Raman spectroscopy, electronic microscopy and SPME-GC-MS to elucidate the mode of action of a new antimicrobial food packaging material

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Abstract

One critical challenge when developing a new antimicrobial packaging material is to demonstrate the mode of action of the antimicrobials incorporated into the packaging. For this task, several analytical techniques as well as microbiology are required. In this work, the antimicrobial properties of benzyl isothiocyanate, allyl isothiocyanate and essential oils of cinnamon and oregano against several moulds and bacteria have been evaluated. Benzyl isothiocyanate showed the highest antimicrobial activity and it was selected for developing the new active packaging material. Scanning electron microscopy and Raman spectroscopy were successfully used to demonstrate the mode of action of benzyl isothiocyanate on *Escherichia coli*. Bacteria exhibited external modifications such as oval shape and the presence of septum surface, but they did not show any disruption or membrane damage. To provide data on the in vitro action of benzyl isothiocyanate and the presence of inhibition halos, the transfer mechanism to the cells was assessed using solid-phase microextraction–gas chromatography–mass spectrometry. Based on the transfer system, action mechanism and its stronger antimicrobial activity, benzyl isothiocyanate was incorporated to two kinds of antimicrobial labels. The labels were stable and active for 140 days against two mould producers of ochratoxin A; *Penicillium verrucosum* is more sensitive than *Aspergillus ochraceus*. Details about the analytical techniques and the results obtained are shown and discussed.

Introduction

Food safety is a global priority and one of the major concerns of consumers, administration and industry. Antimicrobial packaging is an increasing demand of the food industry, but the development of stable and feasible materials at industrial scale is very difficult. Active packaging systems, such as antimicrobial or antioxidant ones, may be launched into the European market if they comply with the restrictions set out in European Regulations (EC) 1935/2004 and (EC) 450/2009. Antimicrobial materials are beneficial to the consumers as well as to the food industry since they can extend the product shelf-life and/or maintain food safety by inhibiting, killing or reducing the growth rate of target microorganisms. However, there are several critical challenges in their development. A key point is the selection of antimicrobial agents which may be incorporated into food packaging materials. Different chemicals such as natural agents, probiotics [1–3] as well as essential oils (EOs) [4] have been explored for this task [1, 5]. It has been demonstrated that EOs represent an interesting source of natural antimicrobials for food preservation [6] and they have also been categorised as generally recognised as safe (GRAS) by the US Food and Drug Administration and published in the Code of Federal Regulations (CFR) [7]. A second critical point is the demonstration of their mode of action by which the new packaging is able to inhibit or even kill bacteria and moulds. For this task, the combination of several analytical techniques together with microbiology is required.

The first goal of this work was to study in-depth the performance of a new antimicrobial packaging material. For this purpose, several antimicrobial agents were screened in order to select the most active one. In addition, the mode of action and the transfer mechanism from the packaging to the cells were studied. Cinnamon and oregano EOs have already been described as powerful antibacterial agents [8–11] and both have been used to develop several active packaging systems [8, 12, 13]. However, the study of new natural alternatives that can improve antimicrobial activity of active packaging is always a challenge, as well as the design of novel active packaging. In this work, two natural active agents with a high antibacterial activity obtained from *Brassicaceae* and *Salvadoraceae* family, mustard essential (AITC) and BITC, respectively [11, 14–21], were selected. The effectiveness of all of them, alone and inside a novel active packaging based on the design of active labels, was evaluated with the aim to select the best candidate. It is important to highlight that the incorporation of isothiocyanate derivatives in food packaging has been informed by European Food Safety Authority (EFSA), as “no safety concern at estimated level of intake as flavouring substances” [22, 23]. EFSA is in concordance with the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and the information is collected in the JECFA/65/SC rules [24]. EOs of cinnamon and oregano have already been described as antibacterial and antifungal agents [8, 9] and they have a positive opinion from EFSA. The substances used have been introduced in a register of approximately 2800 substances, which has been adopted by the Commission decision of 1999/217/EC, as last amended by decision 2005/389/EC [25]. Indications do not appear for any of the substances used in the research carried out. In Spain, the Spanish Food Safety, Consumption, and Nutrition Agency (AECOSAN) stated in a guide the legislation on food flavourings [26]. Cinnamon, oregano and their major

components are listed and used as flavouring substances, but it is true that they have antimicrobial activity as well, what is well known since centuries. Due to the volatile nature of antimicrobial agents, transfer mechanisms were studied by solid-phase microextraction coupled to gas chromatography–mass spectrometry (SPME-GC-MS).

Once the antimicrobial was chosen, the active material was prepared and its efficiency was evaluated. The mode of action of antimicrobials was studied by using scanning electron microscopy (SEM) [8] and Raman spectroscopy [27, 28]. SEM is a very important viewing technique for the study of microorganisms as it allows determining changes in the cell structure of microorganisms and a possible damage produced by the antimicrobial compounds. Raman spectroscopy [27, 28] provides information about molecular vibrations and it can be used to identify possible molecular changes in cell structure. Raman microscopy is a very appropriate technique for this task. Thus, the effects produced by the antimicrobials on foodborne will be evaluated at a molecular level with the use of spectroscopic techniques [29]. This work summarises a complex combination of techniques that will show a complete and relevant set of information necessary to design a new active packaging.

Material and methods

Microbial culture

Aspergillus ochraceus CECT 2093, *Penicillium roqueforti* CECT 2905, *Penicillium verrucosum* CECT 2096, *Aspergillus niger* CECT 2088, and *Salmonella enterica subsp. enterica* CECT556, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were supplied by the Spanish Type Culture Collection (CECT, Valencia, Spain) and the American Type Culture Collection (ATCC, Manassas, VA, USA). Potato dextrose agar (PDA) and tryptic soya agar (TSA) as solid media, and yeast extract broth (YEB) and tryptic soy broth (TSB) as liquid media were supplied by Scharlab (Barcelona, Spain).

Antimicrobial agents

Benzyl isothiocyanate (BITC, 98 %, CAS 622-78-6) and allyl isothiocyanate (AITC, ≥95 %, CAS 57-06-7) were provided by Sigma-Aldrich Química (Madrid, Spain). Oregano EO (CAS 8007-11-2) and cinnamon bark EO (CAS 8015-91-6) fortified with cinnamaldehyde were from Argolide (Barcelona, Spain). The same batch of cinnamon and oregano essential oils used in previous publications [30, 31] was used here. For this reason, the chemical composition is exactly the same as that already published.

Antimicrobial agent selection

Antimicrobial activity in liquid medium

To study the antimicrobial susceptibility of foodborne microorganisms versus the selected active agents, a broth dilution method was used [32, 33]. Ethanol solutions of each antimicrobial agent were prepared. In the case of moulds, an aliquot of 20 µL of antimicrobial agent and 200 µL of a mould spores suspension (10^6 colony forming unit (CFU) per mL) were mixed with a YEB medium to a final volume of 2 mL. For bacteria, an aliquot of 10 µL of antimicrobial agent and 100 µL of a bacteria inoculum (10^6 CFU per mL) were mixed with a TSB medium to a final volume of 1 mL. The final concentration of antimicrobial agents ranged from 0.8 to 1600 µg mL^{-1}. To ensure that ethanol did not inhibit microorganism growth, controls with addition of 20 or 10 µL of ethanol were carried out, depending on the analysed microorganism. Moulds samples were incubated for 48 h at 25 °C and bacteria were incubated at 37 °C overnight, both under continuous shaking. The minimum inhibitory concentration (MIC) was defined as the lowest antimicrobial concentration that did not yield any visible growth [34]. All assays were performed at least in triplicate.

Antimicrobial activity in vapour phase

Vapour phase activity of EOs was studied with a diffusion assay. TSA or PDA was inoculated with 100 µL of bacterial or mould inoculum containing approximately 10^6 $CFU \cdot mL^{-1}$ in physiological saline solution. Then, 10 µL of each pure antimicrobial agent was added to a sterile Whatman® filter paper (diameter 9 mm) placed in the middle of the Petri dish lid which contained 12 mL of adequate medium [35]; blanks were prepared by adding the same amount of ethanol to the filter disks. Petri dishes (90 × 14 mm) were then sealed using Parafilm M® and incubated. The antimicrobial activity was evaluated in terms of the inhibition halo formed [36]. A second experiment was also performed, where low volume aliquots of BITC (10, 5, 3 and 1 µL) were evaluated under the same incubation conditions. The correspondence of milcrolitre to microgram of each active substance was as follows: 1 µL = 1056 µg of cinnamon EO, 1 µL = 1013 µg of mustard EO (AITC) and 1 µL= 1125 µg of BITC.

Antimicrobial activity in agar

The agar diffusion assays were made in the same way as that mentioned above for the vapour phase activity tests, but in these cases the filter disk was placed on top of the agar and only the effect of adding 10 µL of each pure antibacterial agent was studied.

Screening of antimicrobial activity in active packaging

Active agents were incorporated into a 73 $g \cdot m^{-2}$ filter paper (FP) using a water-based adhesive with self-adhesive properties. This way, active labels of 1.0 × 1.5 cm^2 containing cinnamon EO, oregano EO and BITC at 8 % (*w*/*w*) were made. BITC was

integrated in the adhesive using a special formula and then extended to make the active label using a K202 Control Coater 2005 (RK Printcoat Instrument). BITC was homogeneously distributed, since RDSs obtained in the "Chemical evaluation of active material" section were below 5 %. This fact demonstrates a good distribution despite being a laboratory preparation. More details about the packaging system and the formula cannot be disclosed because of confidential reasons. Plates with PDA or TSA, depending on the strain tested, placed at the top of the inner part of the packaging, were inoculated with 100 µL of a physiological saline solution containing 10^6 CFU·mL^{-1} of the microorganism under study [8]. The active labels were placed at the bottom of the plate. Blank labels were prepared following the same procedure above described without active agent addition.

Determination of BITC action mechanism

Scanning electron microscopy

E. coli was treated in liquid media with BITC at sub-MIC and MIC concentrations for 24 h using the method described in section "Antimicrobial activity in liquid medium". Control samples without addition of the antimicrobial substance were also carried out. After that, the samples were prepared for scanning electron microscopy according to the protocol described by Becerril et al. [37]. Morphology of the *E. coli* cells was observed on an Inspect™ F50 scanning electronic microscope (FEI, Hillsboro, OR, USA) working at 15–30 kV and reaching a resolution of 1.5 nm.

Raman spectroscopy analysis

Samples for Raman analysis with liquid media (macro dilution method) were prepared with the minimum concentration of antimicrobial that enabled the growth of *E. coli* (sub-MIC). Samples and controls were transferred to sterile 1-mL Eppendorf tubes and centrifuged at 10,000 rpm for 5 min. Afterwards, the formed pellet was washed with 1 mL of physiological saline solution and this procedure was repeated three times. After the final washing step, the pellet was resuspended in sterile water to a final volume of 1 mL. Finally, 20 µL of each sample was placed in a BaF_2 window and dried in an oven at 40 °C.

Raman spectroscopic analysis was performed using a Thermo Scientific (Madison, WI, USA) DXR™ Raman microscope equipped with a 532-nm laser. Laser power was set at 10 mW (measured at the sample) on the bacteria through a ×100 objective. Raman spectra of each sample were collected with a shift range from 3400 to 50 cm^{-1}. Aperture was set to 25 µm pinhole. Fluorescence correction and cosmic ray rejection were applied. Data analysis and spectral interpretation were made with Thermo Scientific Omnic 9™ software.

With the aim of seeking differences in the Raman spectra between samples and controls, the individual spectrum of eight bacteria was considered for each type. It was considered appropriate in statistical terms, which are discussed in the results section. All experiments were performed in triplicate. Assuming the heterogeneity of

each bacteria group, spectra were normalised using the OriginPro8 SR0® program (OriginLab Corporation, Northampton, MA, USA). Finally, the average spectrum of each set of samples was performed.

Transference mechanisms of BITC

The study of transference mechanisms was performed on the inside of a Petri dish. The top half of the dish contained 12 mL of TSA medium, and a 10-mm sterile blank filter disk spiked with 1 µL of pure BITC was placed on the bottom half. The Petri dish was sealed with Parafilm and incubated in a culture oven at 25 °C. A total of 15 dishes were incubated. Agar plugs of 5-mm diameter were collected with a punch at different incubation times: 3, 6, 24 and 48 h. Plugs were collected from three different areas of the plate (centre, half radius and 90 % radius) in order to study the concentration of BITC across the dish. The test was performed by triplicate. The 10-mm sterile blank filter disk and two Parafilm® tapes were analysed too. The calibration curve was carried out by spiking agar plugs with 1 µL of known concentrations of BITC.

Analysis by SPME-GC-MS

For SPME analysis, agar plugs collected from the transfer mechanism assays were placed in 20 mL glass vials. A 50/ 30 µm divinylbenzene/carboxen/ polydimethylsiloxane SPME fibre, supplied by Supelco (Bellefonte, PA, USA), was used in headspace mode for the analysis.

A CTC Analytics autosampler system from Agilent Technologies (Madrid, Spain) was used. The gas chromatograph system was a HP 6890 series connected to a HP 5973 series mass selective detector. Chromatographic separation was carried out on an HP-5MS column (30 m × 0.25 mm × 0.25 µm) from Agilent Technologies (Madrid, Spain). Sample extraction was performed at 40 °C for 10 min at 500 rpm and desorption was carried out at 250 °C for 2 min. All samples were injected in splitless mode (2 min). The initial column temperature was set at 60 °C held for 4 min, then raised at 15 °C·min^{-1} up to 240 °C held for 2 min. Helium (99.999 %, Air Liquide, Madrid, Spain) was used as carrier gas at a flow rate of 1 mL·min^{-1}. For quantitative analysis, the calibration plot was obtained from appropriate dilutions in methanol of BITC, working under the same conditions as the samples.

Active packaging development

Chemical evaluation of the active material

Active labels were prepared in the same way as section “Screening of antimicrobial activity in active packaging”, but only with BITC and including 30-µm thickness polypropylene (PP) as substrate instead of a FP. The reproducibility of BITC release from the active labels was assessed by HSSPME-GC-MS (n = 6), using the method described previously in the section “Analysis by SPME-GC-MS”. Active labels were

placed in 20-mL glass vials for the analysis. Blanks were prepared with the same composition but without BITC. Before their use, active labels were stored at 4 °C inside a sterile Petri dish sealed with Parafilm. Samples were evaluated periodically up to 140 days after being prepared to confirm their stability.

Antimicrobial evaluation of active material

The antimicrobial activity of active labels was evaluated over the time against two mould strains (*A. ochraceus* and *P. verrucosum*). The procedure was just the same as that described in section “Screening of antimicrobial activity in active packaging”, but here only PDA plates were prepared. In

addition to PF, PP was used too and six replicates of each sample were considered in all cases. Inhibition halo was measured after 7 days of incubation and it was transformed to percentage of inhibition of growth vs. blanks.

Results and discussion

Antimicrobial screening in liquid medium

The antimicrobial activity of BITC, AITC and cinnamon EO was determined against a series of selected foodborne microorganisms. The MIC values obtained are shown in Table 1. AITC and BITC showed higher antimicrobial activity against all moulds and bacteria than cinnamon EO, with MIC values of 0.8–50 $\mu g \cdot mL^{-1}$ for BITC, 0.8–100 $\mu g \cdot mL^{-1}$ for AITC and 25–200 $\mu g \cdot mL^{-1}$ for cinnamon EO.

The three active agents were more active against moulds than bacteria. The high bactericidal activity of AITC was previously reported by Luciano and Holley, who used AITC in their experiments [18]. BITC and AITC have the same active group (R–N=C=S) and this explains the similarity of the values obtained. Nevertheless, MIC values obtained for the three bacteria tested were better for BITC than for AITC. These results are in agreement with those obtained by Aires et al., who studied the antimicrobial activity of BITC and AITC against *S. aureus* [38]. The results obtained for cinnamon EO agree with those described by Becerril et al. for the tested bacteria [33] and with Manso et al. for *A. niger* and *P. roqueforti* using the same EO from the same batch [13].

Antimicrobial activity in vapour phase

Figure 1a shows the percentage of growth inhibition for the three pure active agents in vapour phase when 10 µL of each one was added. BITC as well as AITC showed a total growth inhibition (100 %) in all bacteria and moulds studied, while cinnamon EO showed values between 57.5 ± 0.5 and 92.4 ± 0.8 %, expressed as mean ± standard deviation. These results are consistent with those obtained in liquid phase, and in agreement with those obtained by other researchers, that described the higher antimicrobial activity of AITC against *E. coli*, *S. aureus* [39] and for *A. ochraceus* [40].

The results for *E. coli* and *S. aureus* treated with cinnamon EO are similar to the ones previously reported by Lopez et al. [31].

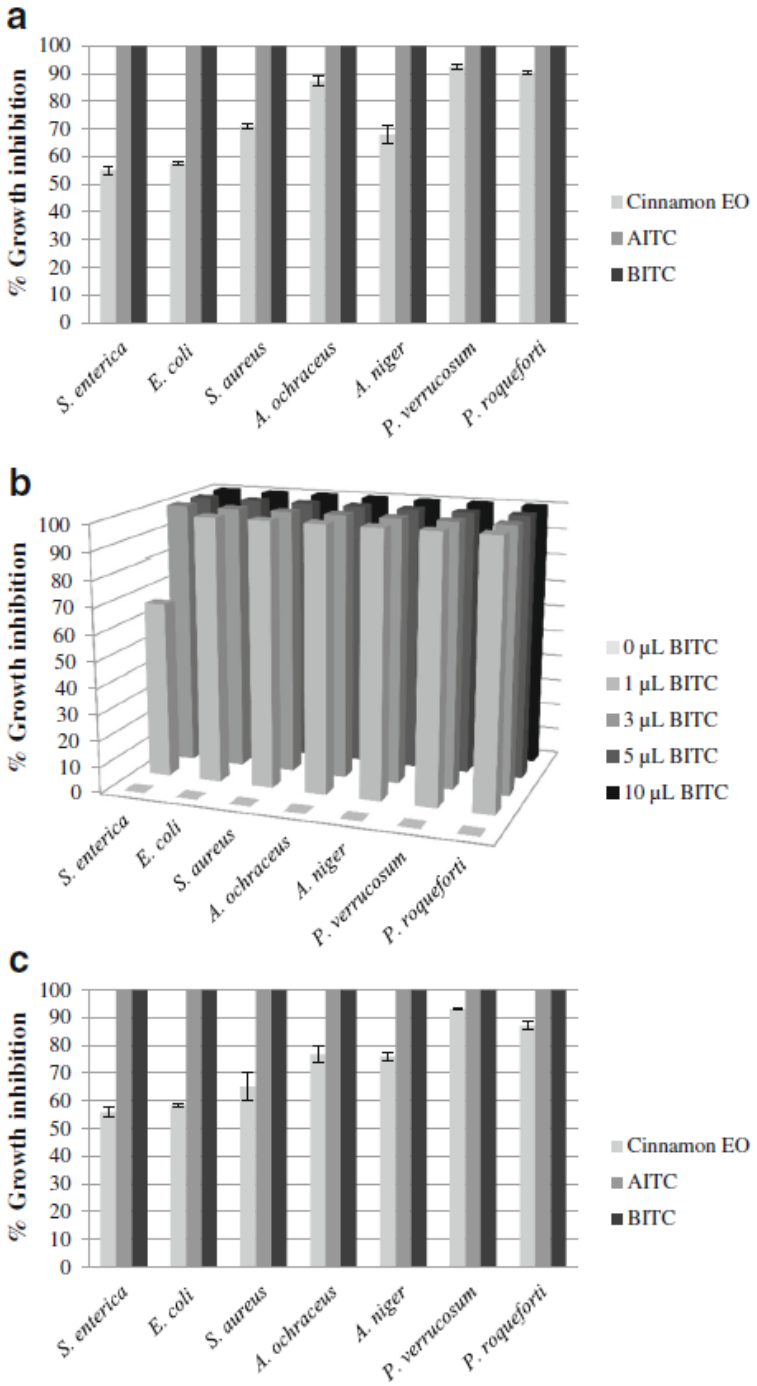


Fig. 1 a Vapour phase activity of 10 µL of cinnamon EO, AITC and BITC; b vapour phase activity of 0, 1, 3, 5 or 10 µL of BITC which was added to a 10-mm sterile blank filter disk at the bottom of the Petri dish; and c agar diffusion assay with 10 µL of cinnamon EO, AITC and BITC

Table 1 Antimicrobial susceptibility, expressed as minimum inhibitory concentration (MIC) values of BITC, AITC and cinnamon EO against foodborne microorganisms

Strain	MIC ($\mu g \cdot mL^{-1}$)		
	BITC	AITC	Cinnamon EO
E. coli	25	25	200
S. aureus	25	100	200
S. enterica	50	100	200
A. ochraceus	6.25	6.25	200
A. niger	3.12	3.12	100
P. verrucosum	12.5	6.25	25
P. roqueforti	0.80	0.80	25
R. stolonifer	100	50	200

Since BITC showed the best results in liquid phase, its antimicrobial activity in vapour phase was evaluated at different concentration levels. Figure 1b shows the percentage of growth inhibition when 10, 5, 3 and 1 µL of pure BITC were added. Total inhibition was obtained in all cases except for *S. enterica* that needed a minimum of 3 µL. This result was obtained measuring the inhibition halo and transforming it to percentage of growth inhibition.

Antimicrobial activity in agar

The results obtained in agar assays were very similar to those obtained in vapour phase (Fig. 1c). When 10 µL of active agents was placed in agar, BITC and AITC caused total inhibition of all microorganisms tested. These results obtained for the moulds agree with those previously shown by Nielsen [19]. In the case of cinnamon EO, the percentage of growth inhibition was between 58.4 ± 0.7 and 93.1 ± 0.3 %, which is in agreement with the results obtained by Lopez [30] and Ghabrie [41]. It can be observed that *S. aureus* was more sensitive to cinnamon EO than *E. coli* in agar as well as in vapour media. In the same way, the results obtained for *A. niger* and *A. ochraceus* agree with those found by Singh et al. [42]. However, the inhibition results cannot be compared, since the cinnamon used in this work was fortified with its major component (cinnamaldehyde).

The results obtained in liquid medium, vapour phase and agar revealed that BITC had the highest antimicrobial activity for the microorganisms under study, and for this reason it was selected for the design of active labels.

Active agent selection

A screening with different active labels using the BITC, AITC, cinnamon and oregano EOs was carried out in order to select the most active label against six microorganisms: four moulds and two bacteria (*E. coli* Gram− and *S. aureus* Gram+). AITC was discarded due to its high volatility and viscosity that resulted in non-reproducible labels. Furthermore, according to the study of transfer mechanism, the diffusion model was better than the convection one for this type of packaging. Oregano EO and cinnamon EO were added to the study for comparison purposes, as they were successfully used in previous studies with another type of active packaging [8, 12].

Figure 2 shows the diverse effects on the microorganism growing depending on the active agent used. It can be seen that the labels containing oregano EO were not efficient against any of the microorganisms tested and the inhibition halo was not observed in any of them. Labels made with cinnamon EO showed antimicrobial activity against the four moulds tested (*A. ochraceus*, *A. niger*, *P. roqueforti* and *P. verrucosum*), but they were inactive against bacteria.

On the other hand, BITC labels presented the best antimicrobial activity. The antifungal activity was much greater than that from cinnamon EO. The moulds *P. verruscosum* and

P. roqueforti were completely inhibited and a higher inhibition halo appeared in *A. ochraceus* and *A. niger*. Furthermore, only active labels with BITC had activity against the tested bacteria, reaching almost their total inhibition.

Although the research previously developed by our group with cinnamon or oregano EO demonstrated acceptable antimicrobial activity, the way of incorporating them in the active packaging was completely different [8, 12, 43]. In this case, the active agents were incorporated into the adhesive that was placed in the rear side of label. This means that the compounds had to diffuse through the material and arrive at the cells via vapour phase. Thus, the formula and the diffusion process play a critical role in the antimicrobial activity of the label. This fact emphasises the importance of the technology to produce active materials, as the results strongly depend on the way and the formula used to incorporate the active agent in the packaging material.

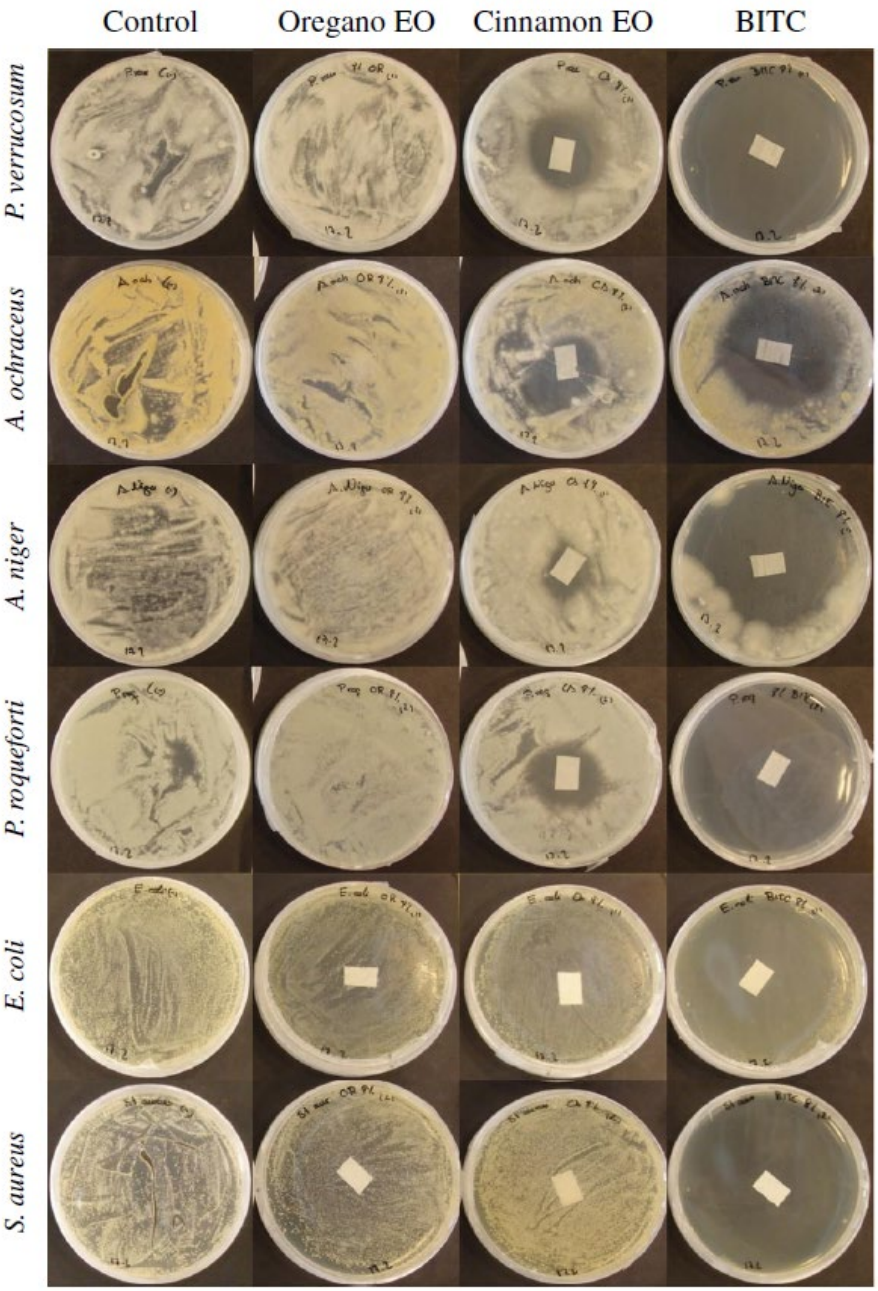


Fig. 2 Controls and treated microorganism with active label (1.0 × 1.5 cm^2) made with 8 % (*w*/*w*) of BITC, cinnamon EO or oregano EO against *A. ochraceus*, *A. niger*, *P. roqueforti*, *P. verrucosum*, *E. coli* and *S. aureus*

Determination of BITC mode of action

Scanning electron microscopy

To gain further knowledge about the mechanism of action of BITC, SEM experiments were carried out. The microphotographs taken by SEM, both with MIC and sub-MIC conditions, showed cellular damage and no viable cells.

Microphotographs showed that in comparison to the control bacteria (Fig. 3a), *E. coli* exposed to BITC at MIC and sub-MIC concentrations displayed considerable morphological alterations. After 24 h of exposure with sub-MIC BITC concentrations (Fig. 3b), the cells exhibited external modifications as oval-shaped bacteria and the presence of septum surface, but no disruption or membrane damage. Nowicki et al. showed that BITC antimicrobial effects did not produce disruption or membrane damage, but they induced the global bacterial stress regulatory system [16]. These results are in concordance with the SEM microphotographs. The oval shape could be explained by some alterations in the exchange of Na^{+}-Ca^{2+} and in the intracellular homeostasis. The septum could be explained by the fact that the cell division could be arrested, as reported by Zhang and partners in their work with isothiocyanates, including BITC [44].

When the incubation was performed at concentration corresponding to the MIC value (Fig. 3c), morphological alterations were more remarkable. Only cell debris was found, and no viable bacteria were identified. This means that the MIC and the minimum bactericidal concentration (MBC) are the same for *E. coli* treated with BITC. This was confirmed by determining the MBC, which turned out to be the same as the MIC. Tyagi and partner had already observed cell debris of *E. coli* when it was treated with lemon EO [45], but in that case, normal cells were mostly observed.

Raman spectroscopy

The different Raman spectra obtained for BITC-treated and non-treated *E. coli* and the active agent BITC can be seen in Fig. 3d. An expanded graphic with the overlapped values of pure BITC and BITC-treated *E. coli* is also shown, where it can be observed that the band detected at 987 cm^{-1} does not correspond to an accumulation of BITC in the treated samples. Even though only eight samples for each type were analysed, the results showed very low variability inside each kind of sample and very clear differences between treated and nontreated samples (Table 2). Regarding to precision, RSD values among the same type of samples were in most cases below 10 %. Student's *t* test was performed and significant differences between both types of samples (treated and nontreated) were obtained, with a value of $p < 0.001$. For this reason, the results can be considered representative of each group of samples.

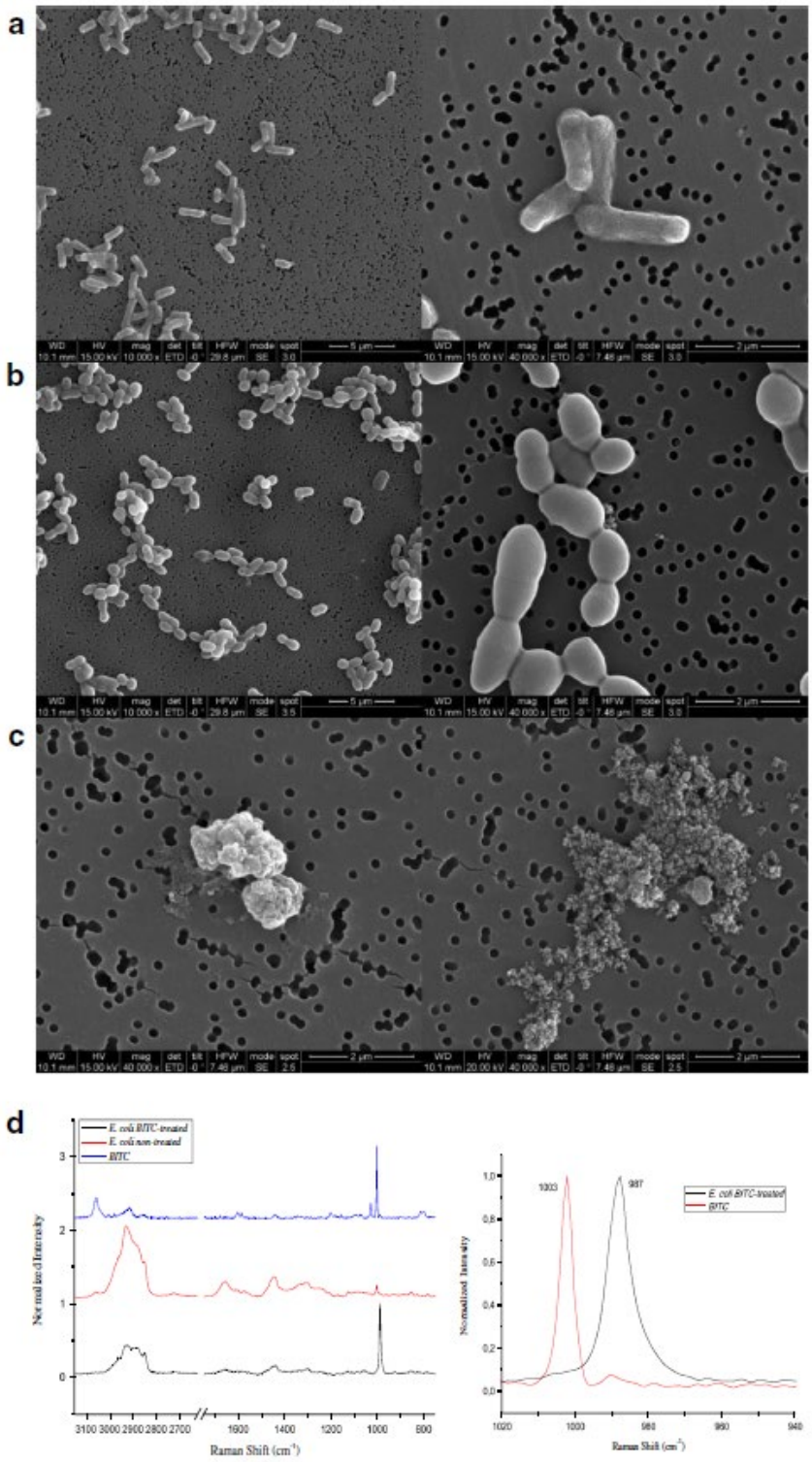


Fig. 3 Scanning electron micrographs of *E. coli* cells. a Untreated cells; b cells after treatment with BITC with subMIC concentration; and c MIC concentration. d Normalised Raman spectra (average of eight samples) of *E. coli* from the study of antibacterial activity in liquid medium: BITC-treated (*bottom*), BITC-treated (*middle*) and pure BITC (*top*)

Table 2 Statistical analysis of Raman band discuss for BITC-treated and non-treated *E. coli*, mean ± devt

Raman band	Non-treated *E. coli*	BITC-treated *E. coli*
987 cm^{-1}	0.098 ± 0.010	0.999 ± 0.002
1002 cm^{-1}	0.180 ± 0.017	0.078 ± 0.001
1336 cm^{-1}	0.206 ± 0.019	0.032 ± 0.000
1441 cm^{-1}	0.287 ± 0.021	0.166 ± 0.002
1658 cm^{-1}	0.234 ± 0.019	0.140 ± 0.001
2932 cm^{-1}	0.992 ± 0.006	0.344 ± 0.002

The biggest difference between non-treated and treated *E. coli* was the appearance of a high intensity Raman shift at 987 cm^{-1}, when bacteria were in contact with BITC (Fig. 3d), which could be due to the presence of phosphate groups [46, 47]. The presence of the phosphate band might be related to the inhibition of ATPase due to the effect of BITC. Inhibition of ATPase would produce an accumulation of phosphate and consequently the interruption of the transport chain of electrons, which could lead to an alteration of intracellular homeostasis. As a result of all these cellular modifications, alterations in the exchange of Na^{+}-Ca^{2+} and in the intracellular homeostasis could take place, which could be associated with the oval size observed for treated *E. coli* in SEM microphotograph (Fig. 3b). The images seen through an optical microscope coincided with those obtained using SEM, both *E. coli* control (usual shape) and those treated under sub-MIC conditions, where oval shape was observed.

Furthermore, this phosphate band might be related to the release of phosphate due to the degradation of DNA and/or RNA backbones [48], and this could be corroborated by the disappearance of the shift at 1336 cm^{-1}. A number of isothiocyanates, including BITC, have been found to induce cell cycle arrest in cultured cells [44] and this fact is in agreement with the effect previously shown in the SEM microphotographs (Fig. 3b) (section “Scanning electron microscopy”),

where the appearance of septum on *E. coli* was observed under sub-MIC condition. The septum could be explained by the fact that the cell division could be arrested.

BITC might be affecting the metabolism of lipids, proteins, carbohydrates, DNA and RNA on *E.coli*, which would explain the decrease or disappearance of the shifts corresponding to these biomolecules in the treated samples such as 1002 cm^{-1} (amino acids) [49–52], 1336 cm^{-1} (polynucleotide chains of DNA purine bases, CH_2/CH_3 wagging and twisting mode in nucleic acid and tryptophan) [50, 51, 53, 54], 1441 cm^{-1} (CH_2 deformation of lipid acyl chains) [55, 56], 1658 cm^{-1} (C=C of lipids, n(C=C)cis of phospholipids and triglycerides) [57] and 2932 cm^{-1} (C-H stretching band, CH_2 and CH_3 groups present in proteins, lipids, nucleic acids and carbohydrates) [28, 58–62]. Nowicki and partner showed that isothiocyanates, such

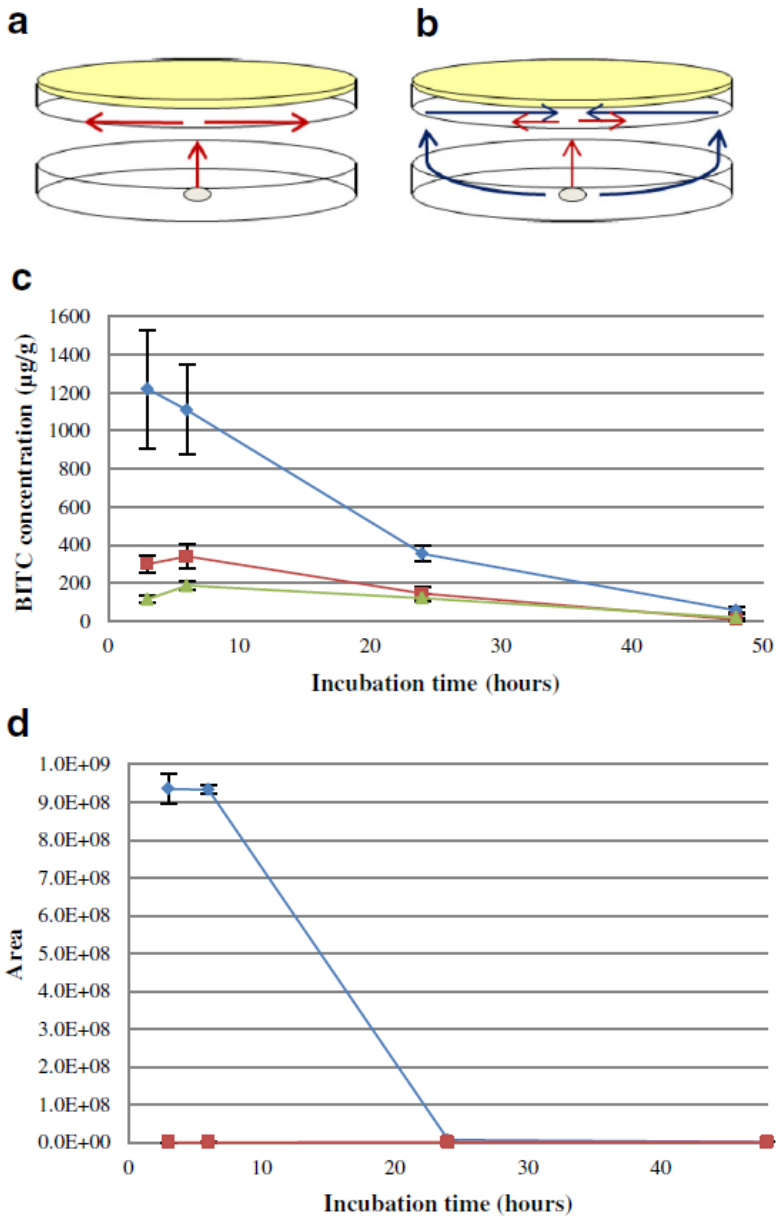
as allyl isothiocyanate (AITC) or benzyl isothiocyanate (BITC), inhibit *E. coli* growth. The mechanism of the mode of action demonstrated by them involved the induction of global bacterial stress regulatory system, and affects major cellular processes too, including nucleic acids synthesis [16]. This was in concordance with the disappearance of the shift corresponding to amino acids. All these results shown on *E. coli* were in agreement with the effect produced by BITC on the mould *A. ochraceus* [27]. Molecular changes observed in Raman spectra and the suggested metabolic changes based on this result agree with those observed by other authors using different techniques as well as with the microphotographs obtained by SEM.

Transfer mechanism of BITC

Transfer mechanisms involve the release of BITC from a filter disk spiked with the EO to the vapour phase, the partition of BITC to the agar phase and finally its diffusion through the agar. This study was carried out to corroborate the presence of inhibition halos in vapour diffusion assays and to know the BITC transfer and diffusion mechanisms. The results help to provide a better understanding of its behaviour within the active packaging developed.

Different transfer mechanisms have been reported in the literature depending on the EO studied. For cinnamaldehyde (major component of cinnamon), the transfer mechanism from the disk to the agar [63] took place from the centre to the exterior of the Petri dish following a normal diffusion model (Fig. 4a). However, a convection model was obtained for AITC (Fig. 4b) [11].

To quantify the concentration of BITC in the different zones of agar, a calibration curve was built by spiking agar plugs with known amounts of BITC. Satisfactory results were obtained for thi s c al ibrat ion curve o f B ITC ($y = 746.34x - 22295$, $r^2 = 0.9973$) with linear range of 12.5– 1250 $mg \cdot kg^{-1}$. Limit of quantification corresponds to the lowest value of the linear range and the limit of detection was 3.75 $mg \cdot kg^{-1}$.



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459 **Fig. 4** a Scheme of normal diffusion model; b scheme of a convection transfer
460 mechanism; c BITC concentration inside the Petri dish in the different areas after
461 incubation at 25 °C with 1 µL of BITC (*diamond*, centre; *square*, half-ratio; *triangle*, 90
462 % ratio); d BITC concentration in the Parafilm tape (*square*) and filter disks (*diamond*)
463 loaded with1 µL of BITC after incubation at 25 °C during different periods of time
464 ranging from 3 to 48 h

465 Figure 4c shows the concentration of BITC in the different zones of the Petri dish
466 (centre, half radius and 90 % radius) at incubation times ranging from 3 to 48 h. After
467 3 h of incubation time, the maximum concentration of BITC was found in the centre of
468 the dish, which means that the transfer of BITC in the vapour phase occurred mainly
469 following direct movements in a linear transfer from the disk to the centre part. After
470 the transfer of compounds from the vapour phase to the agar, BITC diffused across

it, which explained that medium zone had higher concentrations than the exterior zone. This distribution of BITC in the Petri dish is the same as the one previously observed for cinnamon EO, but different from the one observed with AITC, although both compounds have the same isothiocyanate functional group. This different behaviour could be attributed to differences in their volatility (vapour pressure of AITC is 3.697 mmHg at 20 °C and of BITC is 0.0491 mmHg at 25 °C according to ChemSpider chemical database [64]). To sum up, the distribution of the antimicrobial compound followed a gradient with the highest concentration in the centre and a gradual decrease towards the exterior (Fig. 4a). These differences in the transfer mechanisms between AITC and BITC make this later compound a very interesting option as antimicrobial agent because it presents a similar antimicrobial activity and a lower volatility. Thus, a better gradual release control is possible, which makes BITC very appropriate for its incorporation in active packaging.

Distribution of BITC in agar after 6 h of incubation time was the same as that at 3 h but at lower concentrations. When incubation time increased, concentration of BITC decreased, and after 24 h, concentration in all zones was below 20 % of the concentration found at 3 h of incubation time. This decrease of BITC can be attributed to two possible reasons: the loss of compound trough the joints of the Petri dish, even though it was sealed, or the reaction of the compound with agar. In order to test the first hypothesis, a 10-mm sterile blank filter disk spiked with 1 µL of BITC was placed in an empty Petri dish and closed with Parafilm. Disks and Parafilm were separately analysed after 3, 6, 24 and 48 h. Figure 4d shows the evolution of BITC in both materials. No signal of BITC was observed in the sealing Parafilm, what confirmed that there was no loss of BITC through the joints. In the case of a similar experiment previously made using AITC, a high response for AITC was observed in the Parafilm, what confirmed the loss of AITC through the joints due to its high volatility [11]. The results from the disk spiked with BITC showed a decay of the BITC signal at 24 h, confirming that 100 % of the compound was released from the disk and took part in the transfer mechanism. In order to test the second hypothesis, differences in the chromatographic profile (SCAN mode) of BITC overtime were checked. The appearance of a new peak, identified as benzonitrile, was observed. The same experiment carried out previously in our laboratory using AITC did not show any difference in the chromatographic profile across the time. These results indicate that chemical reaction between BITC and agar can take place during the transfer experiment. To our knowledge, this reaction has been never described before.

Active packaging development

According to the results obtained in the previous experiments, BITC at 8 % (*w*/*w*) was selected for the development of new antimicrobial labels using FP and PP as substrate materials. Figure 5a shows the intensity of the BITC signal obtained over time for the two materials. In both cases, high concentrations were observed at least 40 days after their manufacturing. This fact is very interesting because the labels could be used during long time between their manufacturing and their end use.

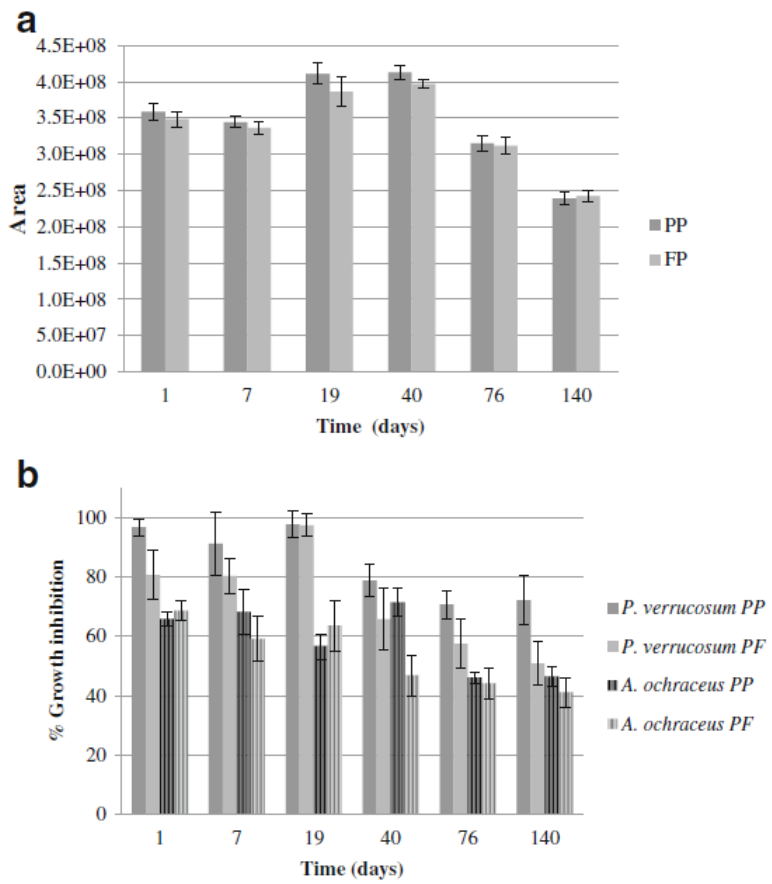

Fig. 5 a Chemical evaluation over time of active labels with 8 % (*w*/*w*) of BITC using two different materials: filter paper (*FP*) and polypropylene (*PP*). b Antimicrobial activity over time of active labels made with PP and FP against *P. verrucosum* and *A. ochraceus*

In order to check possible differences due to the substrates, the antimicrobial activity of BITC labels (8 % *w*/*w*) made with FP and PP was tested against two ochratoxin A-producing moulds (*P. verrucosum* and *A. ochraceus*) [65, 66]. *P. verrucosum* was more sensitive to BITC than *A. ochraceus*, causing greater levels of growth inhibition. This behaviour was observed using either FP (Fig. 5b) or PP (Fig. 5c).

When comparing both substrate materials, active labels manufactured with PP showed a slightly higher antimicrobial activity against the moulds tested, although a similar pattern was observed. Furthermore, a decay of antifungal activity with time was also observed, in agreement with the chemical assessment (Fig. 5a).

Conclusion

This work has shown great differences on the antimicrobial activity among BITC, AITC and cinnamon EO. AITC and BITC exhibited stronger antimicrobial properties than cinnamon EO against foodborne pathogens, in liquid dilution by direct contact, as well as in vapour phase. Besides supporting the activity seen in the broth dilution method, the results of the vapour phase activity open a way for the future application

of BITC in a new antimicrobial label to be incorporated in packaging systems. Screening performed for active labels showed in all cases that moulds were more sensitive than bacteria, and that BITC was the active agent with the highest antimicrobial activity.

SEM and Raman spectroscopy have been shown as powerful techniques for demonstrating the mode of action of antimicrobials on microorganism cells. The action mechanism for BITC on *E. coli* showed its ability to cross the bacterial membrane and exert their bactericidal effect by acting on several cellular targets, but no damage on the membrane was observed. BITC was accumulated in the cells, where it caused alterations in essential cell components such as saccharides, amino acids, proteins, lipids or enzymes, thus impacting several cellular functions such as cell cycle or intracellular homeostasis.

The presence of inhibition halos when BITC was used in vapour diffusion was due to the transfer mechanism from the disk to the agar, which took place from the centre to the exterior of the Petri dish. This was demonstrated by chemical analysis of the agar by HS-SPME-GC-MS.

Due to the type of transfer and action mechanisms, as well as its stronger antimicrobial activity, BITC was incorporated in two kinds of active labels. These labels were stable over the time at least 40 days and still active against two moulds producing Ochratoxin A (OTA), being *P. verrucosum* more sensitive than *A. ochraceus* to the active labels.

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Compliance with ethical standards

Conflict of interest: The authors declare that they do not have potential conflicts of interest.

No human participants or animals are involved in the research

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