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New Cinnamon-Based Active Paper Packaging against *Rhizopusstolonifer* Food Spoilage

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A new active paper package based on the incorporation of cinnamon essential oil to solid wax paraffin as an active coating is proposed, developed, and evaluated. The antifungal activity of the active paper is tested against *Rhizopusstolonifer*, and the results demonstrate that 6% (w/w) of the essential oil in the active coating formulation completely inhibits the growth of *R. stolonifer*, whereas 4% still has strong antimicrobial activity in in vitro conditions. Then, active paper is evaluated with actual food, sliced bread, using different storage times. After 3 days of storage, almost complete inhibition is obtained with 6% cinnamon essential oil. Qualitative analysis by solid-phase microextraction and determination of cinnamaldehyde in the sliced bread were also performed and confirmed the strong correspondence between the inhibition of the mold and the amount of cinnamaldehyde in the bread.

KEYWORDS: Bread spoilage; *Rhizopusstolonifer*; active paper packaging; cinnamon; cinnamaldehyde

INTRODUCTION

Rhizopusstolonifer fungus (black bread mold) constitutes, together with *Aspergillus* and *Penicillium* genera, the most prevalent spoiler of white bread and bakery products (*1, 2*). This fungus is responsible for the production of mycotoxins, off-flavor formation, and disgusting appearance in white bread (*3, 4*). *Rhizopusstolonifer stolonifer* usually grows on bread and fruit and, because its spores are ubiquitous in the air, infests the product within a short time when bread is stored in an enclosed, humid environment. Some strategies have been reported in the literature to preserve bread from *R. stolonifer* (*5*) infestation and to increase the shelf life of bakery goods. Some of them are based on the use of natural essential oils or spices as food preservatives (*6–8*). Essential oils are rich sources of terpenes and phenols (*9*), and for this reason, they have strong antimicrobial properties. Another interesting feature is that these natural compounds do not have any significant medical or environmental impact, so they constitute effective alternatives to conventional antimicrobial agents.

Active packaging is defined as a packaging that changes the condition of the packaged foodstuff to extend its shelf life or improve its safety while maintaining its quality (*10, 11*), and it has become a popular alternative to control undesirable molds in foods (*10, 12*). In a previous communication, a new tailor-made active paper packaging using natural essential oils as antimicrobial solutions was introduced (*13*). In this packaging, some essential oils (clove, cinnamon, and oregano) were used to manufacture an active wax coating using a quality aqueous solution of anionic/nonionic paraffin. The selectivity of cinnamaldehyde-fortified cinnamon essential oil against several fungi (*C. albicans*, *A. flavus*, *P. nalgiovense*, *P. roqueforti*, and *E. repens*) was demonstrated. Nevertheless, a nonaqueous emulsion but solid paraffin was mainly used by coating paper manufacturers in many food-related paper packaging because of its better physical properties. To develop a new active paper based on this solid wax paraffin then became decisive.

The work reported here has two main aims. The first aim is to demonstrate that a new solid active paraffin formulation can be used, in a similar way that the emulsion formulation did, as an efficient antimicrobial active coating in paper or board packaging. For this purpose, this new active paper will be evaluated against mold *R. stolonifer* using a vapor-phase test, as described by López et al. (*14*), to assess the protection provided by the materials in a "worst-case" condition, where no direct contact between the food and the packaging occurs. Other proposed methods, requiring direct contact between the active agent and the microorganism, are not recommended to test commercial packaging in which there is little contact between the food and the packaging material (*15–17*).

The second aim is to test this new active packaging vs white bread as a target food for antimicrobial testing. The rationale for this selection is that, as was previously mentioned, the prevalence of *R. stolonifer* spoilage is remarkable and that there is an industrial interest in this topic. Different approaches have been proposed for controlling microbial growth in sliced bread, including ultraviolet light, aseptic packaging, and use of chemical preservatives (i.e., calcium propionate), but no application of active packaging has been proposed until now. On the other hand, wax-coated paper is commonly used as a packaging material for this type of food; therefore, common acceptance of the proposed

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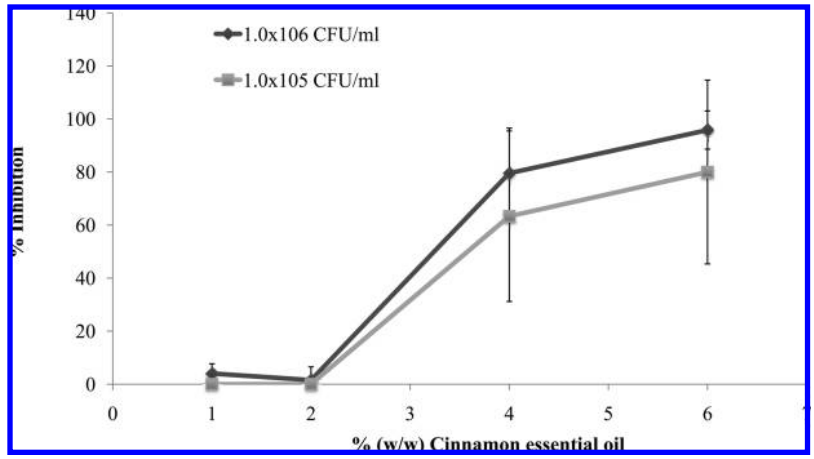

Figure 1. Percent inhibition (100 × viable colonies/viable colonies in the control) vs % of fortified cinnamon essential oil in the active coating obtained after 3 days of storage (25 °C) for two different inocula concentrations. All of the results are based on three replicates.

approach can be granted. As a relevant part, determination of cinnamaldehyde in sliced bread is performed to establish a relationship between the antifungal activity of the active paper and the amount of cinnamaldehyde transferred in the sliced bread. Cinnamaldehyde has been proven to have the strongest antimicrobial activity as compared to the other constituents of the essential oils (*18, 19*). A gas chromatographic–mass spectrometric procedure for the extraction of cinnamaldehyde from commercial cinnamon-containing foods developed by Friedman et al. (*20*) has been adapted for this purpose.

MATERIALS AND METHODS

Microbial Culture. *Rhizopusstolonifer* (aka *R. nigricans*) [Colección Española de CultivosTipo CECT 2344, (www.cect.org), Valencia, Spain] strain was stored at −18 °C in sterilized skimmed milk. Fungal conidia were harvested after inoculation on potato dextrose agar for 5 days at 40 °C and transferred to a test tube with physiological saline solution. Further dilutions to a final working concentration of 10^2 colony-forming unit (CFU)/mL were performed in physiological saline.

Chemicals. *trans*-Cinnamaldehyde (3-phenyl-2-propenal, 99%, CAS 14371-10-9) was supplied by Aldrich (Madrid, Spain); verbenone (4,6,6-trimethylbicyclo[3,1,1]hept-3-en-2-one, >97%, CAS 18309-32-5) was purchased from Fluka (Madrid, Spain); and ethyl acetate [high-performance liquid chromatography (HPLC) grade] was obtained from Scharlau (Barcelona, Spain).The essential oil of *Cinnamomumzeylanicum* (cinnamon, CAS 8015-91-6) was supplied by Argolide (Barcelona, Spain). For the purposes of this contribution, the physical properties of cinnamaldehyde can be considered as follows: water solubility, 3760 mg/L; boiling point, 246.8 °C at 760 mm Hg; enthalpy of vaporization, 48.4 kJ/mol; and vapor pressure, 0.027 mm Hg at 25 °C.

Active Paper Manufacture. The active paper manufacture was as follows: An active paraffin formulation containing the appropriate amount of cinnamon essential oil as an active agent was prepared by Rylesa-Repsol-YPF (Madrid, Spain) in a joint project with the University Research Group GUIA. The methodology is protected by patent no. 200601550. The concentration of the active agent was varied as described in this paper. The manufacture process involved a mixing step of heating at 110 °C for 10 min. Active coating was then applied to a paper (40 g/m^2) provided by Rylesa-Repsol-YPF using a heated manual coating bar supplied by RK Print-Coat Instruments Ltd. (Litlington, United Kingdom). The paper was double coated, but only one side had the active coating, whereas the other one had the ordinary coating. Grammage was controlled by weighting according to the wax manufacturer specifications.

Antimicrobial Testing. Antimicrobial tests in Petri dishes were carried out as follows: A Petri dish with the appropriate solidified agar culture medium was inoculated with 100 μL of a 10^2 CFU/mL solution of the fungi under study. Then, the active paper was placed over the Petri dish and nonhermetically kept in place using a plastic strap. Controls (without paper) and blanks (with paper coated by the paraffin formulation but without the active ingredients) were also prepared for each set of samples. After the cultures were incubated for 48 h and 24 °C (time when *Rhizopus* growing was detectable in the blanks), the inhibition of *R. stolonifer* by a different amount (w/w) of active in the coating was evaluated and was considered as inhibition only when there was no growth at all of the microorganism. All of the tests were performed five times.

Antimicrobial tests in sliced bread were carried out as follows: One side of a slice of bread was inoculated in the center with 200 μL of a 10^5 or 10^6 CFU/mL solution of the microorganism under study (*R. stolonifer*). Then, this inoculated slice was placed in the middle of two noninoculated slices of bread. Finally, these slices of bread were manually packed with the active paper. Blanks (with paper coated by the paraffin formulation but without active ingredients) were also prepared for each set of samples. Bread packages were stored at 25 ± 0.1 °C for 3 and 10 days in a Binder ATP Line KB incubator (Tuttlingen, Germany). Incubation times were selected using the same blank-detectable growing criterion.

After this period, the whole slice was ground in a mortar and 7 g of both the central (inoculated) and the lateral (noninoculated) slice were weighted aseptically in sterile stomacher bags, suspended in 70 mL of sterilized peptone water, and homogenized in a Stomacher-400 Circulator (Seward, United Kingdom) for 1 min at 230 rpm. Microbial counts were determined by seeding 0.1 mL of the homogenized solution on potato dextrose agar plates incubated at 24 °C for 48 h. Inhibition in the bread slice was calculated by counting numbers of viable colonies divided by the numbers on the respective blank dishes. All tests were performed in triplicate.

Analysis of Bread Slices and Active Paper. Analysis of bread slices was as follows: The whole slice was ground in a mortar, and an aliquot of 0.5 g was placed in a 20 mL vial and sealed with a cap. For the analysis of the active paper, a specimen of 0.225 dm^2 of coated paper was introduced into the capped vial. The headspace of the vial was sampled with a 100 μm (nonbonded) polydimethylsiloxane solid-phase microextraction (SPME) fiber obtained from Supelco (Bellefonte, PA), and the compounds were desorbed into the injection port of a gas chromatograph. Extraction conditions were 20 min of absorption time at 30 °C and 5 min of desorption time at 250 °C. For the analysis of the active paper, the absorption time was 5 min. The fiber was conditioned prior to its first use according to the manufacturer's recommendations.

GC/MS analyses were performed using a Hewlett-Packard 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a 5975B inert XL MSD mass spectrometer detector and an A HP-5 MS (30 m × 0.25 mm, 0.25 μm film thickness) capillary

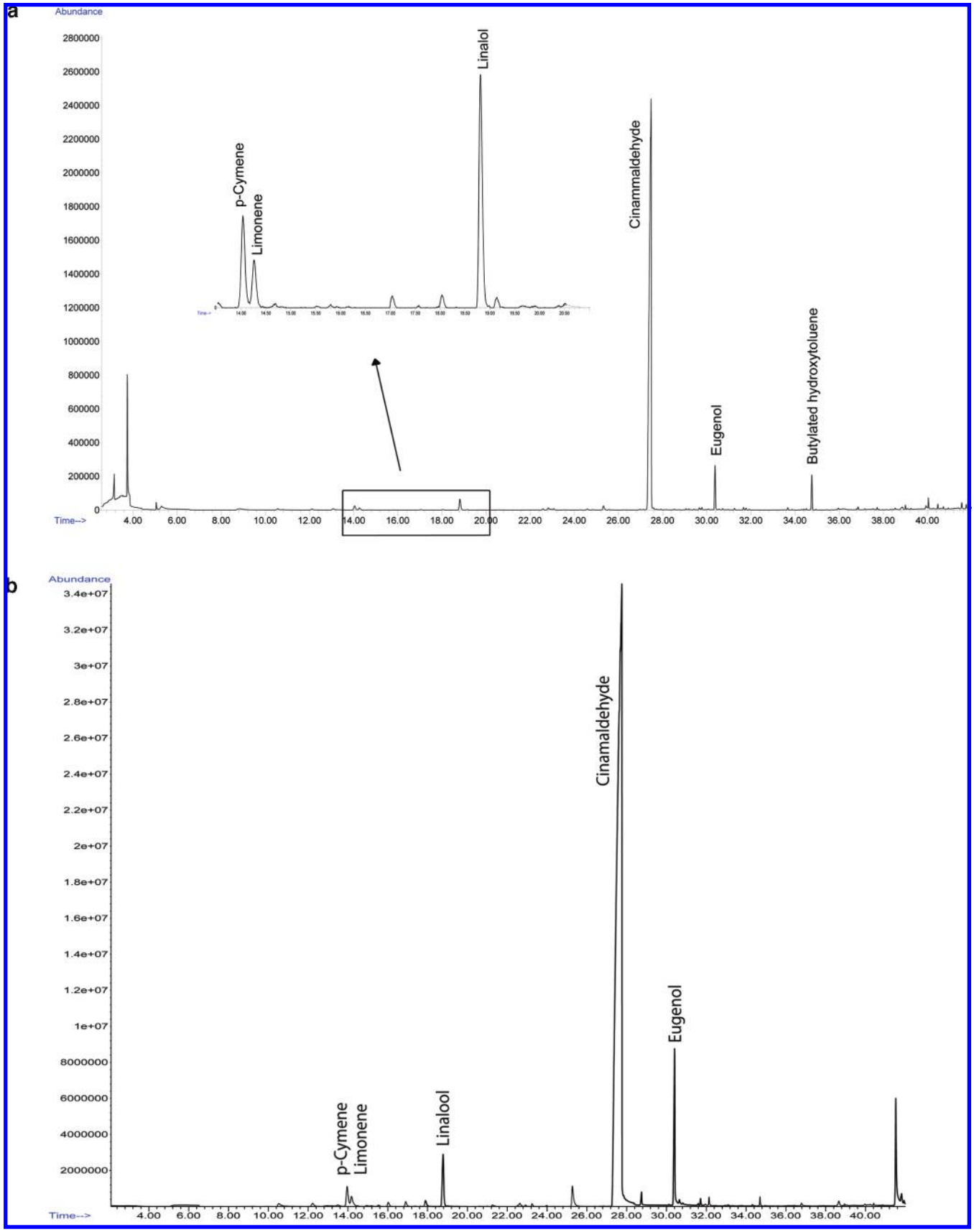

Figure 2. (**a**) Chromatogram of a sample of bread packed with 4% active paper and stored for 3 days extracted by solid-phase microextraction technique in headspace mode. (**b**) Chromatogram of a sample of 0.225 dm^2 of 4% active paper.

column from Agilent. The temperature program for the gas chromatography was as follows: initial temperature, 70 °C held for 1 min, linear gradient of 2 °C/min to 85 °C, second ramp at 5 °C/min to 170 °C, and final at 15 °C/min to 220 °C, which was held for 3 min. The injector temperature was 250 °C, and injection was performed in splitless mode (splitless time, 30 s). The carrier gas

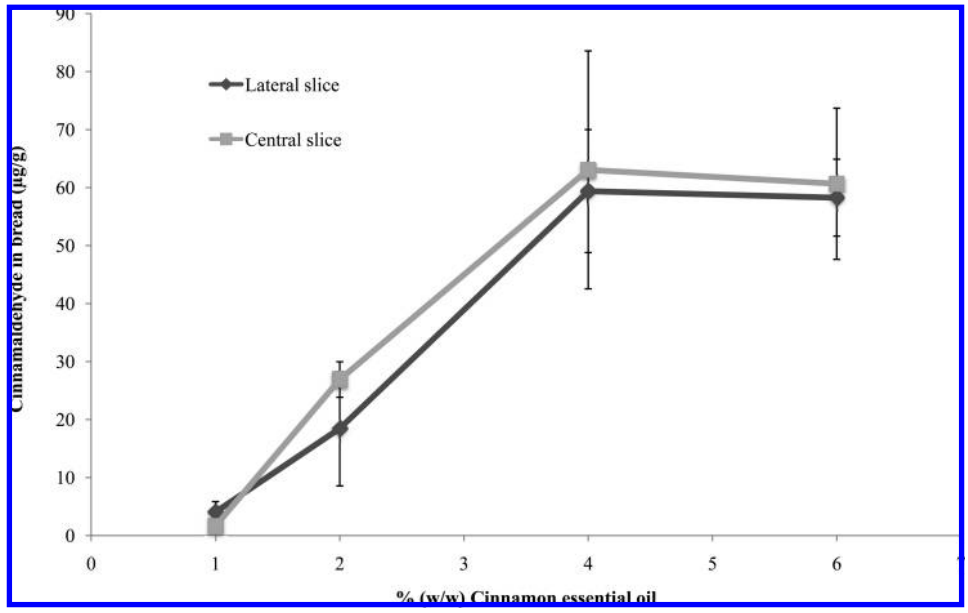

Figure 3. Concentration (μg/g) of cinnamaldehyde in lateral and central slices vs % of fortified cinnamon essential oil after 3 days of storage.

was helium (99.999% purity, 1.0 mL/min) supplied by Carburos Metálicos (Barcelona, Spain). All of the compounds were analyzed in the SCAN (from 40 to 350 *m*/*z*) mode.

Quantification of Cinnamaldehyde Amount in Bread Slices. A procedure for the extraction of cinnamaldehyde from foodstuffs (*20*) has been adapted. The extraction method used 0.3 g of a bread sample, which was crushed in a mortar, placed in a 20 mL vial, and mixed with 3 mL of ethyl acetate. The mixture was vigorously shaken in triplicate for 2 min and then left to settle for 20 min. Then, the three extracts of ethyl acetate were transferred to a vial and directly injected into a GC/MS using the above-mentioned conditions. The internal standard of verbenone was used for quantitative purposes. Sequential extractions of a bread sample spiked with cinnamaldehyde were performed to evaluate the method, and a recovery of 98% and intraday repeatability (relative standard deviation, RSD) of 2.4% were obtained.

RESULTS AND DISCUSSION

Active Paper Evaluation. The antimicrobial activity of the manufactured active paper against *R. stolonifer* with nominal concentration ranging from 1 to 6% (w/w) of the active agent incorporated into the coating was evaluated ($n = 5$). *R. stolonifer* was inhibited in all of the five replicates when exposed to the atmosphere generated with 6% (w/w) of fortified cinnamon essential oil in the active coating. Total inhibition was also obtained in four of the five replicates when 4% of essential oil was used and in two replicates when using 2% (w/w) of the active components, whereas no inhibition was obtained with 1% (w/w) of the essential oil.

Some conclusions can be inferred from this experiment: First, the active paper made with solid paraffin has antifungal activity against *R. stolonifer*, and because there was no direct contact between the active paper packaging and the microorganisms in the experiments, the chemicals responsible for the inhibition must, most probably, have been in the headspace of the Petri dish; therefore, the antimicrobial activity reported here is based on the volatile composition. This is a very interesting achievement taking into account that the manufacture of the coating includes a heating step at 110 °C for 10 min; therefore, significant losses or even degradation of the volatile active chemicals could be reasonably expected. The protective effect of other components of cinnamon essential oil and specifically that of eugenol against the thermal degradation of cinnamaldehyde has been reported in the literature and can be held responsible for the preservation of antifungal activity observed (*17*). Second, the higher the nominal concentration of the essential oil in the active coating, the higher the antifungal activity, being 2% (w/w) the minimum nominal concentration that provides some inhibition against *R. stolonifer*.

Antimicrobial Activity of Active Paper in Bread. Figure 1 shows the results of the fungal count in sliced bread after 3 days of storage and with two different concentrations of inoculation. As can be seen, 80 and 96% of inhibition was obtained with 4 and 6% of cinnamon essential oil in the coating; at 6%, two out of three replicates were totally inhibited, whereas none of the replicates was totally inhibited working at the 4% active compound. With 1 and 2% (w/w) in the wax paraffin, no inhibition at all was obtained. The initial concentration of the inocula at the levels tested has no direct relationship with the antimicrobial activity provided, since no differences were found when 10^6 and 10^5 CFU/mL were used in the inocula.

As in the evaluation of the active paper above-described, there was no direct contact between the active packaging and the inoculated bread slices because they were placed in the middle of two noninoculated slices. For this reason, the inhibition must take place by vapor-phase transfer from the packaging and not by direct migration of the active compounds; therefore, our hypothesis is based on the release of the active chemicals from the active coating to the internal atmosphere in the packaging; this atmosphere is responsible for the antifungal activity.

Identical results with respect to the fungal inhibition were obtained after 10 days of storage. This fact points out that critical inhibition takes place in the first 72 h of the mold growth during its lag phase, when the microorganisms are known to be most susceptible for inhibitory activity, as was previously reported in the scientific literature (*21*).

Analysis of the Bread after Storage with Active Packaging. Figure 2a shows the chromatogram of the extraction of bread packaged with the 4% w/w formulation of active paper. Qualitative analysis of bread slices showed that the active

compounds of the cinnamon essential oil are partially transferred to the bread. Cinnamaldehyde was found to be the major component, which is in agreement with the composition of the essential oil used, with about 80−85% w/w of cinnamaldehyde in the cinnamon essential oil. Some traces of *p*-cymene, eugenol, linalool, limonene, and benzyl benzoate were also detected. For comparison purposes, the same extraction procedure (except absorption time, 5 min) was applied to the active paper, as explained in the Materials and Methods. **Figure 2b** shows a typical chromatogram obtained.

Quantification of cinnamaldehyde in bread slices (3 days of storage) showed that the amount of cinnamaldehyde incorporated to the bread is highly dependent on the natural essential oil concentration in the active wax coating; the amount detected ranges from 5 to 65 μg/g of bread (**Figure 3**). A linear tendency was observed, although a plateau is reached when 4% (w/w) of fortified essential oil is used in the active formulation. Our explanation is that, since the active component has to be released from the coating before it can be transferred to the headspace, this is the rate-limiting step and is therefore responsible for this plateau.

No differences were found when comparing lateral and central slices; this was quite surprising because lateral slices were in direct contact with active packaging and central ones did not; so no direct migration (transfer) processes in the latest were predictable; consequently, a higher cinnamaldehyde concentration in lateral than in central loafs could be expected. Incorporation of cinnamaldehyde from the active packaging to the food can occur through two different ways: First is the migration by direct contact of the packaging and the bread, and second is through the headspace after the active compounds are released from the packaging. An equivalent concentration of cinnamaldehyde in lateral and central slices seems to point out that contribution from direct migration is small as compared to cinnamaldehyde incorporation from the active atmosphere.

It is very interesting to compare the growth of *R. stolonifer* and the amount of cinnamaldehyde transferred to the bread (**Figures 1** and **3**). The final concentration of 30 μg of cinnamaldehyde per g of bread corresponding to 2% (w/w) of the essential oil in the coating is not enough to inhibit *R. stolonifer* growth in bread. However, when the final amount is approximately 60 μg/g of bread, inhibition reaches 70−90% (and many times full inhibition). Very significant is the fact that, despite the amount of cinnamaldehyde in the bread is not statistically different for 4 and 6% of cinnamon in the coating, the antifungal activity is stronger in the second one. One possible explanation of this fact could be the rate of incorporation of cinnamaldehyde to the bread: Because inhibition takes place in the lag phase of the mold growth as was previously explained, the differences between the 4 and the 6% cinnamon will arise not from the final concentration of cinnamaldehyde in bread but from the different incorporation rate during this phase.

A number of conclusions can be drawn from the work reported here. First, it is possible to use active solid paraffin as a way to incorporate active compounds on paper giving antimicrobial activity to this packaging material. The use of this active paper packaging is a smart alternative for protecting bread from fungal infestation since no direct contact between the food and the packaging is required. Inhibition seems to take place during the lag phase of the mold infestation. There is a strong relationship between the inhibitory effect of the active paper and the final amount of cinnamaldehyde found in the bread. As a final point, migration through headspace seems to be much more significant than direct migration through direct packaging−food contact. Further work is in progress to learn more about the rate of migration of the active compound and its relationship with antifungal effects, which could be the key factor to optimize this new active packaging.

ABBREVIATIONS USED

CFU, colony forming unit; HPLC, high-performance liquid chromatography; SPME, solid phase microextraction; GC/MS, gas chromatography/mass spectrometry; RSD, relative standard deviation.

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