Effect of an active label based on benzyl isothiocyanate on the morphology and ochratoxins production of *Aspergillus ochraceus*

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Abstract

The aim of this work was the study of the main effects of benzyl isothiocyanate (BITC) on *A. ochraceus* morphology and on its production metabolism of ochratoxins. This compound was evaluated as active agent of an antimicrobial label in food packaging. Microbiological studies showed a slowdown in mould growth when the active material was applied to *A. ochraceus* and the presence of three different areas of growth. Scanning electron microscopy was successfully used to demonstrate the mode of action of BITC on this strain. *A. ochraceus* exhibited modifications in morphology compared to the control samples such as the disappearance of sclerotia or cleistothecia An extraction protocol and an analytical method by UPLC-MS/MS to determine ochratoxins (OTs) was developed. The results showed that all these morphological changes were related to a decrease on OTs production, both ochratoxin A (OTA) and ochratoxin B (OTB). The presence of BITC caused a great decrease on OTA that modified the OTA/OTB ratio, increasing the OTB proportion. Furthermore, the active packaging also modified the production of other secondary metabolites. The morphological and metabolic effects observed, as well as the relationship between them, are of great interest since they have not been reported before for *A. ochraceus*.

Keywords:

Antimycotoxigenic packaging, active packaging, ochratoxins, benzyl isothiocyanate, *Aspergillus ochraceus*, scanning electronic microscopy.

1. INTRODUCTION

Nowadays, the concern about food contamination by mould has increased, mainly due to two reasons. The first one is the great economic loss, and the second one, the possible production of secondary metabolites, such as mycotoxins, that can be produced by some moulds species (R. A. Samson, Houbraken, Thrane, Frisvad, & Andersen, 2010). The contamination with mycotoxins is produced as a result of inadequate environmental conditions during the harvest operations, storage and product processing. Ochratoxins (OTs), like other mycotoxins, are secondary metabolites produced by some species of the genera *Aspergillus* and *Penicillium*, such as *A. ochraceus* and *P. verrucosum* (Pitt, 1987; R. A. Samson et al., 2010; Vanderme.Kj, Steyn, Fourie, Scott, & Theron, 1965).

A. ochraceus is found predominantly in peanuts, green coffee, grapes and soy beans, as well as sporadically in other stored food commodities, but this fungal species are seldom the cause of a substantial contamination with ochratoxins (J. C. a. T. Frisvad, U. , 2000). Due to its toxicity, OTs maximum concentration limit in several commodities for animal and human consumption is under legal regulation (EFSA, 2004, 2006)Ochratoxins A, B and C (OTA, OTB and OTC) are neurotoxic, nephrotoxic, immunosuppressive, genotoxic, carcinogenic and teratogenic mycotoxins (A. Ravelo Abreu, 2011). The toxicity of OTA has been extensively reviewed by the JECFA (FAO/WHO, 2001) . Its effect towards several animal species has been reported by the International Agency for Research on Cancer, classifying it as possible carcinogen to humans (group 2B) (IARC, 2016).

In the last years, several antifungal packaging with reduction of mould growth had been developed by several authors, but none of them has demonstrated inhibition of mycotoxins synthesis (Martini, Serrano, Barbosa, & Labidi, 2014; Otoni, Soares, da

Silva, Medeiros, & Baffa, 2014; Shemesh et al., 2015; Van Long, Joly, & Dantigny, 2016), and only a few proved to have an antimycotoxigenic effect in relation with the growth inhibition (Manso, 2014; Saladino et al., 2016). For this reason, the results obtained in this work, where the antimycotoxigenic is not only due to the inhibition effect but also to alterations in mould metabolism and morphology, such as the disappearance of sclerotia or cleistothecia, are of great interest for food safety.

Prevention of OTA and other mycotoxins entering the food chain requires specific and rapid diagnostic tools to effectively monitor the critical control points (FAO, 2003). Reliable determination of chemical residues and contaminants from food and feed requires high performance methods, as the compounds are typically present in complex sample matrices at trace levels (Malik, Blasco, & Pico, 2010). For this reason, the analytes extraction from matrices has been stated as the most challenging step in a multi-mycotoxin analysis, as chemically different compounds need to be isolated (Lattanzio, Solfrizzo, Powers, & Visconti, 2007; Sulyok, Berthiller, Krska, & Schuhmacher, 2006).

The present work shows an antifungal and antimycotoxin packaging system, able to act in vapour phase against *A. ochraceus*. The application in vapour phase permits to avoid a direct contact between the packaging material and the food product (Yu-Mei Wu, 2016). The main objective was to reduce or eliminate the ochratoxin production, using an active packaging containing benzyl isothiocyanate (BITC).This compound has been used previously by Troncoso-Rojas et al. to control *Alternaria alternata* growth(Troncoso-Rojas, Sanchez-Estrada, Ruelas, Garcia, & Tiznado-Hernandez, 2005; Troncoso, Espinoza, Sanchez-Estrada, Tiznado, & Garcia, 2005). The new packaging do not only affect the growth inhibition, but also cause some changes in the metabolic pathways of mycotoxins biosynthesis. Due to the strong antifungal activity of BITC,

proved in previous works, and its optimum properties for being incorporated in packaging systems (Clemente, Aznar, Salafranca, & Nerin, 2016), it was selected for evaluating its antimycotoxigenic activity. This way, the application of the active self-adhesive label could be used in any packaging material, which will be converted in an antifungal system.

The experimental plan is divided in two different sections; the first one is the evaluation of the antifungal effect of the active packaging and the study of the action mechanism of BITC on *A. ochraceus*, and the second one contains the chemical evaluation of the antimycotoxigenic effect and the production of metabolites. To achieve this goal, it was necessary to develop a sample extraction technique and a methodology for detection and quantification of ochratoxins, with a high sensitivity that allowed achieving the levels of tolerance of the European Union.

2. MATERIALS AND METHODS

2.1. Mould strain and culture media

Aspergillus ochraceus CECT 2948 from the Spanish Type Culture Collection (CECT), were used. This mould was selected due to its high capacity to produce ochratoxins. As solid media Yeast Extract Agar (YES), Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Czapek-Dox Agar (CZP) provided by Scharlab S.A (Sentmenat, Spain) were used and reconstituted following Samson et al. protocol (R.A. Samson, Houbraken, Thrane, Frisvald, & Andersen, 2010).

2.2. Reagents

Methanol (MeOH) (99.9% LC-MS) and ethyl acetate (HPLC, 99.7%) were purchased to Sigma-Aldrich (Madrid, Spain), dichloromethane (DCM) (GC 99.8%) and formic acid (98-100%) were from Scharlau (Barcelona, Spain) and MilliQ water was obtained from a Millipore system (Milli-Q plus 185).

Ochratoxin A (OTA) (CAS 303-47-9) and Ochratoxin B (OTB) (CAS 4825-86-9), standards were from Sigma-Aldrich. Ochratoxin C (OTC) (CAS 4865-85-4) standard was purchased to LGC Standards (Barcelona, España). All standards were of analytical grade.

Benzyl isothiocyanate (BITC) 98% of quality (CAS 622-78-6), used as active agent, was provided by Sigma-Aldrich. Flow of N_2 (ALPHAGAZ1, 99.999% Global Purity, Air Liquide Zaragoza-Spain) was used to evaporate the extraction solvents.

2.3. Active packaging manufacture

To study the antifungal activity in vapour phase, an active packaging approach was developed. The active agent (BITC) was firstly integrated in a water-based acrylic adhesive using a special formula for self-adhesive labels. Afterwards, a 30 µm polypropylene (PP) film was coated by the active adhesive using a K202 Control Coater 2005 (RK Printcoat Instrument), with a final content of BITC of 8% (w/w). Once the adhesive was dried, a silicone paper was placed over it. Then, the active material was cut into 1.0 x 1.0 cm^2 active labels with self-adhesive properties and these labels were stuck on the lid of the Petri dish when the in vitro tests were performed. More details about the packaging system and the formula cannot be disclosed because of confidential reasons.

2.4. Active and control samples preparation

Mould inoculum was preparated using a previous inoculation on YES incubated during 7 days at 25 ºC of *A. ochraceus.* Fungal conidia was haverested with a sterile swab and transferred into a tube with physiological saline solution (NaCl 0.9%), obtaining a final concentration of 10^6 spores/mL. From this inoculum, 40 µL were seeded into a Petri dish (55 mm) containing 5 mL of the YES culture media. For *active samples*, the active labels were placed at the bottom of the plate, opposite to the culture medium. Then, the plates were sealed using Parafilm® tapes and incubated at 25 °C. The diameter of the inhibition and delay areas was measured using a digital calliper (COMECTA S.A) after the following incubation times: 1, 2, 3, 4, 5, 6, 7, 10, 14 and 21 days. *Control samples* were prepared in the same way as *active samples* but whitout active labels. All assays were performed at least in triplicate, and average and standard deviation were calculated.

2.5. Scanning electron microscopy

Morphological changes in the mould cell structure resulting from the exposure to BITC vapor were studied by Scanning electron microscopy (SEM). Samples were prepared as decribed in section *"2.4. Preparation of active and control samples"*. After 7 days of incubation, plugs of culture medium (YES) were collected with a stainless core (diameter 6 mm) from the agar surface under aseptic conditions. While control samples showed an homogeneus growth, the active samples showed 3 different zones, growth, delay and inhibition, which were separately analyzed. For completion of the SEM observation, the samples were firstly fixed with protein and lipid cross-linking reagents, such as glutaraldehyde, and then dehydrated with a graded ethanol series following the method used by Manso (Becerril, Manso, Nerin, & Gomez-Lus, 2013). The samples

were then dehydrated with a graded ethanol and kept at 4 °C in the last dehydration step. After removing the 100 % ethanol from the samples by evaporation at room temperature, samples were covered with a thin layer of metal (Au/Pd, 15 nm) and examined by SEM at different magnifications. Scanning electron microscopy (SEM) images were acquired using a SEM Inspect™ F50 (FEI Company, Eindhoven, The Netherlands) in an energy range between 0–30 keV.

2.6. Optimization of the OTA extraction protocol from culture media

Optimization of the OTA extraction protocol was performed using Petri dishes (55 mm) that contained 5 mL of the YES culture medium. They were spiked with 50 µL of OTA standard with known concentration. Agar plugs used for the OTA analysis were taken with a stainless core (diameter 6 mm) under aseptic conditions.

The initial procedure was based on Bragulat methodology (Bragulat, Abarca, & Cabanes, 2001) but with slight differences. Different steps were performed to optimize the extraction method:

- Selection of the extraction solvent: Three different extraction solvents were selected according to previous studies: MeOH, MeOH + 1% Formic acid (Bragulat et al., 2001) and the mixture (Ethyl Acetate: DCM: MeOH) (3:2:1) + 1% of Formic acid) (O'Brien et al., 2006).
- Selection of extraction conditions: Solvent extraction of the culture media without agitation (room temperature), with orbital agitation (room temperature) and with sonication (room temperature and 60 ºC), were evaluated.

- Optimization of culture media quantity and solvent volumes: different volumes of agar (3 plugs, 9 plugs and all Petri dish) and extraction solvents (1.5, 3 and 5 mL for 9 plugs of agar) were analysed.
- Optimization of extraction times: 1, 2, 3, 4, 5, 6 and 24 hours were tested.

Controls were prepared following the same procedure described above without OTA addition to the culture media. All assays were performed in quadruplicate.

2.7. Final protocol of OTA extraction from culture media

The whole YES medium present in the Petri dish was collected under aseptic conditions, placed into a vial and weighed (4.98 ± 0.20 g). A volume of 9 mL of the mixture (Ethyl Acetate: DCM: MeOH) (3:2:1) + 1% of Formic acid) was added. The vial was left closed during 4 hours at room temperature. The extract was collected using a syringe and filtered (Acrodisc® Syringe Filter 13mm 0.2 µm GHP). The extracted fraction was transferred into a second vial and it was evaporated under N_2 flow at 50 ºC to dryness. Then, the residue was re-dissolved with 1 mL of MeOH and mixed with 1 mL of milli-Q water.

2.8. Study of matrix effect and OTA recovery

For this study 4 different experiments were performed:

- Culture medium was extracted following the optimized protocol and analyzed by UPLC-MS/MS (A_{blank})
- Culture medium was extracted following the optimized protocol. The final methanol/water extract was spiked with 50 µL of OTA solution (2 µg g^{-1}) and analyzed by UPLC-MS/MS (A_{me})
- Culture medium in liquid state was spiked with 50 µL of OTA solution (2 µg g^{-1}) and then it was extracted following the optimized protocol and analyzed by UPLC-MS/MS (A_{sample})

- A 2 mL aliquot of methanol/water was spiked with 50 µL of OTA solution (2 µg g^{-1}) and analyzed by UPLC-MS/MS (A_{ref})

The next equations were used for matrix effect and recovery percentage calculations:

1. $Matrix\ effect\ (\%) = 100\ x\ \frac{Ame-Ablank}{A\ ref}$ (equation 1)
2. $Recovery\ (\%) = 100\ x\ \frac{Asample-Ablank}{A\ me-Ablank}$ (equation 2)

2.9. Analysis by UPLC-MS(QqQ)

Ultra-high-performance liquid chromatographic (UPLC) analysis was carried out in an Acquity system supplied by Waters (Milford, MA, USA). A Luna Omega Polar C18 1.6 µm particle size (2.1 x 100 mm) column from Phenomenex (Madrid, Spain) was used. Injection volume was 10 µL. Chromatography was carried out at 0.3 mL min^{-1} column flow and 40 ºC column temperature. The mobile phase was water with 0.1 % formic acid (phase A) and methanol with 0.1 % formic acid (phase B). The chromatography started at 30:70 (A: B) (5 minutes), changed to 100:0 (A:B) in 2 minutes and stays at 30:70 for 2 additional minutes. The UPLC was coupled to an ESI probe to the mass spectrometer. A TQ detector with a triple quadrupole mass analyzer supplied by Waters (Milford, MA, USA) was used for the quantification of ochratoxins. Instrumental parameters were as follows: positive ionization, sensitivity mode, capillary at 2.5 kV, sampling cone at 30 V, extraction cone at 4 V, source temperature at 140 ºC, desolvation temperature at 450 ºC, cone gas flow at 50L hr^{-1}, desolvation gas flow at 500L hr^{-1}. Acquisition was carried out in multiple reaction monitoring (MRM) mode. The MRM transitions were optimized infusing individual standard solutions of each analyte in the UPLC-MS(QqQ) system. The transitions used are shown in table 1. For the quantification, calibration curves were built using solutions of the corresponding standards in methanol/water. Data were recorded using MassLynx v4.1 software.

2.10. Analysis by UPLC-QTOF-MS and identification of *Aspergillus* secondary metabolites.

UPLC was performed at the same conditions as in section "*2.9 Analysis by ultra-performance liquid chromatography–mass spectrometry with triple quadrupole mass analyzer [UPLC-MS(QqQ)]".* A Xevo G2 QTOF mass spectrometer supplied by Waters (Milford, MA, USA) was used for the identification of *Aspergillus* secondary metabolites. Instrumental parameters were as follows: positive ionization, sensitivity mode, capillary at 2.5 kV, sampling cone at 30 V, extraction cone at 4 V, source temperature at 120 ºC, desolvation temperature at 450 ºC, cone gas flow at 20L hr^{-1}, desolvation gas flow at 650L hr^{-1}. Acquisition was performed in MS^E mode, as this mode allows both low and high collision energies (CE) in the collision cell during the same run, and thus provides two kinds of mass spectra of the compounds. The low energy (CE at 4 V) spectra provide information about the precursor ion and high energy (CE ramp: from 15 to 30 V) spectra provide information about fragment ions. For the identification of the compounds detected, the following methodology was used. First, the elemental composition of the precursor ion was determined using the low energy spectrum. For this purpose, the exact mass and the isotopic pattern of the precursor ion and the elemental compositions proposed by Masslynx were compared. Those elemental compositions with a low mass error and a good isotopic fit were selected. Afterwards, the elemental composition was linked to a chemical structure using Chemspider database [www.chemspider.com]. The selection of candidates from the chemical database was made according to the chemical criteria and background knowledge of the analyst about microbiology. Finally, the selection of the best candidate was carried out using the high energy spectrum of the compounds detected. For this purpose, the MassFragment tool from MassLynx was used. This tool allowed the comparison of high

energy mass spectra of the unknown peaks and the candidate. For each fragment ion detected in the spectrum, MassFragment provides a fragment structure and a score (S) with values between 1 and 14, the lower the value, the more plausible is the structure proposed. When a candidate structure explained at least two main fragment ions of the spectrum with scores values below 3, the candidate was considered *confirmed by MassFragment*. A complete confirmation is only achieved when the pure standard is injected and the candidate is confirmed by comparison of the retention time and mass spectrum.

3. RESULTS AND DISCUSSION

3.1. Antifungal effect of BITC active labels

The growth of *A. ochraceus* in control and active samples exposed to the BITC active label was evaluated. In active samples, 3 different concentric areas of growth were observed, they presented a different colour depending on the mould growth: the *growth zone*, in brown colour, due to the aerial mycelium and sporulation, was placed in the external area; the *delay zone* was the intermediate area and showed white colour, due to the vegetative mycelium, and the *inhibition zone* was in the internal area and did not shown any visual growth. (Figure 1a –image at left). The different halos were measured and transformed to percentage area related to the overall area of the plate.

In contrast, control samples showed a homogeneous growth throughout the Petri dish at all the tested times (Figure 1a-image at right). It was also observed that the density and the appearance of cleistothecia and sclerotia increased over time. As far as the authors know, this could be the first report of the ascospores or cleistothecia production by *A. ochraceus*. Ascospores are the characteristic spores of the largest group of fungi, the

Ascomycota or Ascomycetes. *Aspergillus ochraceus*, as well as *A. fumigatus* or *A. flavus*, are members of Ascomycota. Figure 1b shows a zoom picture of control sample where sclerotia and cleistothecia can be observed, what makes sense since sclerotia are usually present on *A. ochraceus* (Robert A. Samson, Hoekstra, & Frisvad, 2004; Weber, 2007). Members of the genus *Aspergillus* produce their ascospores in cleistothecia, an spherical structure formed by survival cells under adverse conditions. Each cleistothecia may contain up to 100000 asci, each enclosing (with very rare exceptions) eight spores (Dyer & O'Gorman, 2012) (figure 1b) Afterwards, cleistothecia were isolated, plates were inoculated with the aid of a sterile swab. Mould growth was observed confirming the presence of available spores. The presence of these tissues confirmed that *A. ochraceus* presented a sexual reproduction. Thus, two successive sexual developmental programmes were apparent in the *aspergilli*; the first related to the initial formation of supporting accessory tissues needed for sex and the second to the subsequent production of cleistothecia and ascospore (O'Gorman, Fuller, & Dyer, 2009).

The production of mould secondary metabolites, such as ochratoxins, and morphological moulds modifications have been shown to be intimately associated to the genetic level. Literature is mainly focused on the co-regulation of secondary metabolites production with conidiation, sporulation or formation of sexual fruiting bodies (cleistothecia). However, many of these genetic links also control sclerotial production (Calvo & Cary, 2015).

Figure 1c shows the percentage of each growth area (inhibition, delay and growth) related to the overall Petri dish surface in active samples over time. This kind of growth is in agreement with the transfer mechanism of BITC previously studied (Clemente, Aznar, Salafranca, et al., 2016). The transfer mechanisms of BITC takes place from the centre to the exterior of the Petri dish, for this reason, the central area (inhibition) is

more affected by BITC than the exterior of the plate (growth). In the central part of the plate a lethal concentration was present, as it was observed in the SEM micrographs, where non-viable spores from the inoculum were observed. On the other hand, the concentration in the medium and external zones of the plate was not enough for the lethality as it can be seen in Figure 1a and 2. The outer zones grown and expanded along the plate, so the inhibition decreased over time. It was not seen any sclerotia or clestothecia formation in the treated samples over time. After 1 day, the inhibition area was 100% of the Petri dish, while after the two following days (day 2 and 3) the inhibition area decreased but it still reached values above 90% of the plate. On day 2, it was observed a delay area, while on day 3 a small growth area was also perceived (3.6 %). Between days 4 and 14, the growth area was practically the same (20 %), although there was an increase of the delay area (from 10.8 % to 38.6 %) and therefore a decrease in inhibition. Finally, on day 21, the growth area reached a value of 59.1% of the plate.

3.2. Effects of BITC active labels on *A. ochraceus* cellular morphology

In order to check the presence of morphological alterations caused by the BITC active labels on *A. ochraceus* cellular morphology and therefore to gain further knowledge about the BITC mechanism of action, scanning electron microscopy (SEM) experiments were carried out.

Clestothecia and sclerotia were only observed in control plates (figure 1b). However since dehydration steps are necessary during sample preparation process for SEM fixation, sclerotia may be affected due to their structural weakness. Thus, it was not possible to evaluate it by SEM, only cleistothecia was evaluated by this technology.

Figure 2 shows the scanning electron micrographs of *A.ochraceus* after 7 days of incubation at different magnifications, without the active label (a), and in presence of

the active label containing BITC in the three different areas: the growth area (b), placed in the external area of the plate; the delay area (c), placed in the intermedium area of the plate; and the inhibition area (d), placed in the centre of the plate. In control samples, it was observed a high density of fungal growth as well as the formation of cleistothecia, vegetative mycelium, aerial mycelium with conidiophores and a great amount of spores (a).

In active samples, both vegetative and aerial mycelium were observed in the growth area (Figure 2b), but with lower density than in control plates (figure 2a). The main effect of active agent was the absence of cleistothecia that was present in control samples. This means that the active packaging inhibited the sexual reproduction of *A. ochraceus,* and it would be reprogrammed at genetic level for asexual reproduction.

As a consequence, there will be a decrease on next generation diversity and therefore on its ability for a quick adaptation to environmental changes, going down their chances for long-term survival. This reproductive change could be due to diverse causes. Previous research made using Raman Imaging spectroscopy showed the effect on DNA/RNA (loss of genetic functionality), as well as the effect of the oxidative phosphorylation and the electron transport chain on *A. ochraceus* because of BITC active agent (Clemente, Aznar, & Nerín, 2016). The importance of phosphorus concentration in cell mould has been much less studied, but low phosphorus concentrations suppress sexual development, presumably due to a requirement for phosphorus in the generation of ATP (Bussink & Osmani, 1998). Other authors reported that BITC cause the induction of DNA damage and cell cycle arrest in cultured cells (Kassie, Pool-Zobel, Parzefall, & Knasmuller, 1999; Zhang, 2004).

With regard to the aerial mycelium observed in the microphotographs (figure 2b 12000x), some conidiophores with spores can be observed, but with a different spore shape than in control samples, even presenting holes in its sheath. This new shape could be due to a loss of the cell structure, possibly related to changes in the fluidity of the cellular wall and to some alterations in the exchange of Na^{+}-Ca^{2+} in the intracellular homeostasis (Clemente, Aznar, & Nerín, 2016; Clemente, Aznar, Salafranca, et al., 2016; Dufour, Stahl, & Baysse, 2015; Xiao, Vogel, & Singh, 2006). The holes observed in some spores would not be due to a direct effect of the BITC in its sheath, but to an internal effect that would generate pores in them. This meant that BITC effect on *A. ochraceus* spores was not homogeneous and that the affectation level depended on the spore observed. On the other hand, hyphae of aerial mycelium had spicules covering the whole surface like in control samples (figure 2a 12000x), although some effects can be observed on the thickness and shape of the hyphae (figure 2b 12000x). These results are in agreement with the two different levels of affectation observed in *A. ochraceus* spores and hyphae exposed to BITC in previous work performed with Raman Imaging spectroscopy (Clemente, Aznar, & Nerín, 2016). The results observed are also consistent with other authors, who observed a decrease in the number of conidiophores and sporulation and abnormalities in the thickness and shape of the hyphae. (Becerril et al., 2013; Qian et al., 2016).

In the microphotographs obtained of the delay area (Figure 2c), only vegetative mycelium was observed (hyphae). There were neither conidiophores nor spores and, theoretically, production of secondary metabolites such as ochratoxins was not expected. It was also observed that the hyphae of this area had a very irregular morphology (figure 2c 6000-12000x) and that there was an aggregation between them and also inside them, giving as result some bumps and crevices. Internal aggregation on

hyphae of *A. ochraceus* was previously observed using TEM (Transmission Electronic Microscopy), due to the effect of bacillomycin D (Qian et al., 2016). These results demonstrated that the action mechanism of BITC happened in the inside of the cell, since no disruption or cell wall damage was observed. Nowicki et al. reported that BITC antimicrobial effects did not produce disruption or membrane damage, generated the induction of global stress regulatory system (Nowicki, Rodzik, Herman-Antosiewicz, & Szalewska-Palasz, 2016). These results are in agreement with SEM microphotographs.

The internal zone of Petri dish (figure 2d) corresponded to the inhibition area. The area of agar taken for SEM microphotographs was close to the delay area. It can be observed that vegetative mycelium was poor and with a very similar appearance to that observed in the delay area, but with greater affectation with bumps and crevices due to internal aggregation (figure 2d 6000-12000X). Comparing figure 2c (120000x) and figure 2d (120000x), it can be seen that the internal effects is similar to the one observed in the delay area. The second conclusion was that in the inhibition area, spores were severely damaged; these spores would not come from sporulation, but from the inoculum.

BITC was accumulated in the mould 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 respiration, metabolism or cell cycle (Clemente, Aznar, & Nerín, 2016). All these metabolic changes will affect the production of OTA, what makes BITC a great antifungal and also antimycotoxigenic agent.

3.3. Effects of BITC labels on OTs production

In order to evaluate the antimycotoxigenic effect of the active label, the evolution of ochratoxins production with and without BITC as active agent was studied. First, an

extraction protocol of OT from culture medium and a sensitive analytical method were optimized.

3.3.1. Optimization of the ochratoxins extraction protocol

Optimization of the protocol was performed with OTA since it was the main OT produced by *A. Ochraceus*. In each experiment, the recovery percentage (%) was calculated and used for the selection of the best conditions.

According to the results obtained, the mixture ethyl acetate: DCM: MeOH (3:2:1) with 1% of formic acid was the best extraction solvent for OTA (54.4 ± 1.9 %), since for the other 2 solvents, MeOH or MeOH with 1% formic acid, low recovery and reproducibility were observed.

Once the extraction solvent was determined, several extraction conditions were tested. Recovery percentages obtained were: 59.4 ± 5.7 at room temperature without agitation, 58.6 ± 6.1 at room temperature with orbital agitation, 59.9 ± 6.5 at room temperature with sonication and 62.7 ± 6.0 when sonication was combined with heat. Since there were not significant differences among them ($p>0.1$, student t-test), room temperature without agitation was selected.

The next step was the optimization of the agar quantity in order to achieve higher sensitivity in real samples. For this purpose, three different agar quantities were tested: 3 plugs (0.26g ± 0.03), 9 plugs (0.73g ± 0.06) and all culture medium of the Petri dish (5.44g ± 0.29). The volume of extraction solvent was added ensuring that agar was completely covered by it (0.5, 1.5 and 9 mL). The recoveries obtained were: 59.4 ± 5.7 for three plugs, 73.8 ± 9.3 for nine plugs and 72.4 ± 4.5 for all the plate. The agar volume selected was nine plugs for the following optimization steps and the complete plate for real samples in order to have a better sensitivity.

The next step was to check if better recoveries could be obtained increasing the rate of extraction solvent/agar. Several volumes were tested for 9 agar plugs, 1.5 mL, 3 mL and 5 mL. The following recoveries were obtained: 73.8 ± 9.3% (1.5 mL), 68.1 ± 7.9 % (3 mL) and 74.7 ± 8.6 % (5mL). Since there were not significant differences when solvent volume increased ($p>0.1$, student t-test), the same agar/solvent rate proposed by Smedsgaard and Bragulat was used (Bragulat et al., 2001; Smedsgaard, 1997).

Finally, the optimum extraction time was determined and the results are shown in figure 3. After 4 hours of extraction time, recoveries reached its maximum values, 95.3 ± 4.8 %. After this time the recovery percentage was stable. Final extraction protocol is described in "*section 2.7. Final protocol for OTA extraction from culture media".*

3.3.2. Matrix effect *(*%)

The matrix effect percentage obtained applying the formula described in "*section 2.8 . Study of matrix effect and OTA recovery"* was 96.2 (± 2.0) %, what means that there were minor matrix effects in the experiment. Values above 100% would indicate a signal enhancement of the target analyte due to matrix composition, whereas values below 100% would indicate a signal reduction (Gosetti, Mazzucco, Zampieri, & Gennaro, 2010). Recovery results for ochratoxin A following the extraction protocol and taken into account matrix effect, were 97.8 (± 3.4) %.

3.3.3. Ochratoxins detection and quantification

The next step was to quantify the concentration of ochratoxins produced by *Aspergillus* mould in presence or absence of the active agent (BITC) over time. Three ochratoxins were studied, OTA (ochratoxin A), OTB (ochratoxin B) and OTC (ochratoxin C). The quantification was performed from calibration with their pure standards.

The linearity of the calibration curves was assessed using a twelve-point calibration curve. Table 2 shows the analytical parameters of the method, linear range, determination coefficient, LOQ and LOD expressed as μg/kg. Very satisfactory results were obtained, with detection limits below 1 ppb level (0.11 to 0.29 μg/Kg).

OTA and OTB were previously quantified by other authors using LC-MS (Jung et al., 2015; Meng et al., 2014; Sulyok, Krska, & Schuhmacher, 2007) and HPLC-FLD (Bragulat et al., 2001) in several matrices, but LODs achieved were above the LOD values reached in this work.

Since during the initial stage of fungal growth, the secondary metabolites are present at trace levels, developing a sensitive detection method for ochratoxin A, B, and C is crucial for the study of its metabolism.

Furthermore, the limits of detection achieved with this procedure are below the maximum allowed values in food established by EU legislation (EC, 2005, 2010) and the daily intakes determined by EFSA (EFSA, 2006) that have been also incorporated in the European Commission EC/1881/2006 (EC, 2006).

There is no specific regulation for maximum daily intakes of Ochratoxins B and C, but some authors have shown that they have certain toxicity, although is lower than OTA toxicity (Heussner & Bingle, 2015). For this reason, a high sensitive method for their detection is also necessary.

3.4. Antimycotoxigenic effect of active packaging

Firstly, different culture media were tested (PDA, YES, MEA and CPZ) in order to assure a maximum production of OTA by *A. ochraceus*. The signal obtained for OTA using the optimized extraction protocol was very different depending on the culture

medium used. The order following the OTA production was as follows: YES>>MEA>>CPZ-PDA. Sclerotia development in *A. ochraceus* is greatly affected by the nutrient source (Paster & Chet, 1980) and this fact explains the differences on mycotoxins production. The production of mycotoxins, have been associated with the fungal structure "sclerotia", which in addition to perform biological mould functions it is a place of mycotoxins accumulation (Calvo & Cary, 2015). For this reason, the culture medium selected for evaluation of active packaging was YES that in addition was the medium selected for the optimization according to bibliography. As the growth of *A. ochraceus* was very heterogeneous in the three different zones of the plate, the extraction for the evaluation of ochratoxins production was performed using the full Petri dish.

The results of ochratoxins content in control and active samples are shown in Figure 4. The ochratoxins concentration was expressed as nanograms of OT per colony forming units (cfu) of *A. ochraceus* inoculated at time 0 (ng/cfu). This way the results are related to cfu coming from the inoculum, the same in all cases. The concentration was expressed as average of three independent replicates and very good reproducibility was achieved.

The results for OTA production in both control and active samples can be seen in figure 4a. The data showed a clear reduction in the OTA production due to the use of the new active packaging. Maximum OTA values were found in control samples on days 3 and 4 (30 ng/cfu). After 4 days, OTA production decreased and, at day 14, it stabilized at 16.9 ng/cfu. In active samples, OTA was in all cases below 3 ng/cfu. In addition, the production of OTA in active samples was inhibited during the first incubation days. This fact is remarkable because at day 3, control samples had produced the maximum amount of mycotoxin registered during the experiment. From the beginning till day 10,

the reduction in the production of OTA when the active agent was used was higher than 99%, and from day 10 it was above 85% in all cases.

OTB results can be observed in figure 4b. Control samples showed a similar evolution in the production of OTB to that observed for OTA (Figure 4a), but they showed a lower maximum concentration (15.90 ng/cfu) and they stabilized at a lower value (5 ng/cfu). OTB production in active samples also showed a similar pattern to OTA, with a slowdown in the mycotoxin production and lower values until day 14. Reduction of OTB production in active samples compared to control samples was always above 85%. However, in this case, OTB content after 14 incubation days, was very similar in active and control samples, even slightly superior when the active agent was used.

OTC was in all cases below its LOD (0.29 μg/Kg). This can be due to different reasons, such as a low production of OTC by *A. ochraceus* or a quick transformation of OTC to OTA.

It is also interesting to carry out a joint analysis of both ochratoxins production. Figure 4c shows the evolution of OTs content overtime in control and active samples. The results obtained about the antifungal effect of active packaging showed that even there was a growth delay compared to control samples, the growth area increased overtime. However, this growth should produce a higher OTs content than that measured in this experiment. This difference was due to the cell damage observed in SEM experiments.

In all cases, the overall OTs content was lower in active than in control samples, showing the biggest differences during the initial incubation days. The reduction of OTs production in presence of active agent was superior to the 95% till day 10, 76% at day 14 and 60% at day 21. Another important difference observed between active and control samples was the rate between OTA and OTB content. While in control samples

OTA was the main OT produced, as other authors had previously observed (Harris & Mantle, 2001), in active samples the main OT produced was OTB. This is also a very interesting result, since based on a toxicity ranking reported by Muller et al. (Muller et al., 2004), OTA is at least three times more toxic than OTB. In control samples, the proportion ratio OTA:OTB was 2:1 from day 2 to 10 and 3.5:1 at days 14 and 21. This result was in agreement with the ratio observed by Harris et al. after 80h of incubation (Harris & Mantle, 2001). However, in active samples, the ratio OTA:OTB was mostly 1:12 from day 4 to10, 1:6.5 at day 14 and 1:2 at day 21.

This reversion in the production of OTA and OTB, due to an affectation on *A. ochraceus* caused by the BITC released from the active packaging, could be due to different effects of BITC on the mould. The biosynthesis of these secondary metabolites (OTB and OTA) is complex, since it involves a large number of genes, enzymes and compounds, and both mycotoxins are connected (Gallo et al., 2012).

The effect of active packaging on *A. ochraceus* could be explained according to 2 hypotheses. On one hand, the effects might occur at the level of DNA, modifying the areas related to the metabolic pathway, thus reducing the translation or transcription of key genes (*pks* and *nrps*) (Gallo et al., 2012), that keep the necessary information restricted. This would explain the reduction observed in the overall production of ochratoxins (OTA and OTB). On the other hand, the step of the metabolic pathway where OTB is converted to OTA would be performed by a *chloroperoxidase* (CPO) and a *OTA-shyntase* (enzyme of P450 family) (János Varga, 2009). Furthermore, Harris et al. observed that OTB may be produced by dechlorination of OTA (Harris & Mantle, 2001). BITC could affect the enzymes that produce the conversion of OTB in OTA, *OTA-shyntase* and *CPO*, into a greater or lesser extent. Several authors have reported the effect of BITC on enzymes of the P450 family (*OTA-shyntase*) (Clemente, Aznar, &

Nerín, 2016; Goosen, Mills, & Hollenberg, 2001; Tiznado, Hernández, Troncoso, & Rojas, 2006). In relation to *CPO*, Sundaramoorthy et al. reported that when a ligand SNC (sulphur nitrogen carbon) was linked to the active centre of this enzyme (similar to P450 enzyme), the final complex was unstable (Sundaramoorthy, Terner, & Poulos, 1998) and as consequence, the enzyme would not be functional. The active group of BITC is SCN and it could cause the same effect in both enzymes. This enzymatic effects would explain the inversion in the ratio OTA:OTB, since the conversion of OTB in OTA would be affected.

3.5. Other secondary metabolites

The development of standardized methods for the identification of secondary metabolites not only constitutes a powerful tool in chemotaxonomy, but also in the discovery of new metabolites in fungal extracts. More than 1984 metabolites have been already reported from *Aspergillus sp.* (J. C. Frisvad, 2015). The extracts of *A. ochraceus* in control and active samples were analysed by ultra-performance liquid chromatography-quadrupole time of flight mass spectrometry (UPLC-QTOF-MS). Figure 5 shows the chromatograms of control and active extracts at different incubation times **(**3, 10 and 21 days) and table 3 the main compounds identified and confirmed by MassFragment®.

The compound detected with the highest intensity, was the antibiotic presussin (nº 16), which showed a major production in the extract of *A. ochraceus* exposed to the active packaging from day 14 to 21. This way, the increment on antibiotic production was a further advantage of the active packaging, in addition to the decrease on OTs production. Preussin inhibits human cancer cell growth, within a range of different human cancer cell lines, shows potent growth-inhibitory properties, which are due to an

antiproliferative effect and the induction of programmed cell death (Tatjana V. Achenbach, 2000). Other antibiotics in addition to Preussin were also detected: Avrainvillamide (Antibiotic CJ-17665) (nº 14) and Penicillic acid (nº 3). Avrainvillamide inhibits growth of multi-drug resistant *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Enterococcus faecalis*n (Sugie et al., 2001). Penicillic acid is a well-known antibiotic with neuritogenic, antibacterial and antifungal activity (Kang & Kim, 2004; Komagata D, 1996; Nguyen Van Long & Dantigny, 2016).

Other compound with a great relevance in this work was phenylalanine (nº 2). This amino acid is one of the precursors in the synthesis pathway of OTA and OTB. It was detected in a greater proportion in the active samples than in the controls, supporting the results obtained in section *3.2.3. Antimycotoxigenic effect of active packaging*

Four benzodiazepine alkaloids were also detected, Circumdatin A, C, G and F (nº 13, 5, 7 y 8). They are among the most common peptide derivatives found in nature, and are very common in *Aspergillus ochraceus* (Brase, Encinas, Keck, & Nising, 2009) They have shown to exhibit antiviral, antibacterial, antifungal, and antitumor activities (Graz, Hunt, Jamie, Grant, & Milne, 1999).

Other compounds detected were: Grenadamide (nº 12), a cyclopropil-containing fatty acid metabolites that exhibits modest cannabinoid receptor-binding activity (Sitachitta & Gerwick, 1998); Frehmaglutoside I (nº 11), a glycoside that exhibits protective effects against DOX-induced cardiotoxicity (Li et al., 2015); Cyclo (L-Pro-L-Leu) (nº 4) that inhibits aflatoxin production by *A. parasiticus* (Yan et al., 2004) and shows an antifungal activity on *A. flavus* and *A. niger* (Kumar, Mohandas, & Nambisan, 2013); Ile-phe-thr-pro (nº 10), a hydrophobic tetra-peptide, often showing affinity and specificity for a variety of receptors in protein-protein signalling; Stachydrine or proline

betaine (nº 1), that provides protection from stress by contributing to cellular osmotic adjustment, ROS detoxification, and protection of membrane integrity and enzymes/protein stabilization (Hayat S, 2012). N-dodecyl diethanolamine (nº 17), a bacteriostatic and bactericidal towards *Escherichia coli* (Smith & Lambert, 2008); Oleamide (nº 19), a primary fatty acid that is used industrially as lubricants and antiblocking agents that it can be produced by micoorganisms (Kwon et al., 2001; Levinson, Kuo, & Kurtzman, 2004); N-Phenethylundecanamide (nº 9), a derivate of 2-Phenylethylamine that can be produced by microorganism like *Xenorhabdus doucetiae* (Press, 2016) and finally Apo-10'-licopenol (nº 6) that is a lycopene derivative.

Figure 5 shows that the production of the identified compounds overtime was different in control and active samples. These results corroborated that the active packaging did not only act as an inhibitor of OTs, but also modified mould metabolism related to other metabolites production.

4. CONCLUSION

Active labels based on BITC have demonstrated to have antifungal as well as antimycotoxigenic activity against *A. ochraceus* mould. Microbiological studies showed a slowdown in mould growth over time when the active labels were applied, and SEM images showed a considerable cell damage caused by contact with BITC. The active agent was accumulated in the mould cells where it caused morphological alterations in more or less grade depending on the proximity to the active agent. In the growth area, several effects were observed such as the inhibition of the sexual reproduction, changes in spores shape and apparition of holes in its sheath and the decrease on the number of conidiophores and sporulation. In the delay area, no conidiophores or spores were

observed and therefore, no production of secondary metabolites such as ochratoxins was expected. Finally, inhibition area presented severely damaged spores and, as consequence, no mould growth over time. All these changes affected, among other cellular functions, the production of OTs, what makes BITC a great antimycotoxigenic agent. To measure the OT content in the culture media, a sample extraction method was optimized obtaining very high recoveries (97.3 %). Extracts were analyzed by UPLC-MS/MS achieving LODs below 1 μg/Kg. The presence of active labels caused a decrease on OTs production, especially on the most toxic one, OTA. In addition, it modified the ratio of OTA/OTB production, increasing the OTB proportion. Once the efficiency of the BITC active label as antimycotoxigenic packaging has been demonstrated in *in vitro* studies, the next step would be to perform *in vivo* studies. All these results and the fact that this active label is economic and easy to be incorporated in the packaging, make it a very attractive solution to improve the food safety.

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6. BIBLIOGRAPHY

A. Ravelo Abreu, C. R. A., A. J. Gutiérrez Fernández y A. Hardisson de la Torre. (2011). Revisión La ocratoxina A en alimentos de consumo humano: revisión *Nutrición Hospitalaria, 26*(6), 1215-1226. doi: DOI:10.3305/nh.2011.26.6.5381

Achenbach, T. V., Slater, E. P., Brummerhop, H., Bach, T., & Muller, R. (2000). Inhibition of cyclin-dependent kinase activity and induction of apoptosis by preussin in human tumor cells. *Antimicrobial Agents and Chemotherapy, 44*(10), 2794-2801. doi: Doi 10.1128/Aac.44.10.2794-2801.2000

Akpunarlieva, S. N. (2016). *Quantitative proteomic and metabolomic characterization of glucose transporter mutant promastigotes of Leishmania mexicana.* University of Glasgow.

Becerril, R., Manso, S., Nerin, C., & Gomez-Lus, R. (2013). Antimicrobial activity of Lauroyl Arginate Ethyl (LAE), against selected food-borne bacteria. *Food Control, 32*(2), 404-408. doi: DOI 10.1016/j.foodcont.2013.01.003

Bragulat, M. R., Abarca, M. L., & Cabanes, F. J. (2001). An easy screening method for fungi producing ochratoxin A in pure culture. *Int J Food Microbiol, 71*(2-3), 139-144. doi: Doi 10.1016/S0168-1605(01)00581-5

Brase, S., Encinas, A., Keck, J., & Nising, C. F. (2009). Chemistry and Biology of Mycotoxins and Related Fungal Metabolites. *Chemical Reviews, 109*(9), 3903-3990. doi: 10.1021/cr050001f

Buckingham, J., Baggaley, K. H., Roberts, A. D., & Szabé, L. F. (2010). *Dictionary of alkaloid*.

Bussink, H. J., & Osmani, S. A. (1998). A cyclin-dependent kinase family member (PHOA) is required to link developmental fate to environmental conditions in Aspergillus nidulans. *Embo Journal, 17*(14), 3990-4003. doi: DOI 10.1093/emboj/17.14.3990

Calvo, A. M., & Cary, J. W. (2015). Association of fungal secondary metabolism and sclerotial biology. [Review]. *Frontiers in Microbiology, 6*(62), 1-12. doi: 10.3389/fmicb.2015.00062

Ciegler, A. (1972). Bioproduction of ochratoxin A and penicillic acid by members of the Aspergillus ochraceus group. *Canadian Journal of Microbiology, 18*(5), 631-636. doi: 10.1139/m72-100

Clemente, I., Aznar, M., & Nerín, C. (2016). Raman imaging spectroscopy as a tool to investigate the cell damage on Aspergillus ochraceus caused by an antimicrobial packaging containing Benzyl isothiocyanate *Analytical Chemistry, 88*(9), 4772–4779. doi: 10.1021/acs.analchem.6b00116

Clemente, I., Aznar, M., Salafranca, J., & Nerin, C. (2016). Raman spectroscopy, electronic microscopy and SPME-GC-MS to elucidate the mode of action of a new antimicrobial food packaging material. *Analytical and Bioanalytical Chemistry, 409*(4), 1037-1048. doi: 10.1007/s00216-016-0022-y

Cheng Wang, X. T., Qiao Yang, Yanan Lu, Liyan Ma, Hangliang Huang, Chengqi Fan. (2014). Diversity of Secondary Metabolites from Two Antartic Microbes Rhodococcus sp. NJ-008 and Pseudomonas sp. NJ-011. *Open Journal of MArine Science, 4*, 214-220. doi: http://dx.doi.org/10.4236/ojms.2014.43020

Dai, J. R., Carte, B. K., Sidebottom, P. J., Yew, A. L. S., Ng, S. B., Huang, Y. C., & Butler, M. S. (2001). Circumdatin G, a new alkaloid from the fungus Aspergillus ochraceus. *Journal of Natural Products, 64*(1), 125-126. doi: Doi 10.1021/Np000381u

Delgado de la Torre, M., Priego-Capote, F., & Luque de Castro, M. (2015). Tentative identification of polar and mid-polar compounds in extracts from wine lees by liquid chromatography-tandem mass spectrometry in high-resolution mode. *Journal of Mass Spectrometry, 50*(6), 826-837. doi: 10.1002/jms.3592.

Deng, W., & Overman, L. E. (1994). Synthesis Applications of Cationic Aza-Cope Rearrangements .27. Enantioselective Total Synthesis of Either Enantiomer of the Antifungal Antibiotic Preussin (L-657,398) from (S)-Phenylalanine. *Journal of the American Chemical Society, 116*(25), 11241-11250. doi: Doi 10.1021/Ja00104a005

Dufour, V., Stahl, M., & Baysse, C. (2015). The antibacterial properties of isothiocyanates. *Microbiology+, 161*(2), 229-243. doi: doi:10.1099/mic.0.082362-0

Dyer, P. S., & O'Gorman, C. M. (2012). Sexual development and cryptic sexuality in fungi: insights from Aspergillus species. *Fems Microbiology Reviews, 36*(1), 165-192. doi: 10.1111/j.1574-6976.2011.00308.x

EC. (2005). *Commission Regulation (EC) No 123/2005*. Official Journal of the European Union: Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32005R0123.

EC. (2006). *Reglamento (CE) N o 1881/2006 de la Comisión*. Official Journal of the European Union: Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32005R0123.

EC. (2010). *Commission Regulation (EC) No 105/2010*. Official Journal of the European Union: Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32005R0123.

EFSA (2004) Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission related to ochratoxin A (OTA) as undesirable substance in animal feed.

EFSA (2006) Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to Ochratoxin A in food, 365 C.F.R. .

FAO, R. (2003). *Manual sobre la aplicación del sistema de análisis de peligros y de puntos críticos de control (APPCC) en la prevención y control de las micotoxinas* (Vol. 73): Estudio FAO: Alimentación y Nutrición.

FAO/WHO. (2001). Ochratoxin A. In W. H. Organisation (Ed.), *Safety evaluation of certain mycotoxins in food* (Vol. 47, pp. 281-387). Geneva, Switzerland: WHO Food Additives Series

Frisvad, J. C. (2015). Taxonomy, chemodiversity, and chemoconsistency of Aspergillus, Penicillium, and Talaromyces species. *Frontiers in Microbiology, 5*(773), 1-7. doi: Artn 77310.3389/Fmicb.2014.00773

Frisvad, J. C. a. T., U. . (2000). Mycotoxin production by common filamentous fungi. In E. S. H. R.A. Samson, J.C. Frisvad and O. Filtenborg (Ed.), *Introduction to food- and airborne fungi* (Sixth Edition ed., pp. 389). Utrecht, The Netherlands: Centraalbureau voor Schimmelcultures.

Furtado, N. A. J. C., Pupo, M. T., Carvalho, I., Campo, V. L., Duarte, M. C. T., & Bastos, J. K. (2005). Diketopiperazines produced by an Aspergillus fumigatus Brazilian strain. *Journal of the Brazilian Chemical Society, 16*(6B), 1448-1453. doi: Doi 10.1590/S0103-50532005000800026

Gallo, A., Bruno, K. S., Solfrizzo, M., Perrone, G., Mule, G., Visconti, A., & Baker, S. E. (2012). New Insight into the Ochratoxin A Biosynthetic Pathway through Deletion of a Nonribosomal Peptide Synthetase Gene in Aspergillus

carbonarius. *Applied and Environmental Microbiology, 78*(23), 8208-8218. doi: 10.1128/Aem.02508-12

Goosen, T. C., Mills, D. E., & Hollenberg, P. F. (2001). Effects of benzyl isothiocyanate on rat and human cytochromes P450: Identification of metabolites formed by P4502B1. *Journal of Pharmacology and Experimental Therapeutics, 296*(1), 198-206.

Gorcica, H. J., Peterson, W. H., & Steenbock, H. (1934). The chemistry of mould tissue: Fractionation of the nitrogen in the mycelium of Aspergillus fischeri. *Biochemical Journal, 28*(2).

Gosetti, F., Mazzucco, E., Zampieri, D., & Gennaro, M. C. (2010). Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A, 1217*(25), 3929-3937. doi: 10.1016/j.chroma.2009.11.060

Graz, M., Hunt, A., Jamie, H., Grant, G., & Milne, P. (1999). Antimicrobial activity of selected cyclic dipeptides. *Pharmazie, 54*(10), 772-775.

Harris, J. P., & Mantle, P. G. (2001). Biosynthesis of ochratoxins by Aspergillus ochraceus. *Phytochemistry, 58*(5), 709-716. doi: Doi 10.1016/S0031-9422(01)00316-8

Hayat S, H. Q., Alyemeni MN, Wani AS, Pichtel J, Ahmad. (2012). Role of proline under changing environments: A review. *Plant Signaling & Behavior, 7*(11), 1456-1466. doi: doi:10.4161/psb.21949

Heussner, A. H., & Bingle, L. E. H. (2015). Comparative Ochratoxin Toxicity: A Review of the Available Data. *Toxins, 7*(10), 4253-4282. doi: 10.3390/toxins7104253

Hong, H. X., Han, Y. L., Xin, X. Q., & Shi, Y. F. (1995). Essesa - an Expert-System for Structure Elucidation from Spectra .6. Substructure Constraints from Analysis of C-13-Nmr Spectra. *Journal of Chemical Information and Computer Sciences, 35*(6), 979-1000.

Hong, H. X., & Xin, X. Q. (1994). Essesa - an Expert-System for Structure Elucidation from Spectra .5. Substructure Constraints from Analysis of First-Order H-1-Nmr Spectra. *Journal of Chemical Information and Computer Sciences, 34*(6), 1259-1266. doi: Doi 10.1021/Ci00022a006

IARCS Monographs on the Evaluation of Carcinogenic Risks to Human (2016).

János Varga, S. K., Zsanett Péteri and Robert S. Samsom (2009). An Overview of ochratoxins research. In M. Rai (Ed.), *Apply mycology* (pp. 38-55).

Jung, S. Y., Choe, B. C., Choi, E. J., Jeong, H. J., Hwang, Y. S., Shin, G. Y., & Kim, J. H. (2015). Survey of mycotoxins in commonly consumed Korean grain products using an LC-MS/MS multimycotoxin method in combination with immunoaffinity clean-up. *Food Science and Biotechnology, 24*(4), 1193-1199. doi: 10.1007/s10068-015-0153-5

Kang, S. W., & Kim, S. W. (2004). New antifungal activity of penicillic acid against Phytophthora species. *Biotechnology Letters, 26*(9), 695-698. doi: 10.1023/B:BILE.0000024090.96693.a4

Kassie, F., Pool-Zobel, B., Parzefall, W., & Knasmuller, S. (1999). Genotoxic effects of benzyl isothiocyanate, a natural chemopreventive agent. *Mutagenesis, 14*(6), 595-603. doi: DOI 10.1093/mutage/14.6.595

Komagata D, F. S., Yamashita N, Saito S, Morino T. (1996). Novel neuritogenic activities of pseurotin A and penicillic acid. *Journal of Antibiotics, 49*(9), 958-959.

Kumar, S. N., Mohandas, C., & Nambisan, B. (2013). Purification of an antifungal compound, cyclo(L-Pro-D-Leu) for cereals produced by Bacillus cereus subsp thuringiensis associated with entomopathogenic nematode. *Microbiological Research, 168*(5), 278-288. doi: 10.1016/j.micres.2012.12.003

Kwon, H. J., Hwang, S. E., Han, J. T., Kim, C. J., Rho, J.-R., & Shin, J. (2001). Production of oleamide, a functional lipid, by Streptomyces sp. KK90378. *Journal of microbiology and biotechnology, 11*(6), 1018-1023.

Lattanzio, V. M. T., Solfrizzo, M., Powers, S., & Visconti, A. (2007). Simultaneous determination of aflatoxins, ochratoxin A and Fusarium toxins in maize by liquid chromatography/tandem mass spectrometry after multitoxin immunoaffinity cleanup. *Rapid Communications in Mass Spectrometry, 21*(20), 3253-3261. doi: 10.1002/rcm.3210

Levinson, W. E., Kuo, T. M., & Kurtzman, C. P. (2004). *Production of Fatty Amides via Biocatalysis–A Brief Overview.* Paper presented at the The 36th ACS Great Lakes Regional Meeting 2004.

Li, M., Wang, X. L., Zheng, X. K., Wang, J. C., Zhao, W., Song, K., . . . Feng, W. S. (2015). A New Ionone Glycoside and Three New Rhemaneolignans from the Roots of Rehmannia glutinosa. *Molecules, 20*(8), 15192-15201. doi: 10.3390/molecules200815192

Malik, A. K., Blasco, C., & Pico, Y. (2010). Liquid chromatography-mass spectrometry in food safety. *Journal of Chromatography A, 1217*(25), 4018-4040. doi: 10.1016/j.chroma.2010.03.015

Manso, S. (2014). *Combinación de técnicas analíticas y microbiológicas para el estudio de un envase activo antifúngico, frente a mohos alterantes de alimentos.* Zaragoza.

Martini, R., Serrano, L., Barbosa, S., & Labidi, J. (2014). Antifungal cellulose by capsaicin grafting. *Cellulose, 21*(3), 1909-1919. doi: 10.1007/s10570-014-0219-1

Meng, H., Wang, Z. H., De Saeger, S., Wang, Y., Wen, K., Zhang, S. X., & Shen, J. Z. (2014). Determination of Ochratoxin A in Cereals and Feeds by Ultra-performance Liquid Chromatography Coupled to Tandem Mass Spectrometry with Immunoaffinity Column Clean-up. *Food Analytical Methods, 7*(4), 854-864. doi: 10.1007/s12161-013-9692-7

Muller, G., Burkert, B., Moller, U., Diller, R., Rohrmann, B., Rosner, H., & Kohler, H. (2004). Ochratoxin A and some of its derivatives modulate radical formation of porcine blood monocytes and granulocytes. *Toxicology, 199*(2-3), 251-259. doi: 10.1016/j.tox.2004.01.040

Nguyen Van Long, N., & Dantigny, P. (2016). Fungal Contamination in Packaged Foods. In J. Barros-Velázquez (Ed.), *Antimicrobial Food Packaging* (pp. 45-64). Elsevier: Nikki Levy.

Nowicki, D., Rodzik, O., Herman-Antosiewicz, A., & Szalewska-Palasz, A. (2016). Isothiocyanates as effective agents against enterohemorrhagic Escherichia coli: insight to the mode of action. *Scientific Reports, 6*. doi: Artn 2226310.1038/Srep22263O'Brien, M., Nielsen, K. F., O'Kiely, P., Forristal, P. D., Fuller, H. T., & Frisvad, J. C. (2006). Mycotoxins and other secondary metabolites produced in vitro by Penicillium paneum Frisvad and Penicillium roqueforti Thom isolated from baled grass silage in Ireland. *Journal of Agricultural and Food Chemistry, 54*(24), 9268-9276. doi: 10.1021/jf0621018

O'Gorman, C. M., Fuller, H. T., & Dyer, P. S. (2009). Discovery of a sexual cycle in the opportunistic fungal pathogen Aspergillus fumigatus. *Nature, 457*(7228), 471-U475. doi: 10.1038/nature07528

Otoni, C. G., Soares, N. D. F., da Silva, W. A., Medeiros, E. A. A., & Baffa, J. C. (2014). Use of Allyl Isothiocyanate-containing Sachets to Reduce Aspergillus flavus Sporulation in Peanuts. *Packaging Technology and Science, 27*(7), 549-558. doi: 10.1002/pts.2063

Paster, N., & Chet, I. (1980). Effects of Environmental-Factors on Growth and Sclerotium Formation in Aspergillus-Ochraceus. *Canadian Journal of Botany-Revue Canadienne De Botanique, 58*(16), 1844-1850.

Pitt, J. I. (1987). Penicillium-Viridicatum, Penicillium-Verrucosum, and Production of Ochratoxin-A. *Appl Environ Microb, 53*(2), 266-269.

Press, C. (2016). Dictionary of natural products. Retrieved 15/12/2016 http://dnp.chemnetbase.com/dictionary-search.do?method=view&id=11963955&si=

Qian, S. Q., Lu, H. D., Sun, J., Zhang, C., Zhao, H. Z., Lu, F. X., . . . Lu, Z. X. (2016). Antifungal activity mode of Aspergillus ochraceus by bacillomycin D and its inhibition of ochratoxin A (OTA) production in food samples. *Food Control, 60*, 281-288. doi: 10.1016/j.foodcont.2015.08.006

Rahbaek, L., Breinholt, J., Frisvad, J. C., & Christophersen, C. (1999). Circumdatin A, B, and C: Three new benzodiazepine alkaloids isolated from a culture of the fungus Aspergillus ochraceus. *Journal of Organic Chemistry, 64*(5), 1689-1692. doi: Doi 10.1021/Jo981536u

Raimo Mikkola, M. A., Maria Hautaniemi and Mirja Salkinoja-Salonen. (2015). Toxic indole alkaloids avrainvillamide and stephacidin B produced by a biocide tolerant indoor mold Aspergillus westerdijkiae. *Toxicon, 99*, 58-67. doi: 10.1016/j.toxicon.2015.03.011

Reynaud, E. (2009). *Synthese organique d'apo-lycopenoïdes etude des proprietes antioxydantes et de complexation avec l'albumine de serum humain.* Universite D'Avignon et des Pays de Vaucluse.

Saladino, F., Manyes, L., Luciano, F. B., Manes, J., Fernandez-Franzon, M., & Meca, G. (2016). Bioactive compounds from mustard flours for the control of patulin production in wheat tortillas. *Lwt-Food Science and Technology, 66*, 101-107. doi: 10.1016/j.lwt.2015.10.011

Samson, R. A., Hoekstra, E. S., & Frisvad, J. C. (2004). Identificstion of the common food-borne fungi. In R. A. Samson, E. S. Hoekstra & J. C. Frisvad (Eds.), *Introduction to Food-Borne Airborne Fungi* (Seventh Edition ed., pp. 1-282). The Nertherlands.

Samson, R. A., Houbraken, J., Thrane, U., Frisvad, J. C., & Andersen, B. (2010). *Food and Indoor Fungi*.

Samson, R. A., Houbraken, J., Thrane, U., Frisvald, J. C., & Andersen, B. (2010). *Food and Indoor Fungi*: CBS-KNAW Fungal Biodiversity Centre.

Schwartz, R. E., Liesch, J., Hensens, O., Zitano, L., Honeycutt, S., Garrity, G., . . . Monaghan, R. (1988). L-657,398, a Novel Antifungal Agent - Fermentation, Isolation, Structural Elucidation and Biological Properties. *Journal of Antibiotics, 41*(12), 1774-1779.

Shemesh, R., Krepker, M., Goldman, D., Danin-Poleg, Y., Kashi, Y., Nitzan, N., . . . Segal, E. (2015). Antibacterial and antifungal LDPE films for active packaging. *Polymers for Advanced Technologies, 26*(1), 110-116. doi: 10.1002/pat.3434

Sitachitta, N., & Gerwick, W. H. (1998). Grenadadiene and grenadamide, cyclopropyl-containing fatty acid metabolites from the marine cyanobacterium Lyngbya majuscula. *Journal of Natural Products, 61*(5), 681-684. doi: Doi 10.1021/Np970576a

Smedsgaard, J. (1997). Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. *Journal of Chromatography A, 760*(2), 264-270. doi: Doi 10.1016/S0021-9673(96)00803-5

Smith, A. R. W., & Lambert, P. A. (2008). Antimicrobial action of N-(n-dodecyl)diethanolamine on Escherichia coli: effects on enzymes and growing cultures. *Journal of Applied Microbiology, 105*(6), 2161-2168. doi: 10.1111/j.1365-2672.2008.03963.x

Sugie, Y., Hirai, H., Inagaki, T., Ishiguro, M., Kim, Y. J., Kojima, Y., . . . Kojima, N. (2001). A new antibiotic CJ-17,665 from Aspergillus ochraceus. *Journal of Antibiotics, 54*(11), 911-916.

Sulyok, M., Berthiller, F., Krska, R., & Schuhmacher, R. (2006). Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Communications in Mass Spectrometry, 20*(18), 2649-2659. doi: 10.1002/rcm.2640

Sulyok, M., Krska, R., & Schuhmacher, R. (2007). A liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application to semi-quantitative screening of moldy food samples. *Analytical and Bioanalytical Chemistry, 389*(5), 1505-1523. doi: 10.1007/s00216-007-1542-2

Sundaramoorthy, M., Terner, J., & Poulos, T. L. (1998). Stereochemistry of the chloroperoxidase active site: crystallographic and molecular-modeling studies. *Chemistry & Biology, 5*(9), 461-473. doi: Doi 10.1016/S1074-5521(98)90003-5

Tatjana V. Achenbach, E. P. S., Harm Brummerhop, Thorsten Bach and Rolf Müller. (2000). Inhibition of Cyclin-Dependent Kinase Activity and Induction of Apoptosis by Preussin in Human Tumor Cells. *Antimicrobial Agents and Chemotherapy, 40*(10), 2794-2801. doi: 10.1128/AAC.44.10.2794-2801.2000

Tiznado, Hernández, M.-E., Troncoso, & Rojas, R. (2006). Control of fungal diseases with isothiocyanates. *Stewart Postharvest Review, 2*(1), 1-14. doi: 10.2212/spr.2006.1.4

Troncoso-Rojas, R., Sanchez-Estrada, A., Ruelas, C., Garcia, H. S., & Tiznado-Hernandez, M. E. (2005). Effect of benzyl isothiocyanate on tomato fruit infection development by Alternaria alternata. *Journal of the Science of Food and Agriculture, 85*(9), 1427-1434. doi: 10.1002/jsfa.2129

Troncoso, R., Espinoza, C., Sanchez-Estrada, A., Tiznado, M. E., & Garcia, H. S. (2005). Analysis of the isothiocyanates present in cabbage leaves extract and their potential application to control Alternaria rot in bell peppers. *Food Research International, 38*(6), 701-708. doi: 10.1016/j.foodres.2005.02.004

Tseng, M. C., Yang, H. Y., & Chu, Y. H. (2010). Total synthesis of asperlicin C, circumdatin F, demethylbenzomalvin A, demethoxycircumdatin H, sclerotigenin, and other fused quinazolinones. *Organic & Biomolecular Chemistry, 8*(2), 419-427. doi: 10.1039/b910545j

Vadla, N. C., Davalagar, V. D., & Sripadi, P. (2013). Detection and characterization of N-alkyl diethanolamines and N-2-alkoxyethyl diethanolamines in milk by electrospray ionization mass spectrometry. *Metabolomics, 9*(3), 623-630. doi: 10.1007/s11306-012-0492-7

Van Long, N. N., Joly, C., & Dantigny, P. (2016). Active packaging with antifungal activities. *International Journal of Food Microbiology, 220*, 73-90. doi: 10.1016/j.ijfoodmicro.2016.01.001

Vanderme.Kj, Steyn, P. S., Fourie, L., Scott, D. B., & Theron, J. J. (1965). Ochratoxin a a Toxic Metabolite Produced by Aspergillus Ochraceus Wilh. *Nature, 205*(4976), 1112-1113. doi: Doi 10.1038/2051112a0

Visagie, C. M. V., J.; Houbraken, J.; Meijer, M.; Kocsubé, S.; Yilmaz, N.; Fotedar, R.; Seifert, K.A.; Frisvad, Jens Christian; Samson, R.A. (2014). Ochratoxin production and taxonomy of the yellow aspergilli (Aspergillus section Circumdati). *Studies in Micology, 78*, 1-61. doi: 10.1016/j.simyco.2014.07.001

Weber, J. W. a. R. W. S. (2007). Introduction. In Cambridge (Ed.), *Introdution to Fungi* (Third ed., pp. 1-32).

Wen, J., Kong, W. J., Hu, Y. C., Wang, J., & Yang, M. H. (2014). Multi-mycotoxins analysis in ginger and related products by UHPLC-FLR detection and LC-MS/MS confirmation. *Food Control, 43*, 82-87. doi: 10.1016/j.foodcont.2014.02.038

Witt, A., & Bergman, J. (2001). Total syntheses of the benzodiazepine alkaloids circumdatin F and circumdatin C. *Journal of Organic Chemistry, 66*(8), 2784-2788. doi: Doi 10.1021/Jo001696h

Xiao, D., Vogel, V., & Singh, S. V. (2006). Benzyl isothiocyanate-induced apoptosis in human breast cancer cells is initiated by reactive oxygen species and regulated by Bax and Bak. *Molecular Cancer Therapeutics, 5*(11), 2931-2945. doi: Doi 10.1158/1535-7163.Mct-06-0396

Yan, P. S., Song, Y., Sakuno, E., Nakajima, H., Nakagawa, H., & Yabe, K. (2004). Cyclo(L-leucyl-L-prolyl) produced by Achromobacter xylosoxidans inhibits aflatoxin production by Aspergillus parasiticus. *Applied and Environmental Microbiology, 70*(12), 7466-7473. doi: 10.1128/Aem.7012.7466-7473.2004

Yu-Mei Wu, Z.-W. W., Chang-Ying Hud & Cristina Nerín. (2016). Influence of Factors on Release of Antimicrobials from Antimicrobial Packaging Materials. *Critical Reviews in Food Science and Nutrition, online: 13 Oct 2016,* . doi: DOI:10.1080/10408398.2016.1241215 2016

Zhang, Y. S. (2004). Cancer-preventive isothiocyanates: measurement of human exposure and mechanism of action. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 555*(1-2), 173-190. doi: DOI 10.1016/j.mrfmmm.2004.04.017

Table 1: MS/MS parameters for the analysis of target analytes in positive ionization mode, precursor ion, voltages in the sample cone (CV) and in the collision cell (CE) and MRM (multiple reaction monitoring) transitions

Target compounds	Precursor Ion	CV-CE (V)	MRM transitions
Ochratoxin A	404.3	30 - 25	404.3 > 221.1
			404.3 > 239.2
			404.3 > 341.3
Ochratoxin B	370.2	30 - 25	370.2 > 324.6
			370.2 > 205.2
Ochratoxin C	432.9	30 - 25	432.9 > 239.18

Table 2: Analytical parameters of OTs analysis by UPLC-MS/MS, retention time (RT), equation of the regression lines, determination coefficient, linear range and limits of detection (LOD) and quantification (LOQ).

Compound	RT	Equation of the regression line	Determination coefficient (R^2)	Linear range µg/Kg	LOQ µg/Kg	LOD µg/Kg
Ochratoxin A	5.48	y = 104.17 x + 272.38	0.9995	4400.16 - 0.36	0.36	0.11
Ochratoxin B	5.04	y = 479.13 x - 1042.2	0.9993	884.04 - 0.54	0.54	0.16
Ochratoxin C	6.07	y = 116.82 x + 958.17	0.9996	1390.71 - 0.97	0.97	0.29

Table 3: Secondary metabolites detected in control and treated samples with the active packaging using UPLC-QTOF-MS, retention time (RT), Exact mass, molecular formula, possible candidate, daughter, MassFragment score and References.

Nº	RT	Mass (Da)	Molecular formula	Candidate	Daughter (Da)	Score	References
1	0.87	$[M-H]^+$ 144.1025	$C_7H_{13}NO_2$	Stachydrine	144.1015 118.0851	0.0 3.0	(Buckingham, Baggaley, Roberts, & Szabé, 2010; Gorcica, Peterson, & Steenbock, 1934)
2	1.20	$[M-H]^+$ 166.0868	$C_9H_{11}NO_2$	L-phenylalanine (phe)	120.0806 91.0530	1.0 1.0	(Delgado de la Torre, Priego-Capote, & Luque de Castro, 2015)
3	1.83	$[M-H]^+$ 171.0654	$C_8H_{10}O_4$	Penicillic acid	125.0603 153.0561	1.0 0.5	(Ciegler, 1972; J. C. Frisvad, 2015; Visagie, 2014)
4	2.21	$[M-H]^+$ 211.1439	$C_{11}H_{18}N_2O_2$	Cyclo-(L-Pro-L-Leu)	114.0905 98.0590	2.0 2.0	(Furtado et al., 2005). (Yan et al., 2004)
5	2.70	$[M-H]^+$ 308.1037	$C_{17}H_{13}N_3O_3$	Circumdatim C	146.0259 93.0345	3.0 3.0	(Rahbaek, Breinholt, Frisvad, & Christophersen, 1999; Tseng, Yang, & Chu, 2010; Witt & Bergman, 2001)
6	2.75	$[M-H]^+$ 380.3085	$C_{27}H_{39}O$	Apo-10'-lycopenol			(Reynaud, 2009)
7	2.88	$[M-H]^+$ 308.1033	$C_{17}H_{13}N_3O_3$	Circumdatim G	265.0618 291.0783	3.0 3.0	(Dai et al., 2001; Visagie, 2014)
8	3.25	$[M-H]^+$ 292.1086	$C_{17}H_{13}N_2O_2$	Circumdatim F	249.0675 275.0862	3.0 3.0	(Tseng et al., 2010; Witt & Bergman, 2001)
9	3.37	$[M-H]^+$ 290.2483	$C_{19}H_{31}NO$	N-Phenethylundecanamide	272.2373 142.1587	2.0 3.0	(Hong, Han, Xin, & Shi, 1995; Hong & Xin, 1994)
10	3.44	$[M-H]^+$ 477.2717	$C_{24}H_{36}N_4O_6$	Ile-phe-thr-pro	261.1240 346.1754	2.0 1.0	(Akpunarlieva, 2016)
11	3.54	$[M-Na]^+$ 447.2719	$C_{22}H_{38}O_9$	Frehmaglutoside I			(Li et al., 2015)
12	3.74	$[M-H]^+$ 316.2638	$C_{21}H_{33}NO$	Grenadamide	298.2531 224.1955	2.0 1.0	(Buckingham et al., 2010; Sitachitta & Gerwick, 1998)
13	3.80	$[M-H]^+$ 394.1391	$C_{21}H_{19}N_3O_5$	Circumdatim A	379.1153 123.0442	0.5 2.5	(Rahbaek et al., 1999; Tseng et al., 2010)
14	3.87	$[M-H]^+$ 446.2086	$C_{26}H_{27}N_3O_4$	Avrainvillamide Antibiotic CJ 17665	414.1810 429.2050	3.0 1.0	(Raimo Mikkola, 2015; Sugie et al., 2001)
15	3.90	$[M-H]^+$ 370.1277	$C_{20}H_{19}NO_6$	Ochratoxin B	205.0494 187.0388	0.5 2.5	(Sulyok et al., 2007)
16	4.00	$[M-H]^+$ 318.2794	$C_{21}H_{35}NO$	Preussin Antibiotic L-657,398	226.2171 91.0533	1.0 1.0	(Achenbach, Slater, Brummerhop, Bach, & Muller, 2000; Deng & Overman; Schwartz et al., 1988)
17	4.25	$[M-H]^+$ 274.2729	$C_{16}H_{35}NO_2$	N-dodecyl diethanolamine	256.2624 88.0777	0.5 1.0	(Vadla, Davalagar, & Sripadi, 2013)
18	4.39	$[M-H]^+$ 404.2308	$C_{20}H_{18}ClNO_6$	Ochratoxin A	358.0833 386.0786	1.0 0.5	(Visagie, 2014; Wen, Kong, Hu, Wang, & Yang, 2014)
19	6.02	$[M-H]^+$ 282.2787	$C_{18}H_{35}NO$	Octadecenoic amide (oleamide)	69.0690 111.1031	2.0 3.0	(Cheng Wang, 2014)

Figure captions

Figure 1: a) *A. ochraceus* plate after 7 days of incubation, with and without an active label containing BITC, **b)** magnification of the control plate and **c)** evolution of percentages of inhibition, delay and growth areas over time in plates with *A. ochraceus* and active label.

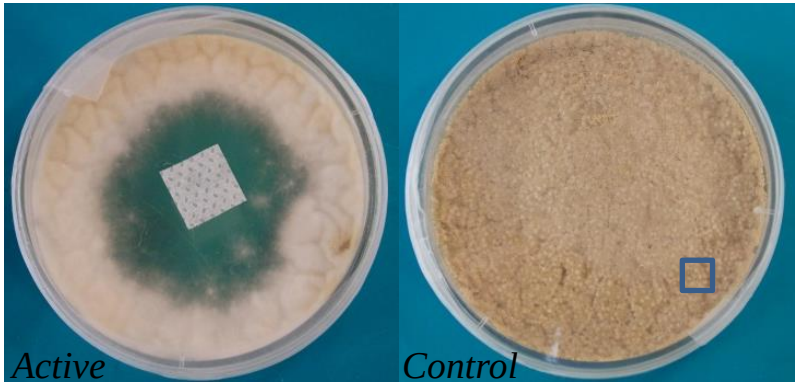
Figure 2: Scanning electron micrographs of *A.ochraceus* after 7 days of incubation. Untreated (**a)**, and treated with the active packaging containing BITC, growth area **(b),** delay area **(c)** and growth inhibition area **(d)**

Figure 3: OTA recovery percentage at different extraction times..

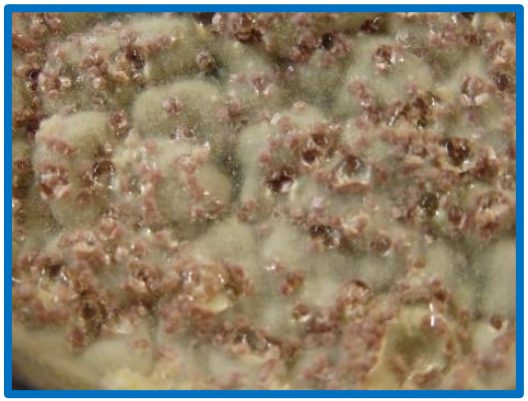
Figure 4: a) Ochratoxin A concentration (ng/CFU) in control (♦) and active (■) samples at different incubation times **b)** Ochratoxin B concentration (ng / CFU) on control (♦) and active (■) samples at different incubation times **c)** overall ochratoxins concentration (ng/CFU) at different incubation times in control (dark colours) and active samples (light colours).

Figure 5: Chromatograms of control and active extracts obtained by UPLC-QTOF-MS at different incubation times **a)** 3 days, **b)** 10 days and **c)** 21 days. Green colour corresponded to control samples and red colour to active samples. The number corresponds with the candidates proposed: 1) Stachydrine, 2) L-phenylalanine (phe), 3) Penicillic acid. 4) Cyclo-(L-Pro-L-Leu), 5) Circumdatim C, 6) Apo-10'-lycopenol, 7) Circumdatim G, 8) Circumdatim F. 9) N-Phenethylundecanamide, 10) Ile-phe-thr-pro, 11) Frehmaglutoside I, 12) Grenadamide, 13) Circumdatim A, 14) Avrainvillamide., 15) Ochratoxin B, 16) Preussin, 17) N-dodecyl diethanolamine, 18) Ochratoxin A and 19) Octadecenoic amide (oleamide).

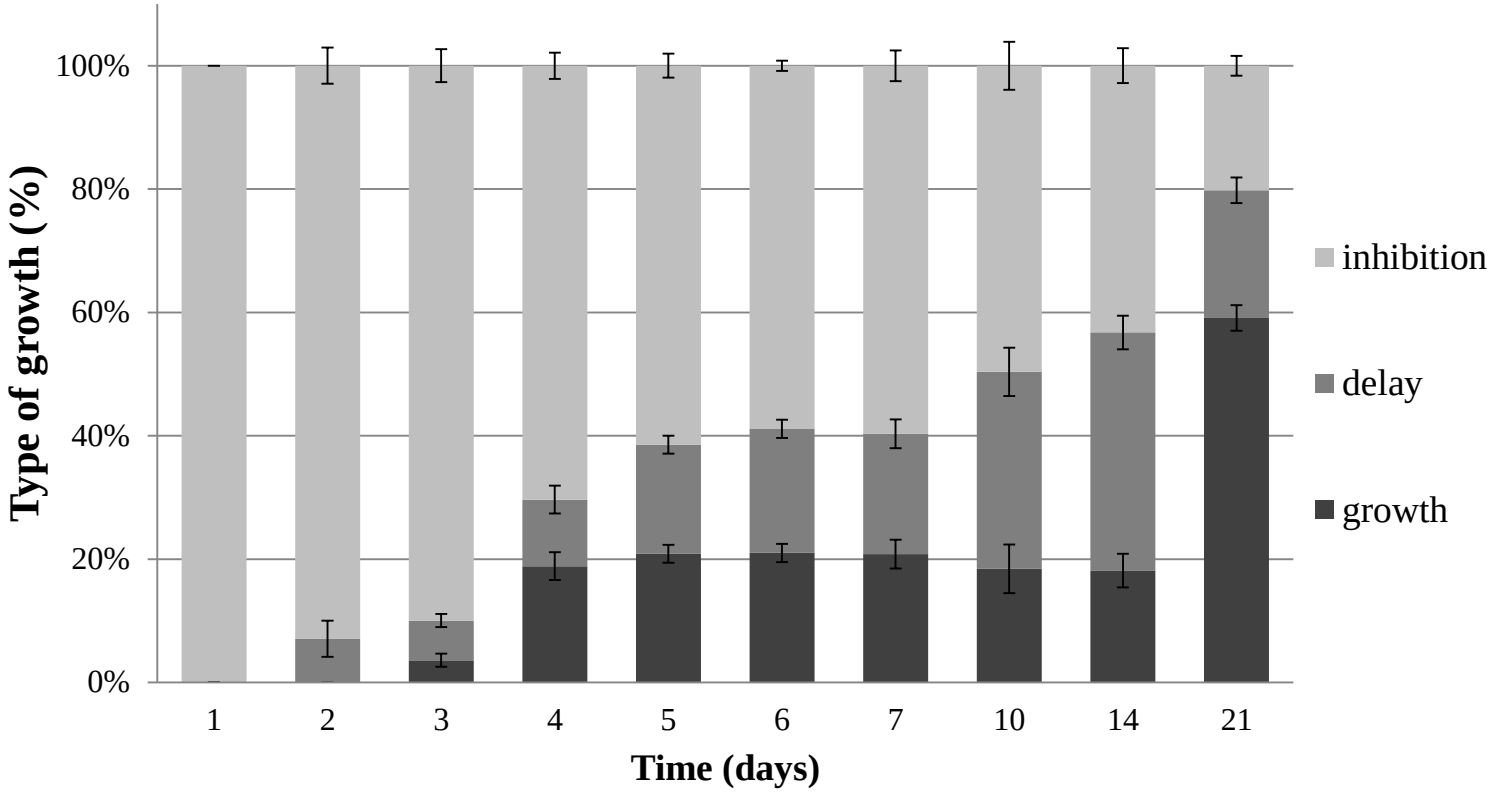
a)

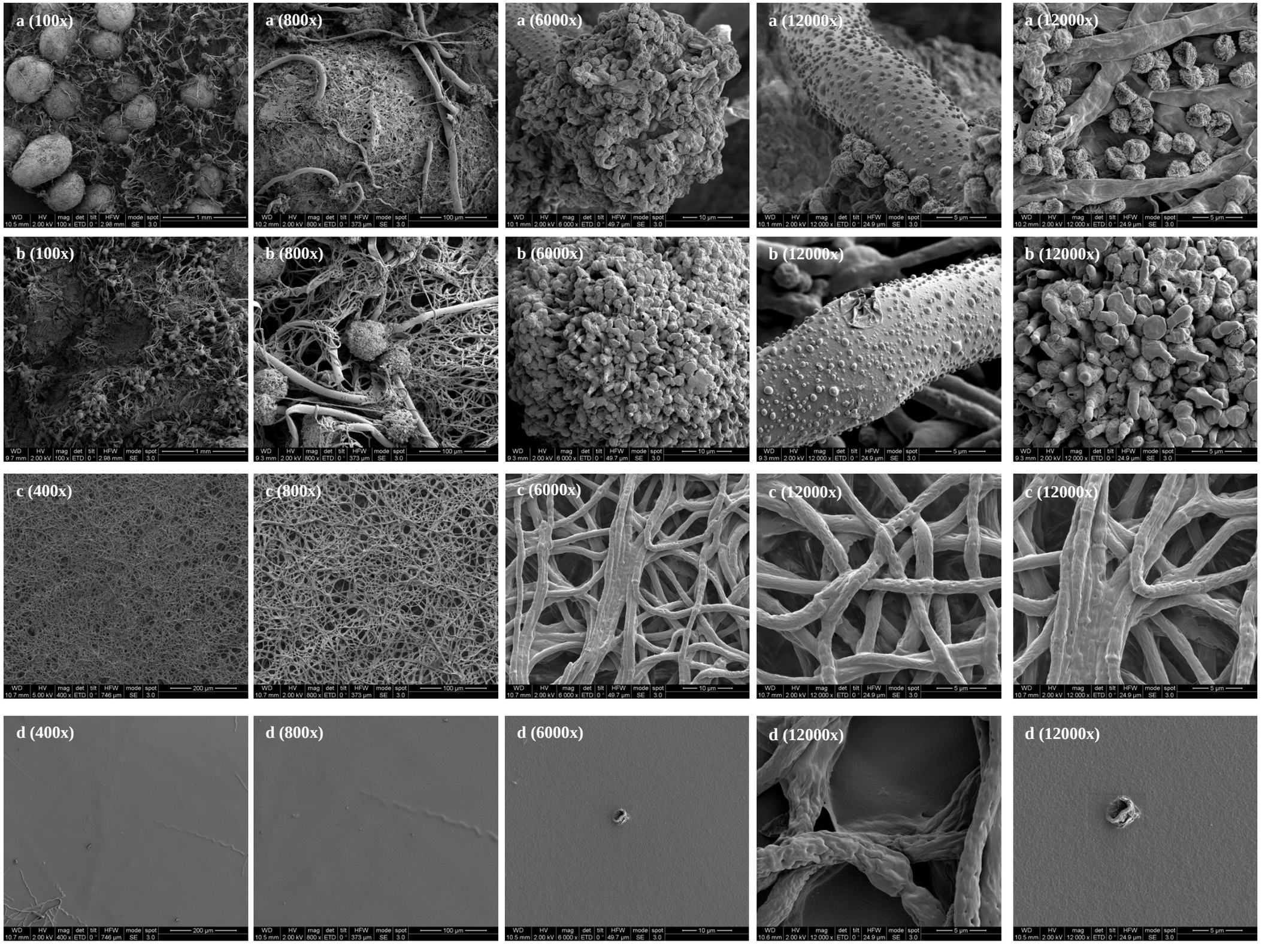

Active
Control

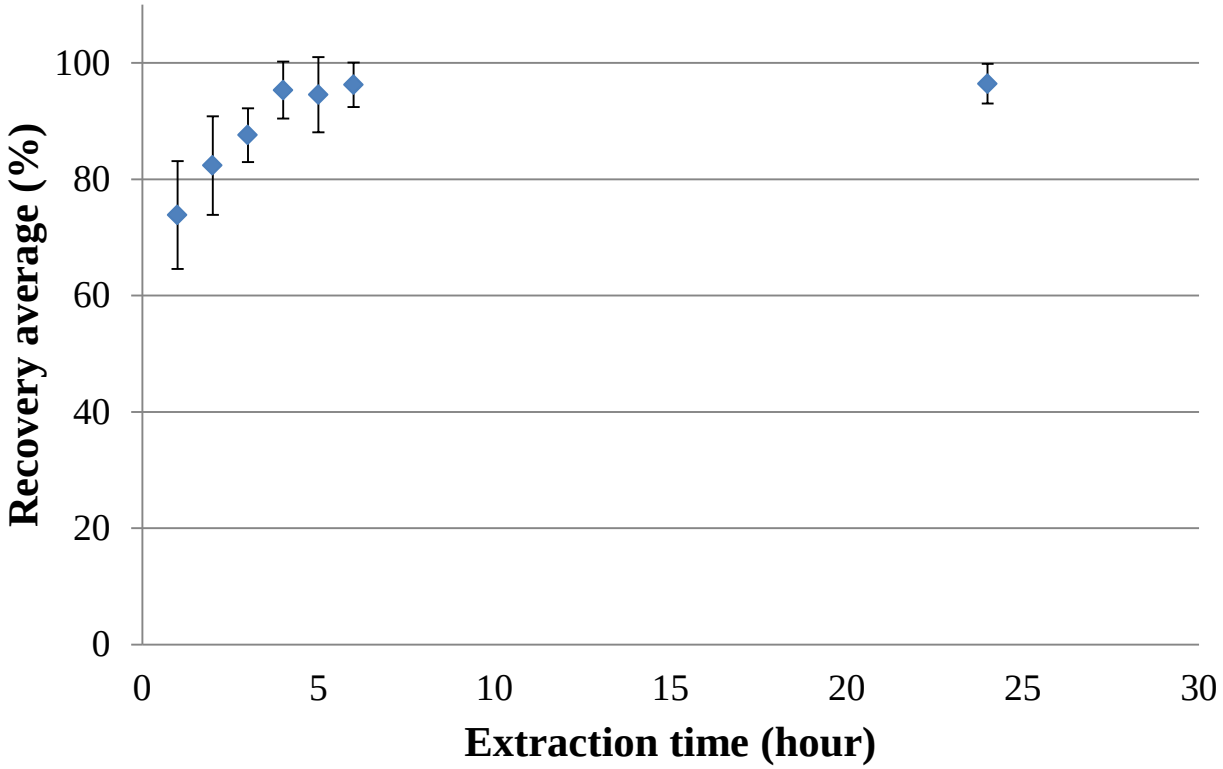
b)



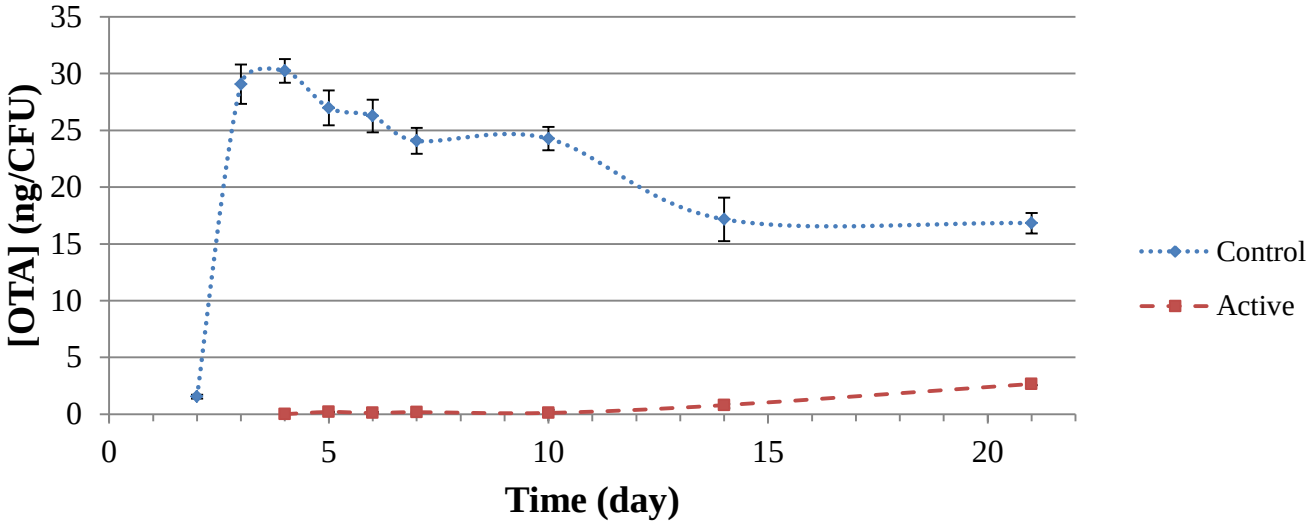
c)


Type of growth (%)
100%
80%
60%
40%
20%
0%
1
2
3
4
5
6
7
10
14
21
Time (days)
inhibition
delay
growth

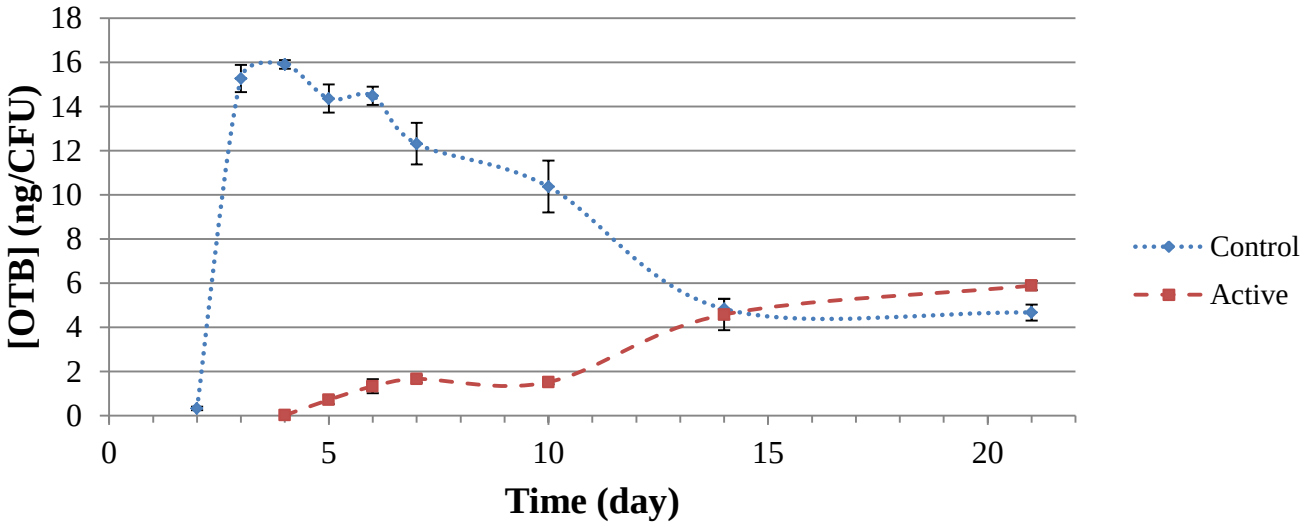

a (100x)
a (800x)
a (6000x)
a (12000x)
a (12000x)
b (100x)
b (800x)
b (6000x)
b (12000x)
b (12000x)
c (400x)
c (800x)
c (6000x)
c (12000x)
c (12000x)
d (400x)
d (800x)
d (6000x)
d (12000x)
d (12000x)


Recovery average (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
Extraction time (hour)

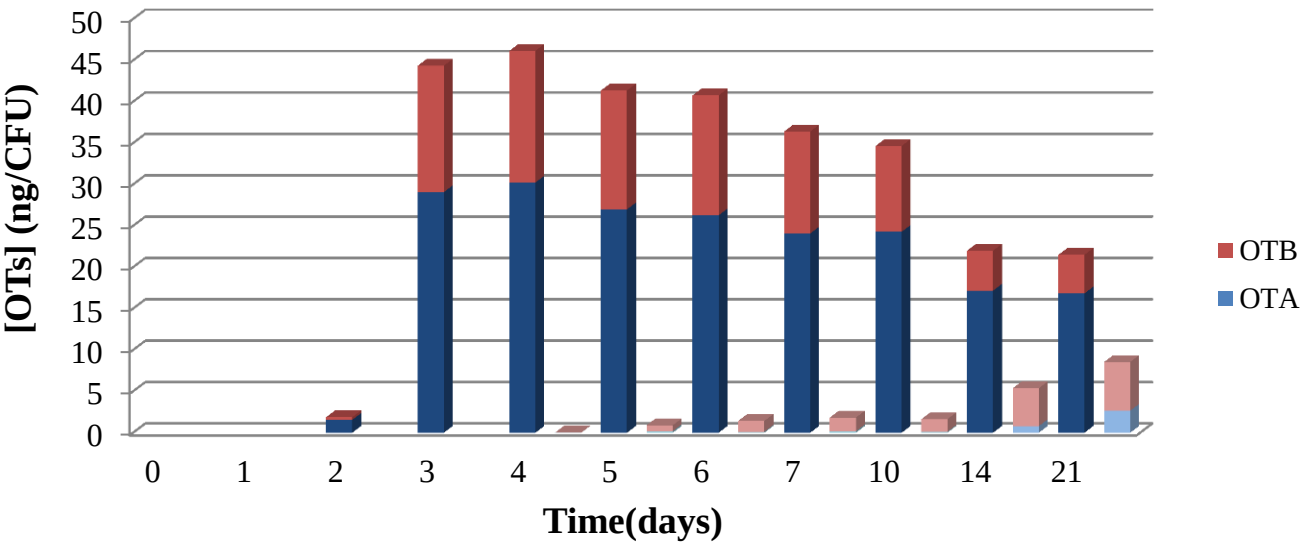
a)


35
30
25
20
15
10
5
0
[OTA] (ng/CFU)
0
5
10
15
20
Time (day)
Control
Active

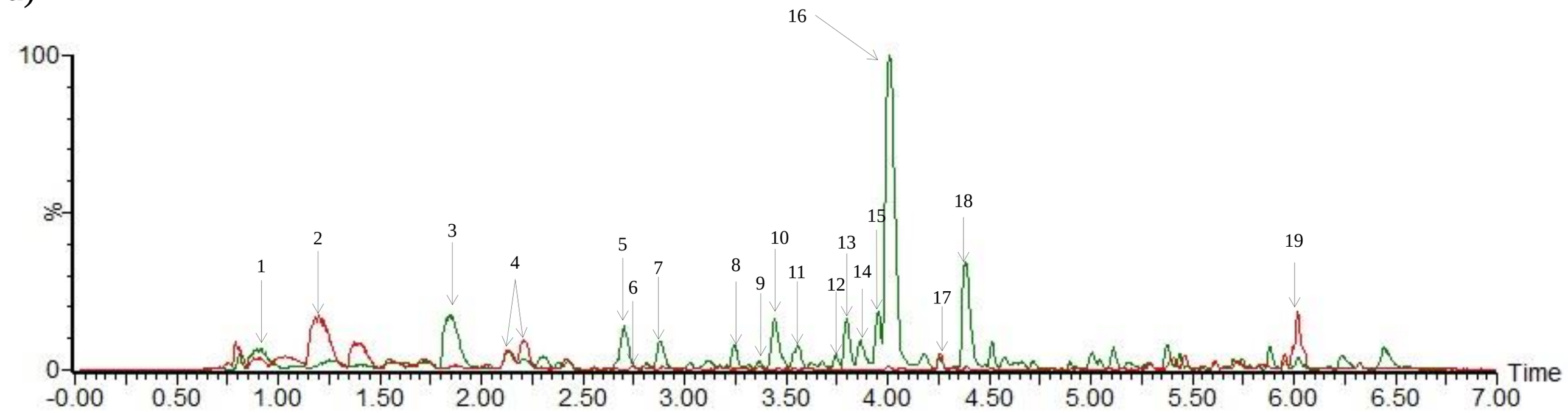
b)


18
16
14
12
10
8
6
4
2
0
[OTB] (ng/CFU)
0
5
10
15
20
Time (day)
Control
Active

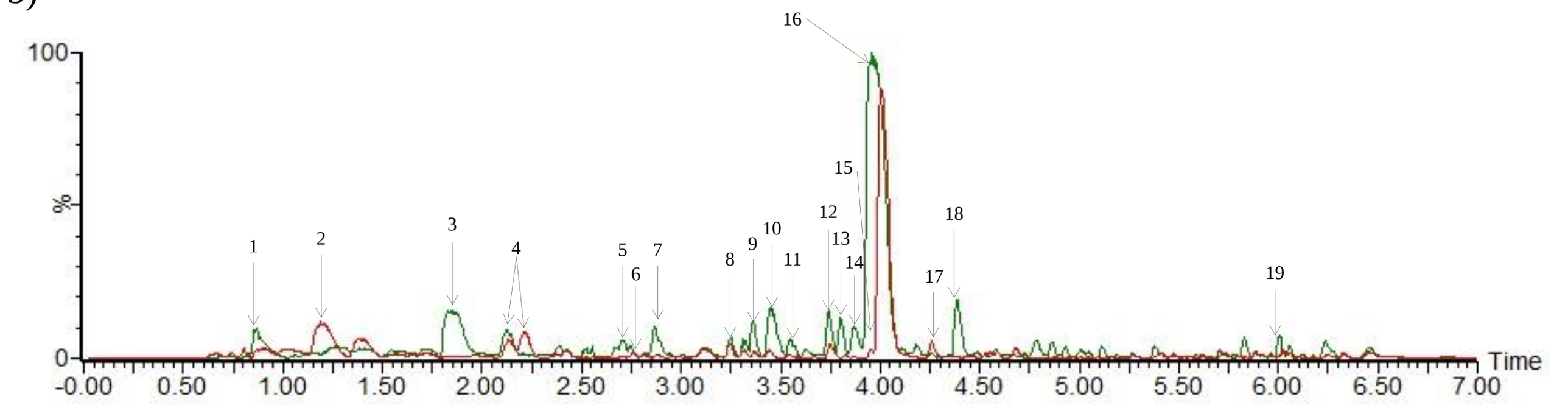
c)


50
45
40
35
30
25
20
15
10
5
0
[OTs] (ng/CFU)
0
1
2
3
4
5
6
7
10
14
21
Time(days)
OTB
OTA

a)


100
%
0
Time
-0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
5.50
6.00
6.50
7.00
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

b)


100
%
0
Time
-0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
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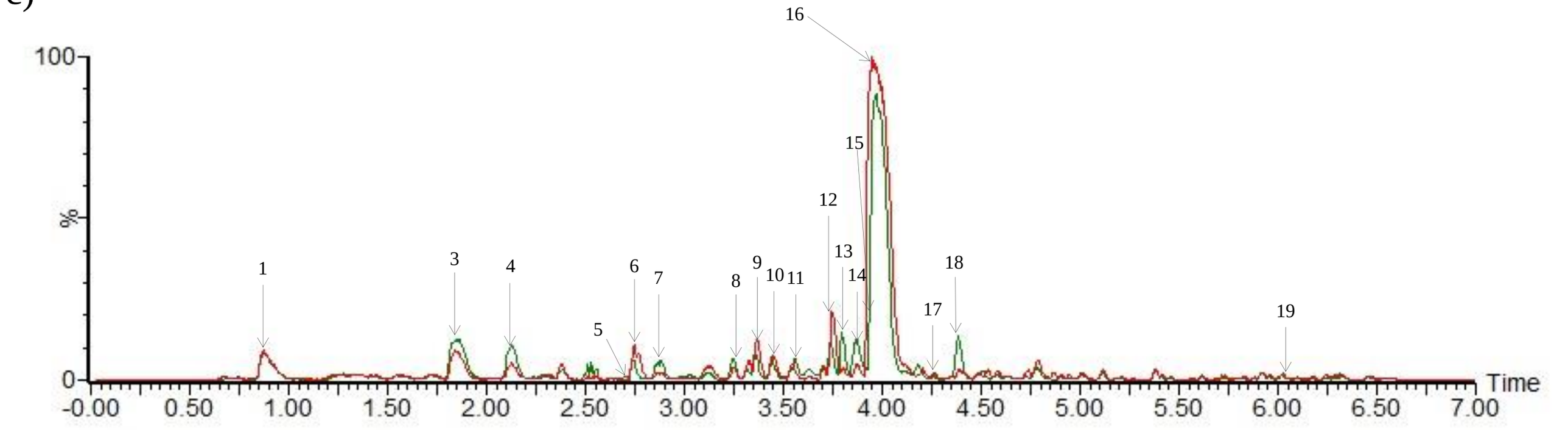
5.00
5.50
6.00
6.50
7.00
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

c)


100
%
0
Time
-0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
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5.00
5.50
6.00
6.50
7.00
1
3
4
5
6
7
8
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10
11
12
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19