Evaluation of fungal- and photo-degradation as potential treatments for the removal of sunscreens BP3 and BP1

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ARTICLE INFO

ABSTRACT

Photodecomposition might be regarded as one of the most important abiotic factors affecting the fate of UV absorbing compounds in the environment and photocatalysis has been suggested as an effective method to degrade organic pollutants. However, UV filters transformation appears to be a complex process, barely addressed to date. The white rot fungus *Trametes versicolor* is considered as a promising alternative to conventional aerobic bacterial degradation, as it is able to metabolise a wide range of xenobiotics. This study focused on both degradation processes of two widely used UV filters, benzophenone-3 (BP3) and benzophenone-1 (BP1). Fungal treatment resulted in the degradation of more than 99% for both sunscreens in less than 24 h, whereas photodegradation was very inefficient, especially for BP3, which remained unaltered upon 24 h of simulated sunlight irradiation. Analysis of metabolic compounds generated showed BP1 as a minor by-product of BP3 degradation by *T. versicolor* while the main intermediate metabolites were glycoconjugate derivatives. BP1 and BP3 showed a weak, but significant estrogenic activity (EC50 values of 0.058 mg/L and 12.5 mg/L, respectively) when tested by recombinant yeast assay (RYA), being BP1 200-folds more estrogenic than BP3. Estrogenic activity was eliminated during *T. versicolor* degradation of both compounds, showing that none of the resulting metabolites possessed significant estrogenic activity at the concentrations produced. These results demonstrate the suitability of this method to degrade both sunscreen agents and to eliminate estrogenic activity.

1. Introduction

UV filters are a diverse group of chemical additives used in everyday products with a continuously increasing rate of use. These substances are used as protection against solar UV radiation and can be found not only in sunscreen cosmetics but also in other personal care products, food packaging, pharmaceuticals, domestic and industrial commodities or vehicle maintenance products (Ash and Ash, 2004). Sunscreen agents enter the aquatic environment as a direct release or through sewage, where monitoring data of wastewater treatment plants (WWTPs) indicates that current techniques are not effective at removing UV filters since several of them were found in treated wastewater (Snyder et al., 2006; Li et al., 2007; Rodil et al., 2008; Negreira et al., 2009), and sewage sludge (Plagellat et al., 2006; Gago-Ferrero et al., 2011a). UV filters are considered environmental contaminants of increasing concern since most of the commonly used are known to cause endocrine disrupting effects (Schreurs et al., 2005) and bioaccumulate in both aquatic and terrestrial organisms (Balmer et al., 2005).

The organic UV filters most frequently found in water samples are 2,4-dihydroxybenzophenone (BP1), benzophenone-3 (BP3), 2-Hydroxy-4-methoxybenzophenone-5-sulphonic acid (BP4), 2-phenylbenzimidazole-5-sulfonic acid (PBSA), 4-methyl-benzylidene camphor (4-MBC), ethylhexyl methoxycinnamate (EHMC), isoamyl methoxycinnamate (IAMC), octocrylene (OC) and octyl dimethyl-p-aminobenzoate (OD-PABA) (Rodil et al., 2008; Kasprzyk-Hordern et al., 2008; Negreira et al., 2009). The widespread use of BP3 in personal care products was recently documented in an extensive survey (Calafat et al., 2008). Maximum values reported in raw wastewaters

for this sunscreen agent are 462 ng/L in Spain (Negreira et al., 2009), 722 ng/L in China (Li et al., 2007) and 2930 ng/L in USA (Snyder et al., 2006). BP3 was also detected in sludge, soils, sediments and industrial drainage (Jeon et al., 2006; Gago-Ferrero et al., 2011b). Regarding its toxicity, BP3 is under study by the European Commission (EU, 2007) as substance with potential evidence of endocrine disrupting effects and it is regulated by the 2002/72/EC Directive, relating to compounds in contact with food.

Since biological conventional treatments in WWTP are not effective at degrading most UV filters, new approaches are being developed. Two of them are assessed in the present work, one physicochemical and one biological. Because of UV filters are substances designed to absorb solar radiation, their fate in natural waters might be driven by sun radiation mediated mechanisms. To date, very few studies have examined UV filters response under UV radiation when exposed in aqueous samples (Vanquerp et al., 1999; Rodil et al., 2009) and in none of them degradation products were identified. On the other hand, it has been widely proved that white rot fungi (WRF) have the ability to oxidise a large number of organic contaminants (Pointing, 2001; Blánquez et al., 2004; Auriol et al., 2008; Marco-Urrea et al., 2009) due to their powerful enzymatic system, characterised by a high unspecificity given by the oxidation mechanism, partly based on the generation of free radicals and the presence of extracellular enzymes such as laccases and peroxidases. The basidiomycete *Trametes versicolor* is one of the most studied WRF. Nevertheless, these degradation processes are only useful if they do not lead to the formation of new compounds with higher toxicity or bioaccumulation capacity. Therefore, it is necessary to identify and characterise the derivatives formed during the transformation processes and to assess the potential toxicity (or any potentially deleterious biological activity) not only of the parental compounds, but also of their degradation products, in order to draw a complete picture of the process.

The present work was aiming to study the photolysis of BP3 and one of its main derivatives (BP1, also used as UV filter) in aqueous samples using artificial UV radiation, and to determine the feasibility of the ligninolytic fungus *T. versicolor* to degrade them and the intermediate products generated during fungal degradation. Further identification and structural characterization of these metabolites was performed by HPLC-MS/MS. Finally, an assessment of the total estrogenic activity after and before both treatments was carried out using the recombinant yeast assay (RYA).

2. Materials and methods

2.1. Reagents and fungal and yeast strains

The UV filter BP3 was kindly given by Merck (Darmstadt, Germany). BP1 (also known as DHB), 4-hydroxybenzophenone (4HB) and 4,4′-dihydroxybenzophenone (4DHB) were purchased from Sigma-Aldrich (Munich, Germany), and 2-2′dihydroxy-4-methoxybenzophenone (DHMB) and 2,3,4-trihydroxybenzophenone (THB) were from Dr Ehrenstorfer (Augsburg, Germany). The isotopically labelled compound 2-hydroxy-4-methoxy-2′,3′,4′5′,6′-d$_5$ (BP3-d$_5$) used as internal standard in HPLC-MS/MS analyses, was obtained from CDN isotopes (Quebec, Canada). All other chemicals used were of analytical grade.

T. versicolor (ATCC#42530) was from the American Type Culture Collection and was maintained by subculturing on petri dishes in malt extract (2%) and agar (1.5%) medium at 25 °C.

For Estrogen Receptor Assay (ER-RYA), yeast strain BY4741 (MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0) from EUROSCARF (Frankfurt, Germany) was transformed with plasmids pH5HE0 (hER) and pVitBX2 (ERE-LacZ) as described in Noguerol et al., 2006.

2.2. Biodegradation by T. versicolor

2.2.1. Media and cultures for pellets production

Pellets production was done as previously described by Font et al., 2003. For the experiments, a defined medium was used (Blánquez et al., 2004). For the bioreactor experiments, few drops of antifoam (204, Sigma, USA) were also added to the media.

2.2.2. Experiments of UV filter degradation at bioreactor scale

A glass bioreactor (1.5 L) at experimental conditions described in Blánquez et al. (2004) was used for the degradation experiments in batch operational mode. Pellets were added in an amount equivalent to a final concentration of 2 g/L dry weight (dw) and UV filters at 250 μg/L from a concentrated stock standard solution in ethanol. Samples taken at scheduled times were filtered by vacuum with Withman GF/C filters and concentrated by solid phase extraction (SPE) with Oasis HLB cartridges (Waters, Mildford, USA) as described elsewhere (Radjenovic et al., 2007). The extracts were reconstituted with 0.5 ml 40:60 v/v ethanol:water 1% acetic acid. The percentage of recovery was 78.2 ± 7.5 for BP3 and 96.3 ± 1.3 for BP1. For ER-RYA assay, samples were evaporated and reconstituted with 0.5 ml of methanol (MeOH).

2.2.3. Experiments of UV filter degradation at Erlenmeyer scale

The experiments were performed on 125 mL amber serum bottles (Wheaton, Mealville, NJ) with cotton plugs in a total reaction volume of 25 mL. Pellets were added to a final concentration of 5 g/L dw and BP3 or BP1 at 10 mg/L. The experiments were carried out at 25 °C and 130 rpm of orbital agitation. Treatments were done by triplicate and fresh bottles were sacrificed at each sample time point. Apart from the experimental bottles (EB), uninoculated controls (UNI), without fungi but with UV filter to take into account potential natural photodegradation or other abiotic processes; heat killed fungal control (HK), with the same amount of fungi than the EB but killed by the autoclave (30 min at 121 °C) to quantify the amount of adsorbed contaminant; and blank controls (BC), with solely alive fungi to detect diminishing in the fungal growing due to toxicity of the compound or to compare laccase production activation by the contaminant, were also included. At each sample time, 1 mL of liquid medium was filtered with a Millex-GV (Millipore) 0.22 μm syringe filter in order to determine the glucose concentration and the laccase enzymatic activity. Then, ethanol at a final concentration of 40% v/v was added to each bottle to achieve the total solubilisation of the UV filters. Finally, the bottle content was filtered by vacuum, and then analyzed by either HPLC-UV or HPLC-MS/MS.

Degradation was determined by comparing BP3 or BP1 concentration in the UNI controls with that in the EB flasks. All the degradation values were corrected for the sorption concentration values determined in HK control flasks, whereas UV filter's removal were only referred to UNI concentration values.

2.3. Photodegradation experiments

Photolysis studies were carried out in 25 mL Duran glass UV reactor by exposing 20 mL of the aqueous solutions spiked with BP3 or BP1 at 250 μg/L in HPLC grade water under simulated UV radiation. Irradiation was performed using a SunTest apparatus from Heraeus (Hanan, Germany) equipped with a Xenon arc lamp providing a light intensity of 400 W/m^2. Aliquots of 1 mL at scheduled times were taken for analysis. In order to ensure that observed transformations were only due to photochemical processes, sunscreens stability in aqueous solutions was verified, by storing 500 mL spiked solutions in HPLC grade water under the same experimental conditions at initial concentrations, and a blank control for each sample in the dark and at room temperature (dark controls). For ER-RYA assay, irradiated unitary

samples were taken at the scheduled times, and further concentrated as described in Section 2.5.

2.4. Analytical methods

2.4.1. HPLC–UV analysis

The samples of biodegradation experiments were placed in amber HPLC vials to avoid natural photodegradation during the analysis. A Dionex 3000 Ultimate HPLC equipped with UV detector and autosampler Dionex were used. The chromatographic separation was achieved on a LiChrosphere RP-18 (125 mm × 4 mm, 5 μm) LC column from Merck. The method used was based on that of Salvador and Chisvert (2005). Flow rate and injection volume were set up to 0.5 mL/min and 20 μL, respectively. The mobile phase consisted of ethanol (A) and acetic acid 1% in MilliQ water (B). The eluent gradient started with 20% A, from 5 to 10 min, increased to 50% and from 10 to 15 until 70%. Then, from 15 to 20 it was set to 100% A and finally, returned to initial conditions within the next 10 min.

2.4.2. HPLC-MS/MS analysis

Target analysis of BP3 and its known metabolites, namely BP1, 4HB, 4DHB, DHMB and THB, in samples from biodegradation and photodegradation experiments were performed by HPLC using a hybrid triple quadrupole-linear ion trap mass spectrometer (HPLC–QqLIT-MS/MS) from Applied Biosystems-Sciex (Foster City, California, USA). The chromatographic separation was achieved on a Hibar Purospher® STAR® HR R-18 ec. (50 mm × 2.0 mm, 5 μm) LC-column from Merck. In the optimised method, the mobile phase consisted of a mixture of HPLC grade water and acetonitrile (ACN), both with 0.15% formic acid. Analyte elution was achieved by increasing the organic composition of the mobile phase from 5% to 25% in 7 min, and then to 100% in the following 3 min. Pure organic conditions were kept constant for 2 min and then initial conditions were reached in the next 2 min. The injection volume and the mobile phase flow-rate were set to 10 μL and 0.3 mL/min, respectively.

MS/MS detection was performed in positive (PI) electrospray ionization (ESI) mode operating under the selected reaction monitoring (SRM) mode. Two major characteristic fragments of the protonated molecular ion $[M + H]^+$ were monitored for improved sensitivity and selectivity (see Table 1). The most abundant transition was used for quantification, whereas the other was used for confirmation. Fragmentation voltage and collision energy were optimised for each transition. The experimental conditions were investigated by infusion experiments of single UV filter standard solutions at 500 ng/L in ACN. The optimised values were selected as a compromise using the optimum values for the majority of the analytes. This procedure was in compliance with the European Council Directive, 2002/657/EC, that although it was initially conceived for food residue analysis, it has been accepted by the scientific community for environmental analysis. Molecular structures of target analytes, their chromatographic retention times (t_R) and the optimum MS/MS acquisition parameters are shown in Table 1.

Non-target analyses for the identification of BP3 unknown metabolites produced during the fungal treatment were performed by high performance liquid chromatography coupled to quadrupole-time of flight-tandem mass spectrometry (HPLC-QqTOF-MS/MS) using a Waters Acquity UPLC™ system attached to a Waters/Micromass QqToF-Micro™ (Waters/Micromass, Manchester, UK). Chromatographic conditions were the same as those described above for the HPLC–QqLIT-MS/MS method.

For identification of biodegradation products full-scan analyses were carried out on selected samples in the PI and in the negative (NI) ESI ionization modes in the range m/z 50–700 at different cone voltages (15–35 V). PI mode allowed higher sensitivity as compared to NI mode, and as NI mode did not provide additional information further MS and MS/MS analyses were carried out in PI mode. Collision

Table 1
SRM experimental conditions used in the HPLC–MS/MS determination of UV filters and proposed product ions.

Target compound	Chemical structure	Retention time (min)	Transition[a]	Fragmentation voltage (V)	Collision energy (eV)	Proposed product ion
4,4′-Dihydroxybenzophenone (4DHB)		5.63	215 > 121 215 > 93	8	27 45	$[M - C_6H_5OH]^+$ $[C_6H_4OH]^+$
2,3,4-Trihydroxybenzophenone (THB)		6.68	231 > 153 231 > 105	10	31 31	$[M - C_6H_5OH]^+$ $[C_6H_5C≡O]^+$
4-Hydroxybenzophenone (4HB)		7.07	199 > 121 199 > 105	8	25 27	$[M - C_6H_5]^+$ $[C_6H_5C≡O]^+$
2,4-Dihydroxybenzophenone (BP1)		7.70	215 > 137 215 > 105	8	27 29	$[M - C_6H_5]^+$ $[C_6H_5C≡O]^+$
2,2′-Dihydroxy-4-methoxybenzophenone (DHMB)		8.20	245 > 151 245 > 121	10	27 29	$[M - C_6H_5OH]^+$ $[C_6H_4(OH)C≡O]^+$
Benzophenone 3 (BP3)		9.31	229 > 151 229 > 105	15	25 27	$[M - C_6H_5]^+$ $[C_6H_5C≡O]^+$

[a] All compounds were determined in ESI(PI) mode. Parent ion correspond in all cases to $[M + H]^+$.

induced fragmentation (CID) of selected m/z ions was evaluated at different collision energies (10–40 eV), using argon as collision gas at 1.5 bars. Data were collected in the centroid mode, with a scan time of 0.3 s and an inter scan delay time of 0.1 s, with a full width at half maximum (FWHM) resolution of 5000. Other MS parameters were set as follows: 600 L/h for the desolvation gas at 350 °C, 50 L/h for the cone gas and 120 °C as source temperature. A valine–tyrosine–valine (Val-Tyr-Val) reference solution (m/z 380.2185 of $[M+H]^+$) was used to tune the instrument and also as lock mass to achieve mass accuracy. This solution was analyzed every 4 s by infusion through an independent reference probe (LockSpray™). Elemental compositions and accurate masses of the molecular ions and their fragments were determined using MassLynx V4.1 software.

2.4.3. Additional analyses

Glucose concentration was measured with a biochemical analyser YSI 2700 SELECT (Yellow Spring Instruments) in the concentration range 0–20 ± 0.04 g/L.

Laccase activity can be measured through the oxidation of 2,6-dimetoxyphenol (DMP) by the enzyme laccase. The analysis process is based on the measure of the absorbance variance at 468 nm and 30 °C during 2 min in a Varian Cary 3 UV/Vis spectrophotometer. The reaction was done with 600 µl of sample, 200 µL of sodium malonate 250 mM pH 4.5 and 50 µl of DMP 20 mM. Activity units per litre (U/L) are defined as the amount of DMP in micromoles per litre which are oxidised per minute (µmol DMP/L min). The molar extinction coefficient of DMP is 24.8 mM^{-1} cm^{-1} (Wariishi et al., 1992).

Biomass amount was determined as the constant weight at 100 °C.

2.5. Estrogenic Recombinant Yeast Assay (ER-RYA)

This yeast-based bioassay, which harbours the human estrogen receptor (hER), is able to monitor and quantify the interactions between the ER and the compounds present in the medium by activation of the *lacZ* gene. The protocol carried out is described elsewhere (Noguerol et al., 2006). The incubation period was 6 h before adding Y-PER®. Samples from photodegradation and biodegradation experiments for ER-RYA assays were concentrated by SPE using Oasis HLB cartridges as previously described (Radjenovic et al., 2007) and further evaporated to 0.5 mL MeOH, which corresponded to a concentration of 40-fold the original concentration (250 µg/L).

3. Results and discussion

3.1. Comparison of photodegradation and biodegradation of BP3 and BP1

As shown in Fig. 1A, BP3 was not photodegraded after 24 h of irradiation. This result is in accordance with previous studies (Rodil et al., 2009). In contrast its derivative BP1 was readily photodegraded, disappearing after 24 h of irradiation (Fig. 1B).

High biodegradation rates were observed by *T. versicolor* during 24 h batch operating. Initial levels of BP3 dropped to non-detectable levels in 8 h (Fig. 1C). In the case of BP1, about 95% of the initial concentration was removed after 2 h of treatment and completely eliminated at 24 h (Fig. 1D). During the biodegradation treatment, new peaks were observed in the chromatograms indicating that these contaminants were degraded to some extent by the fungus and that their elimination from the medium was not only driven by their adsorption onto the biomass. For BP3 the new peak was observed at $t_R = 13.4$ min (M1), which reached a 40% of the initial area of BP3 ($t_R = 18.5$ min) upon 24 h of treatment. Similarly, for BP1 the new peak ($t_R = 13.7$ min, N1) reached a maximum (50% of initial BP1 signal at $t_R = 15.5$ min after 8 h, to further decrease. Any of the detected compounds presented toxicity to the fungus since glucose consumption was in the usual range.
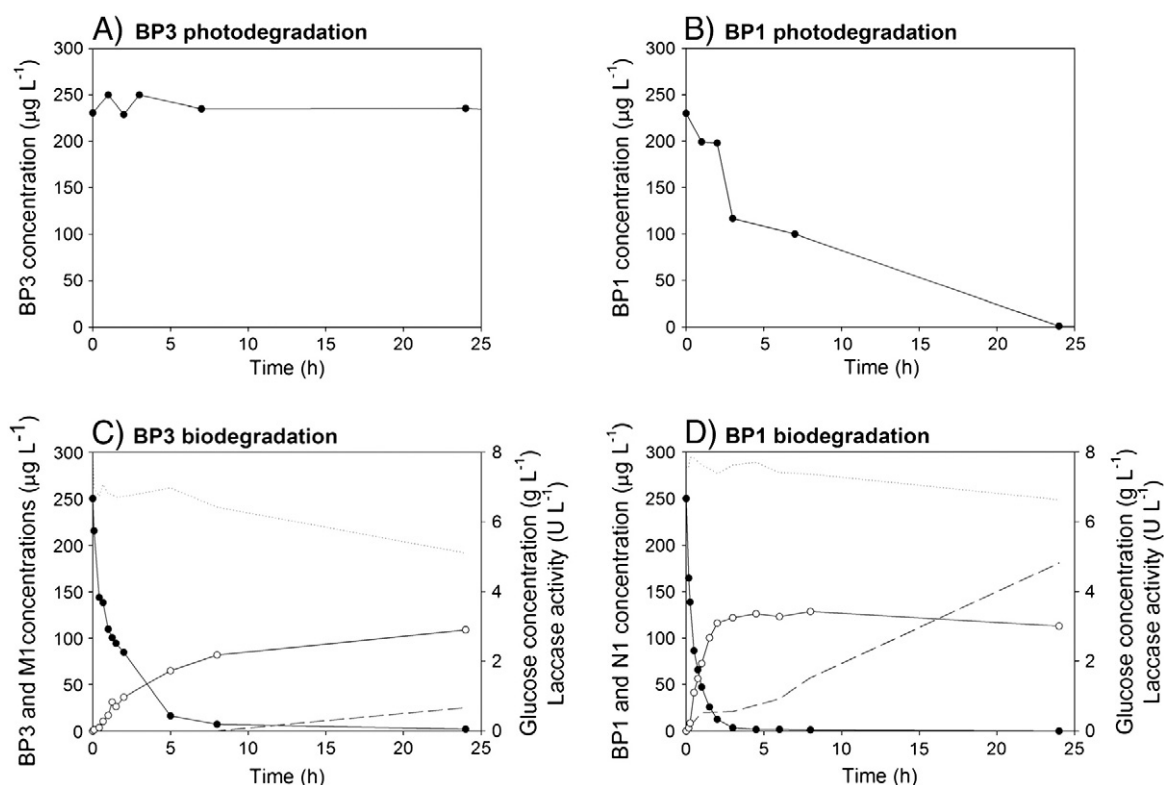

Fig. 1. Concentration profiles of A) BP3 and B) BP1 during UV irradiation and C) BP3 and D) BP1 during fungal degradation in the bioreactor at initial concentration of 250 µg/L (close circles). Glucose concentration and laccase activity in bioreactors are shown in dotted line and long dashes, respectively, and concentrations of main metabolites M1 and N1 in white circles.
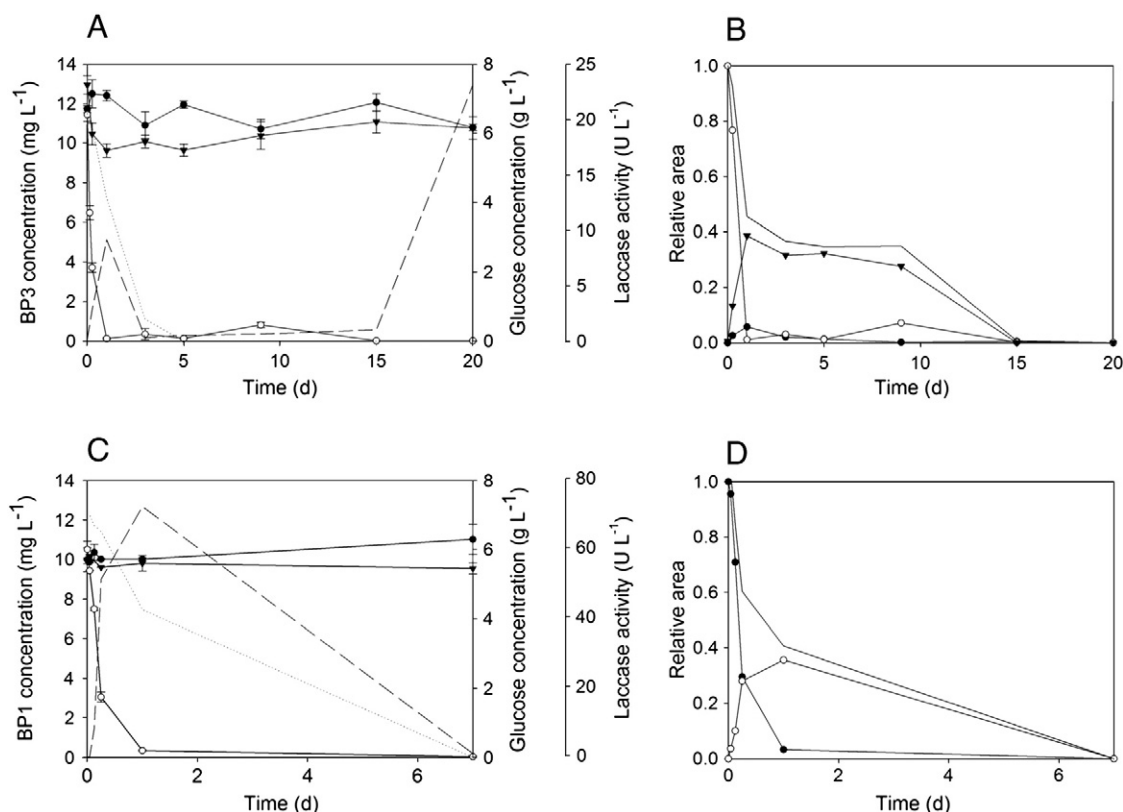
Fig. 2. A) BP3 concentration profile degradation experiment by *T. versicolor* at Erlenmeyer scale at 10 mg/L BP3 initial concentration and B) evolution of peak areas in HPLC chromatograms. Legend: (○) BP3 peak, at t_R 18.5 min; (▼) M1 peak, at t_R 13.4 min; (●) M2 peak, at t_R 12.7 min. C) BP1 concentration profile in degradation experiment by *T. versicolor* at Erlenmeyer scale and at 10 mg/L BP1 initial concentration and D) evolution of peak areas in HPLC chromatograms. Legend: (○) BP1 peak, at t_R 15.5 min; (●) N1 peak, at t_R 13.7 min. In A) and C): Treatments: (●) UNI, (○) EB and (▼) HK. Glucose concentration and laccase activity in EB are also plotted in a dotted line and long dashes, respectively. Values plotted are means ± standard error for triplicates. In B) and D): Sum of all areas of the main peaks is plotted in a solid line.

3.2. Target analysis of BP3 and BP1 metabolites

In order to characterise the biodegradation products formed, and to assess the capacity of the fungus to degrade them, experiments at Erlenmeyer scale were carried out. To facilitate metabolites identification, higher concentrations of UV filters (10 mg/L) and fungus (5 g/L dw) were used. The results obtained in long term experiments for BP3 are shown in Fig. 2A. Abiotic degradation processes were discarded, since no decrease of the BP3 concentration in the UNI controls was observed. Dead fungi controls were also performed in order to ensure that the sunscreen agent's elimination was not only produced by its adsorption onto the biomass. The little adsorption observed (between 0 and 19.6%) was in agreement with the relative low hydrophobicity of BP3 (log K_{OW} = 3.79, experimental value from database of physico-chemical properties. Syracuse Research Corporation: http://www.syrres.com/esc/physdemo.htm) and the step of the solubilisation applied.

In the experimental bottles BP3 exhibited a high degree of elimination, reaching >99% between 6 and 24 h. During 20 days (Fig. 2A), elimination of BP3 was maintained near 100% without abiotic elimination or high adsorption in HK controls. Therefore, taking into account that amount of BP3 adsorbed on the biomass, at least 80.4% of the initial concentration could be assigned to degradation processes at 24 h. During the biodegradation the M1 peak appeared again and another one at a t_R = 12.7 min (M2) not detected in bioreactor experiments (Fig. 2B). None appeared at HK controls. The maximum concentration of both was observed at 24 h of culture, M1 with a relative area of 40% compared to the initial concentration of BP3, while M2 reached only a relative area of 5%. Thus, the sum of all degradation products areas did not achieve the corresponding initial

concentration of BP3. As between 10 and 15 days all peaks disappeared (Fig. 2B), it can be assumed that, finally, the aromatic ring was broken. It must be remarked that this biotransformation occurred approximately 10 days after glucose depletion in the media. Thus, fungus was still alive even at 20 days of culture, as it is corroborated by the production of laccase at that moment (Fig. 2A).

Similarly, 7 days-long fungal degradation experiments were performed for BP1. Results showed a similar but faster degradation profile to that of BP3 (see Fig. 2C and D).

The formation of derivatives during biodegradation of BP3 by the fungus *T. versicolor* was investigated. Firstly, target compounds, namely BP1, 4HB, 4DHB, DHMB and THB, which have been previously identified as metabolites in rats and humans, were considered (Felix et al., 1998; Díaz-Cruz et al., 2008; Jeon et al., 2008). These compounds were analysed in the samples by HPLC-MS/MS according to the methodology described in Section 2.4.2. The developed procedure exhibited excellent linearity (R^2 > 0.99) in a wide range of concentrations (0.1–300 µg/L). Good instrumental precision was obtained, with intraday RSD values between 0.5% and 2% and interday RSD values ranging from 1% to 4%. Method limits of detection (MLODs) and method quantification limits (MLOQs) were calculated in spiked samples as the concentration corresponding to a signal-to-noise ratio of 3 and 10, respectively. Estimated values were in the ranges 0.4–11.1 µg/L for MLOD and 1.3–37.0 µg/L for MLOQ.

BP1, 4DHB and 4HB were identified as metabolites produced during the degradation experiments of BP3 with the fungus. BP1 was detected up to 6 h of treatment at 3.6 µg/L maximum concentration, indicating a high fungal degradation rate. 4DHB and 4HB were determined after 3 days of treatment. 4DHB was detected at 3, 5 and 9 days at a concentration of 11.5, 50.7 and 31.3 µg/L, respectively, while 4HB
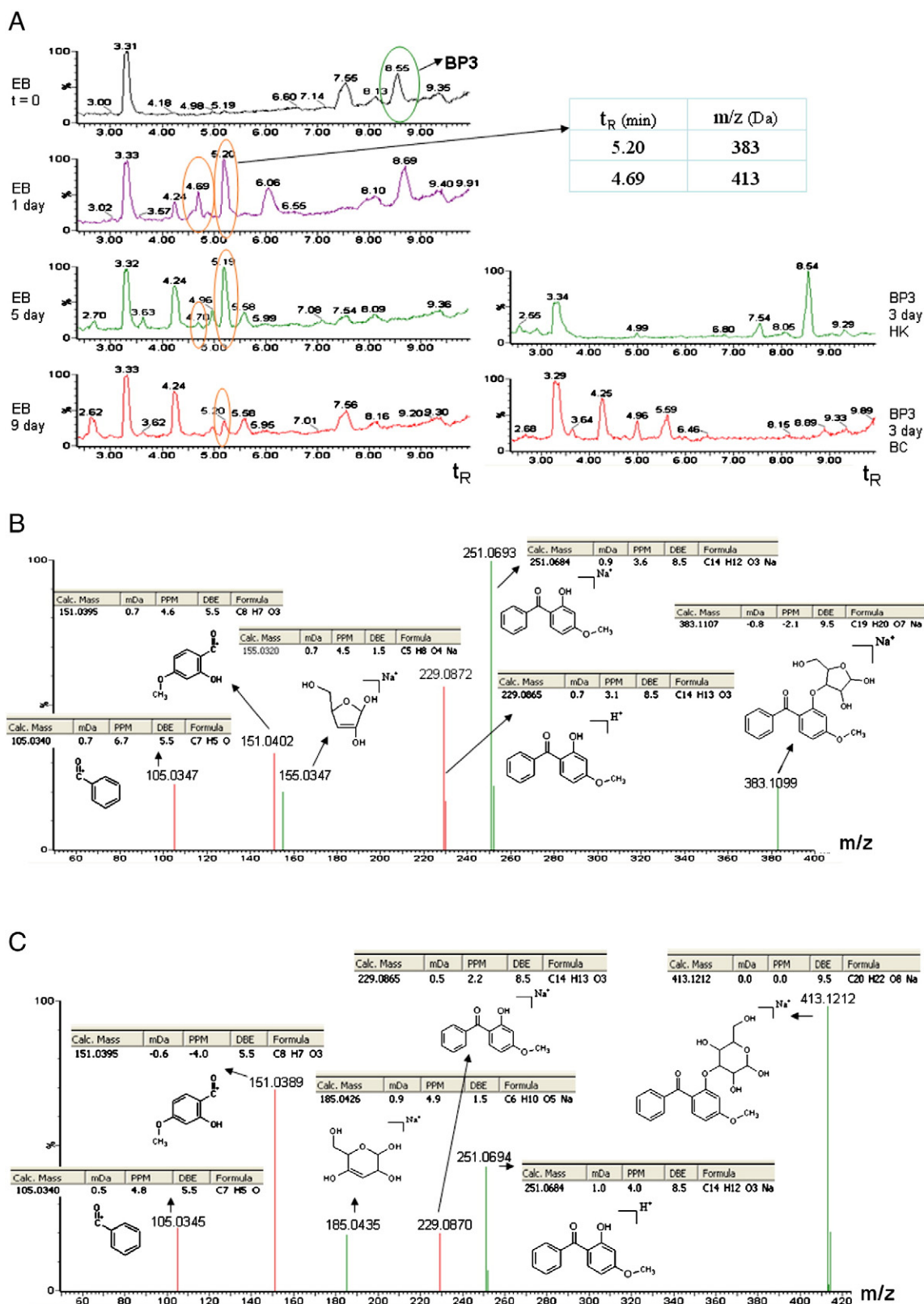
Fig. 3. A) Total ion current chromatograms (TIC) of samples collected at t = 0, 1, 5 and 9 days. HK and BC TIC are also shown. B) and C) ESI(PI)-MS/MS spectra of the molecular ions (as sodium adduct) of the metabolites at B) m/z: 383 and C) m/z 413 in t = 1 day samples (cone voltage = 20 V, collision energies = 10 eV (green) and 35 eV (red)). Additionally, the experimental and theoretical exact masses obtained for the molecular and fragment ions, expressed as m/z, the proposed elemental composition, together with recalculated mass errors (in mDa and mg/g) and double bond equivalents (DBEs) given by the software (mass measurements accuracy threshold of 5 mg/g) are provided.

was always below MLOQ. THB and DHMB were apparently not produced by the treatment with the fungus, since they were not detected in any sample.

Fungal degradation of BP1 resulted in the formation of 4HB and 4DHB. Their concentrations after 3 and 6 days of treatment were similar to those reported for BP3 degradation experiments. Likewise in

BP3 degradation tests, THB and DHMB were not detected in the analysed samples.

Since the relative concentrations of these metabolites were quite low suggesting that other major metabolites might be formed, further studies were carried out in order to identify them.

3.3. Identification and characterization of non-target metabolites of BP3 and BP1

As depicted in the chromatograms of Fig. 3A, solely BP3 ($t_R = 8.55$ min) was present before starting the biodegradation experiment but readily decreased to be undetectable upon 24 h of fungal treatment. At this time two new chromatographic peaks at t_R 4.69 and 5.20 min were observed in the chromatogram, corresponding to m/z ratios of 413 and 383 Daltons (Da), respectively. Maximum levels of these metabolites were determined after 1 day, to almost disappear between 9 and 15 days of treatment.

Fig. 3B and C summarises the MS/MS information obtained and the fragmentation patterns proposed for these two degradation products (with m/z 383 and 413). In both cases, higher sensitivity was obtained for the sodium adduct of the molecule ($[M + Na]^+$) as compared to that of the protonated molecule $[M + H]^+$, which was hardly detected. Thus, further MS/MS experiments were performed taking the sodium adduct as the precursor ion.

As shown in Fig. 3B, fragmentation of m/z 383 at high collision energy completely fragmented the molecular ion and provided the m/z 229.0865 as the major fragment ion, corresponding to the molecular formula $C_{14}H_{13}O_3$, which fits with the formula of the BP3 molecular ion $[M + H]^+$. Additionally, two other product ions were generated with m/z 151.0402 and m/z 105.0347, which gave the best-fit formula $C_8H_7O_3$ and C_7H_5O, respectively. The first fragment, is produced by the loss of the phenyl group of BP3 $[M-C_6H_5]^+$, whereas the other one corresponded to the benzoyl cation $[C_6H_5C=O]^+$. In the light of these results, it appears that BP3 is part of the molecular structure of the unknown metabolite with m/z 383. In the spectrum obtained at low collision energy the molecular ion at m/z 383 was still observed ($C_{19}H_{20}O_7Na$) together with two product ions at $m/z = 251.0693$ ($C_{14}H_{12}O_3Na$), which likely corresponds to the adduct $[BP3 + Na]^+$, and to m/z 155.0327 ($C_5H_8O_4Na$), which would not correspond to any part of the BP3 molecule, unless aromatic ring cleavage occurred, which is not likely at low collision energy. Therefore, those data suggested that the metabolite at m/z 383 might be BP3 conjugated with a molecule of 132 Da. Several studies claim that the formation of conjugated metabolites with pentoses (mainly xylose and ribose) and hexoses (mainly glucose) is a predominant pathway in the degradation of chemicals by WRF, especially in presence of phenolic hydroxyl groups (Kondo et al., 1993; Gesell et al., 2004). In this case, the addition of a pentose via glycosidic bond to the BP3 with the consequent loss of one molecule of water corresponds to an increase in the molecular mass of 132 Da. Thus, the metabolite at m/z 383 may be produced by the addition of one pentose molecule to BP3.

CID spectra obtained for the metabolite at m/z 413 was similar to that of the previously discussed metabolite (m/z 383). At high collision energy the fragments with m/z 229.0870 ($C_{14}H_{13}O_3$), m/z 151 and m/z 105 were also detected. In the spectrum obtained at low collision energy, besides the sodium adduct of BP3 (m/z 251.0694, $C_{14}H_{12}O_3Na$ ($[BP3 + Na]^+$), two other fragment ions were observed. One at m/z 413.1212, which gave the best fit formula $C_{20}H_{22}O_5$ Na, corresponding to an increase of 162 Da of the BP3 molecular weight, and the other at m/z 185.0435 ($C_6H_{10}O_5Na$). Following the rationale discussed above, it appears that the metabolite at m/z 413 might be the result of the addition of one hexose molecule to BP3, likely glucose, via glycosidic bond and consequent loss of one molecule of water.

The results obtained in a similar study carried out with BP1 were pretty similar. After 24 h of treatment, BP1 disappeared and only one new chromatographic peak ($t_R = 5.09$ min), was observed. This compound reached the maximum concentration upon 24 h of treatment, and then, decreased until not detectable levels after 6 days of treatment. Its MS/MS spectra at m/z 347 were very similar to those obtained for BP3, however, in this case the most abundant molecular ion was found to be the protonated form $[M + H]^+$. Its CID fragmentation allowed to observe the ion $[BP1 + H]^+$ and its two main fragments $[BP1-C_6H_5]^+$ (m/z 137.0233), and $[C_6H_5C=O]^+$ (m/z 105.03043). Additionally the molecular ion m/z 347.1131 ($C_{18}H_{19}O_7$) and the fragment m/z 133.0507 ($C_5H_9O_4$) were observed. As it was reported for BP3 a difference in 132 Da in the molecular mass suggests the addition of one pentose molecule to BP1 (m/z 347). In a previous study on fungal degradation of the sunscreen agent 4-MBC similar conjugated metabolites were identified (Badia-Fabregat et al., 2012).

Non-target identified metabolites constitute conjugated forms of BP3 and BP1, and thus, further enzymatic activity of the fungus might revert them to the parent compound. Nevertheless, the fact that all identified metabolites, together with BP3 and BP1, disappeared along the treatment confirms further fungal degradation with potential cleavage of the aromatic rings.

The increase in the polarity of the identified metabolites caused by the addition of sugars is in agreement with the lower t_R associated to them in comparison to those of the respective parental compounds. Taking into account that absorbance of conjugates is similar to that of the corresponding parent, the metabolite generated in a higher amount in BP3 degradation (M1) would be the pentose conjugate, while that less abundant (M2) would be the glucose conjugate.

These findings support the hypothesis that conjugation processes constitute one of the defensive mechanisms that fungi have against toxic hydroxylated compounds (Hundt et al., 2000). Thus, glycoconjugation appears to be the first step in the BP3 and BP1 metabolism. The active enzymes appear to be UDP-xylosiltransferase when conjugation occurs with a xylose (Kondo et al., 1993) or UDP-glucosyltransferase if the added molecule is a glucose, as it has been previously reported for other xenobiotics upon the action of *T. versicolor* (Hundt et al., 2000). Conjugation with ribose, also described for other fungi (Gesell et al., 2004), constitutes a possibility that cannot be ruled out since the molecular weight of this conjugate fits with the results obtained in the HPLC-MS/MS analyses. Sugar residues would bind to the molecule through an O-glycosidic bond to the unique free hydroxyl of BP3 and to one of the two free hydroxyl groups present in BP1. Both approaches must be considered since the fragmentation of the conjugate yield BP3 and BP1 molecules, but any other breakdown product.

Later, O-glycosidic bond would break down and other fungal enzymes would act. Likely, the monooxygenase cytochrome P450 would oxidise BP3 and BP1 by adding hydroxyl groups (Hammer et al., 2001) or eventually demethylation (Campoy et al., 2009) for BP3. This would lead to the formation of BP1, 4HB and 4DHB, following a metabolic pathway similar to the one reported for mammals (Jeon et al., 2008). In fact, similar experiments of cytochrome P450 inhibiton described at Marco-Urrea et al. (2009) pointed to the possible involvement of this intracellular enzymatic system in the first steps of BP3 degradation (data not shown). Laccase could oxidise BP3 as well, although only with the help of mediators (Garcia et al., 2010).

Summarizing, BP3 and BP1 are rapidly transformed in their glycoconjugated forms to decrease its toxicity and to increase its bioavailability. Then, conjugation would steadily revert and oxidation of compounds and further ring cleavage would occur.

3.4. Estrogenic activity of BP3 and BP1

Estrogenic activity of BP3 and BP1 was determined by ER-RYA obtaining EC50 values of 12,5 mg/L and 0.058 mg/L for each compound and a LOEC of 1,6 mg/L and 0.015 mg/L, respectively. BP1 was three orders of magnitude less estrogenic than 17β-estradiol and 200-fold

higher estrogenic than BP3. Similar values have been described in the literature (Kunz et al., 2006).

3.5. Endocrine disruption evaluation of the treatments

In order to evaluate the evolution of the endocrine disruption during the degradation processes either by photodegradation or by *T. versicolor* at bioreactor-scale, the estrogenic activity was monitored at the same scheduled times than chemical analyses. During BP3 treatments, estrogenic activity was below the detection limit in all checked points. This indicates that putative estrogenic metabolites formed by *T. versicolor* degradation, such as BP1 were readily metabolised and, therefore, they were not present at sufficiently high concentration to elicit biological response. This is in agreement with the low BP1 concentrations found during fungal degradation.

The estrogenic activity of BP1 was reduced by both degradation processes, although *T. versicolor* treatment was faster and more effective than photodegradation. Fig. 4 shows that *T. versicolor* eliminated almost completely the estrogenic activity of BP1 after 4 h of treatment, whereas photodegradation needed more than 7 h to show a significant decrease of the estrogenic activity, and did not fully remove the activity until 24 h of incubation. These results are in agreement with the chemical analyses of BP1 (see Fig. 1B and D). These outcomes suggest that neither bidegradation by *T. versicolor* nor photodegradation of BP3 and BP1 produced significant amounts of estrogenic metabolites.

4. Conclusions

Comparison between photodegradation and biodegradation studies of one of the most widely used sunscreen agents, BP3, and one of its degradation products, BP1, under both high and environmental-like concentrations evidenced that high degradation rates were achieved by both approaches for BP1 ($>95\%$ at 3 h in bioreactor and 100% after 24 h UV irradiation). Conversely, BP3 was only degraded by the fungus but not by UV irradiation. Therefore, we conclude that the biodegradation approach appears as the most effective treatment in degrading both BP1 and BP3. BP1 was formed during the BP3 degradation process by *T. versicolor*, but it was readily degraded and never reached significant concentrations. The developed UPLC-ESI(PI)-QqTOF-MS/MS method allowed the univocal identification of BP3 and BP1 fungal metabolites by providing their exact mass and molecular formula. Up to 6 derivatives were identified; BP1, 4DHB, 4HB, and the conjugates BP3-pentose (xylose or ribose) and BP3-hexose (glucose) for BP3, and the conjugate
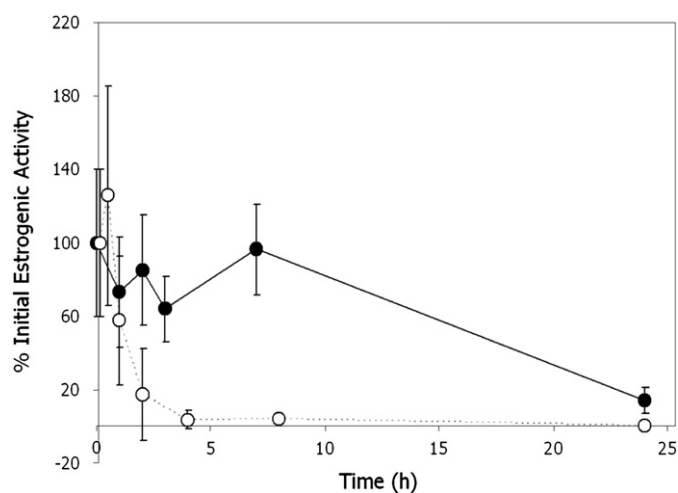
BP1-pentose for BP1. Finally, estrogenic disruption capacity profiles of the biodegradation and photodegradation treatments indicated that none of the evaluated treatments induced the formation of estrogenic metabolites at concentrations sufficiently high to exceed the activity of the parental compound.

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Fig. 4. Estrogenic activity profiles of BP1 degradation mixture by either fungal biodegradation (○) or photodegradation (●).

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