-inhibitory concentration". As explained in Section 2.8.1, a growth scale was established in order to extract the sample in relation to the growth observed. Considering the three replicates of each culture media, PP 2% CIN achieved a 41.67% of mold reduction in YES, while 66.67% and 95.83% diminution was obtained for CYA and CZP. Finally, MEA and PDA suffered the same growth degree and 79.17% of fungal reduction was obtained in both cases.

On the other hand, the antimycotoxigenic action of the PP 2% CIN was evaluated following the same statistical analyses as above and a significant reduction of AFB1 accumulation was observed in all cases ($P < 0.05$), comparing PP 2% CIN and PP 0% samples. However, as observed in Fig. 2, a different degree was obtained in the different media. As explained before, in this experiment MEA and PDA were more influenced by the closure with blank film (PP 0%) than the rest of culture media. This fact explains why in these cases, despite the concentrations of AFB1 from PP 2% CIN were significantly reduced, the differences in the bars between PP 0% and PP 2% CIN are not so evident for MEA and PDA compared to the rest culture media. AFB1 concentrations obtained in the PP 2% CIN samples were: 7.9 μg/g (YES), 6.46 μg/g (CYA), 0.73 μg/g (MEA), 0.56 μg/g (PDA) and 2.84 μg/g (POOL). In the case of CZP, AFB1 concentration was not detected (<LOD).

In addition to the sub-inhibitory concentration, active PP with 4% and 6% CIN were tested on YES and CYA culture media. Due to the inhibition of mold growth, these concentrations were considered as MIC (Minimal Inhibitory Concentration). However, at the moment of the extraction, 6 supplementary plugs were taken from active plates with PP 4% and 6% CIN and placed onto new fresh PDA plates. No growth was observed in any case after 7 days of incubation at 25 °C, confirming the fungicidal action of these concentrations. For this reason, PP 4% and PP 6% CIN were referred as "fungicidal concentrations". Due to the absence of growth mentioned, all plugs from these concentrations were extracted from the entire plate, considering the whole Petri dish as clear area. Analytical data revealed that AFB1 concentrations for PP 4% CIN and PP 6% CIN were below LOD, hence reducing the 100% of mycotoxin content.

Finally, differences on visual growth development between all types of samples can be seen in Supplementary Fig. 3. The minor changes between controls (control 1 and PP 0%), the clear reduction of mold growth caused by sub-inhibitory concentration (PP 2% CIN) and total inhibition caused by the fungicidal concentrations (PP 4% CIN and PP 6% CIN) are shown. YES and CYA have been chosen as visual example, due to the fact that fungicidal concentrations were applied to these two culture media. Images corresponding to PP 6% CIN are not presented, because the same total inhibition was obtained for PP 4% CIN and PP 6% CIN as explained above.

Passone et al. (2013) analyzed the antifungal and antimycotoxigenic activity of the vapor phase of *Peumus boldus* essential oil on *A. flavus*. They obtained a clearly retard in the lag phase at dose higher than 1000 μL/L, as well as a total inhibition of AFB1 content (Passone et al., 2013). In another work (López-Malo, Barreto-Valdivieso, Palou, & Martín, 2007) cinnamon extract seemed to act more as fungistatic rather than fungicidal agent. This observation is also in agreement with our results obtained for the sub-inhibitory PP 2% CIN, because despite it presented a clear diminution on AFB1 content, the mold is able to grow beyond 4 days. The correlation between antifungal action and essential oil concentration has been also pointed out by other authors (Bluma, Landa, & Etcheverry, 2009; Matan et al., 2006; Rodríguez, Nerín, & Batlle, 2008; Velázquez-Nuñez, Avila-Sosa, Palou, & López-Malo, 2013). Also, in a previous work (Manso et al., 2013), active PET (Polyethylene Terephthalate) films with CIN in the range of 2 %–8% concentration were evaluated under three different *A. flavus* suspensions (10^6, 10^5 and 10^4 sp/mL). As well as in the present experiment, PET 2% CIN was determined as sub-inhibitory concentration at 10^6 sp/mL of *A. flavus* suspension, while antifungal effect was found between PET 4 %-8% CIN. However, an important outcome from that study, where PET 2% CIN showed complete inhibition at 10^4 sp/mL, revealed not only the importance of the CIN concentration on the antifungal action, but also the initial fungal suspension. Hence, extrapolating the present results to this mentioned situation, we could positively achieve a 100% reduction on AFB1 accumulation from 2% CIN at low fungal concentration. This dependence on mold suspension has also been concluded by other researchers (Rasooli, Rezaei, & Allameh, 2006), and more specifically, the increased on conidia production has been related to the sequence steps in the biosynthesis of AFB1(Wilkinson, Ramaswamy, Sim, & Keller, 2004). Because of that, the first step for an efficient and durable antifungal effect should be to avoid the spore germination, which prevents the posterior mycelium development, decreasing the risk of possible conidia formation and hence, the consequent AFB1 production.

3.4. *Mechanism of action: antifungal and antioxidant activity of CIN vapor phase on the aflatoxin production*

It seems probable that damage caused by volatile compounds from *cinnamon* essential oil (cinnamaldehyde and probably eugenol) may affect the enzymatic mold activity. Cinnamaldehyde has demonstrated to inhibit the elastase and keratinase activity, two extracellular enzymes associated with their virulence factor, in *Aspergillus fumigatus* and *Trichophyton rubrum* (Khan & Ahmad, 2011). Most of the reactions in aflatoxin biosynthesis are mediated by oxygenases enzymes (Dutton, 1988). The antioxidant properties of essential oils lead to an antiaflatoxigenic action (Patil, Nimbalkar, Jadhav, Dawkar, & Govindwar, 2010). Hence, blocking the enzymatic activity of a biosynthetic enzyme constitutes one of the three possible levels to inhibit AFB1 biosynthesis,as secondary metabolism is sensitive to stress, due to the correlation between oxidative stress and cellular development and differentiation (Alpsoy, 2010). Lipid peroxidation, antioxidant enzyme activity and ROS (reactive oxygen species) produced during the oxidative stress are also related to aflatoxin formation (Holmes, Boston, & Payne, 2008). Inhibition of LOX (Lypoxygenase), the enzyme responsible for lipid peroxidation, in peach fruits, caused by an active packaging containing CIN, has been demonstrated (Montero-Prado, Rodriguez-Lafuente, & Nerin, 2011). Besides, Alpsoy (2010) pointed out that essential oils can decrease the pathogenic effect of aflatoxins by two different ways: reducing the formation of DNA binding, and reacting with ROS that aflatoxins produce. The radical scavenging activity of cinnamaldehyde has been proved (Pezo, Salafranca, & Nerin, 2008). Consequently, diminution of AFB1 reached by both antifungal and antioxidants properties highlights

the option to include cinnamon essential oil in the packaging material as a protective agent against fungal development and mycotoxin production, thus contributing to the food safety.

4. Conclusions

The results obtained in the present work demonstrated that the vapor phase of CIN essential oil achieved a significant reduction ($P < 0.05$) of AFB1 production. The same conclusion was confirmed in all experiments (disc samples and PP films), despite the different methodology applied. CIN concentration has been pointed out to play an important role on the antifungal and antimycotoxigenic actions, showing a significant reduction of AFB1 production at PP 2% CIN and total inhibition at PP 4% CIN and PP 6% CIN. As we have seen, these two later active concentrations confirmed the fungicidal behavior, so the complete AFB1 reduction is expected to be constant. This indicates the impossibility of spores inoculated to grow, and therefore, to produce the secondary metabolite above the LOD.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2014.04.031

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