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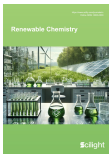

Article

Laccase-Mediated Transformation of Trazodone Hydrochloride and Its By-Products

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Abstract: The increasing drug consumption worldwide raises environmental and health concerns, as pharmaceutical residues entering the environment pose risks to both ecosystems and human health. Enzyme remediation has been emerging in recent decades as a possible solution to eliminate recalcitrant pharmaceutical pollutants in wastewater or contaminated sites, offering a faster alternative to microbial remediation, e.g., without the need for microbial growth or adaptation. Moreover, enzymes operate under a wider range of conditions, avoiding biomass formation and disposal, and antibiotic resistance risks. In the present study, high amounts (up to 5000 mg/L) of trazodone hydrochloride and its by-products, namely Impurity H and Compound F, were enzymatically treated using a laccase-mediator system consisting of the laccase from *Trametes hirsuta* (ThL) and the mediator 1-hydroxybenzotriazole (HBT). Different concentrations of an organic solvent system composed of acetone and isobutyl alcohol in a 1:1 ratio and up to 20% (*v*/*v*) were employed to simulate the matrix of a real industrial production waste stream. Thereby, the enzymatic oxidation of trazodone hydrochloride and its by-products was not significantly affected by the varying solvent concentrations, resulting in maximum conversions of 62%, 73% and 62% of trazodone hydrochloride, impurity H, and impurity F, respectively. Liquid chromatography—high-resolution mass spectrometry (LC-HRMS) indicated preferential oxidation of the piperazine group in all three molecules. In vivo ecotoxicity experiments must be carried out in the future to assess the toxicological and environmental behaviour of the obtained degradation products. This work emphasised the potential role of enzymes in supporting the transition towards a more sustainable pharmaceutical industry.

Keywords: trazodone hydrochloride; enzyme remediation; pharmaceutical wastewater; laccase-mediator system

1. Introduction

The global rise in medicine usage, driven by ageing populations and increased reliance on pharmaceutical products, makes the pharma industry a priority area from an environmental health standpoint. Pharmaceutical residues can enter the environment through manufacturing effluents, natural excretion, or improper disposal, and have been shown to accumulate in fish, vegetables, livestock, and drinking water. In fact, over 990 pharmaceuticals and their metabolites have been already identified in the environment worldwide [1] raising concerns about

potential harm to human health and ecosystems. Notable examples include the lethal impact of diclofenac on Asian vultures [2] through exposure to contaminated cattle carcasses, as well as the recent discovery of nitrosamine impurities in some drugs [3], underscoring the need for effective quality control and compliance systems. Recognising these challenges, the European Commission adopted a strategic approach in 2019 [4] to manage pharmaceuticals' environmental impact, covering their entire lifecycle from design to disposal.

Enzymes, environmentally-friendly biocatalysts, have been utilized in recent years not only for the manufacturing of active pharmaceutical ingredients (APIs) [5] but also for the removal of pharmaceutical residues or end-of-life products in wastewater, both municipal [6] and effluents from hospitals [7], as they can transform pharmaceutical pollutants into more bioavailable or even innocuous products [8]. Despite the growing interest in enzyme remediation, there is a general lack of literature on the removal of pharmaceutical impurities directly from waste streams from industrial API producers. This is potentially due to several factors: high organic solvent concentrations usually result in low enzyme stability, higher cost of effluent treatment, and inability to completely eliminate many pharmaceutical pollutants [8,9]. Should these limitations be overcome, enzyme remediation of industrial pharmaceutical waste could lead to a significant reduction in total waste and carbon dioxide emissions, considering that a major proportion of these streams is currently incinerated [10].

Oxidoreductases are the most widely investigated class of enzymes for remediation thanks to their high capability to convert a diverse range of organic pollutants e.g., through radical reactions into smaller molecules that may possess less toxicity [6]. In particular, laccases (EC 1.10.3.2) require only oxygen as a cofactor and have been widely reported to be effective in the removal of different pharmaceutical pollutants, such as analgesics [11–13], antibiotics [14,15], antiepileptics [16], antirheumatic drugs [17], hormones [17], anxiolytics and sympatholytic drugs [18–19]. Moreover, their remediation activity can be further boosted in the presence of certain molecules, namely mediators, acting as electron shuttles between the laccase and the substrate to be oxidized, forming the so-called laccase-mediator system (LMS) [19,20]. The laccase from *Trametes hirsuta* (ThL) has recently gained attention for its ability to degrade chloramphenicol in combination with different mediators in only 48 h, confirming its remarkable bioremediation capability even towards one of the most persistent micro-pollutants in pharmaceutical waste [21]. Likewise, the potential of an immobilised laccase from *Myceliophthora thermophila* for the elimination of morphine has been demonstrated [13].

Antidepressant drugs are one of the groups of pharmaceutical residues frequently found in the environment [22,23]. Among these, Trazodone hydrochloride, an antidepressant prescribed worldwide to treat anxiety, depressive disorders and insomnia, has been detected in municipal wastewater and rivers [24]. It was the 21st most prescribed drug in the US in 2023, with a global market value in the same year estimated at 460.5 million USD and projected to reach 882.9 million by 2032, highlighting a global trend of increasing consumption [25,26]. Despite its bioremediation appears as of utmost importance, only very few studies have focused on the enzymatic remediation of trazodone, and none on its synthesis by-products. García-Zamora et al. (2018) [27] reported the degradation of microdoses of trazodone and other pharmaceutical micropollutants using a chloroperoxidase from *Caldaromyces fumago*, resulting in the oxidation of only 12%, highlighting the recalcitrant nature of the compound. Furthermore, ESI-mass spectrometry revealed that enzymatic treatment resulted in the addition of chlorine atoms to the benzene ring. Although the resulting compound was more biodegradable, chlorinated organic compounds, in general, are often mutagenic and carcinogenic [28], and therefore should not be released into the environment.

In this study, the potential of a laccase-mediator system comprising the laccase from *Trametes hirsuta* and the mediator HBT was assessed to degrade high concentrations (5000 mg/L) of the API trazodone hydrochloride and impurities resulting from its synthesis (impurity H and related compound F). Varying concentrations of the organic solvents acetone and isobutyl alcohol were also included in the study to mimic the matrix in a real production effluent [29,30], hence testing enzyme stability in various co-solvent mixtures. These conditions make our work one of the few studies demonstrating the feasibility of enzymatic oxidation under challenging conditions—namely, high concentrations of pharmaceutical residues and an organic solvent-rich setting, reflecting real industrial manufacturing and waste stream scenarios. Furthermore, the oxidation products were characterized by high-resolution mass spectrometry.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and solvents were purchased from Sigma-Aldrich (Vienna, Austria) or Carl Roth (Karlsruhe, Germany) and used without further purification unless stated otherwise. Trazodone-HCl (CAS: 25332-39-2) and the compounds trazodone EP impurity H (CAS: 6323-09-7, referred to as "impurity H") and trazodone USP related

compound F (CAS: 39577-43-0, referred to as "compound F") were kindly provided by Angelini Pharma S.p.a. (Rome, Italy). *Trametes hirsuta* laccase (ThL) was produced and purified in-house as previously described [31].

2.2. Protein Concentration Determination

Protein concentration (Bradford assay) was measured using Bio-Rad solution (Coomassie brilliant blue G-250 dye, Bio-Rad, Vienna, Austria) diluted 1:5 with ddH_2O. Bovine serum albumin was used as a standard (Bovine Serum Albumin protein standard, 2 mg/mL, Merck-Sigma-Aldrich). 10 µL of the sample were put in the plate and 200 µL of 1:5 BioRad solution were added (in triplicates). The mixture was shaken at 21 °C for 5 min at 400 rpm. The absorbance at 595 nm was measured at 30 °C with a Tecan Reader (Tecan, Grödig, Austria) using a 96-well microtiter plate (Greiner 96 Flat Bottom Transparent Polystyrene). A blank was included using only the buffer. The protein concentration was calculated using the calibration curve obtained using the BSA standard dilutions.

2.3. Laccase Activity Assay

The activity of the laccase was determined monitoring the oxidation of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)). 10 mM of ABTS in 10 mL of ultra-pure water were prepared. 50 µL of ABTS solution were then added together with 170 µL of enzyme solution in 100 mM succinic acid buffer pH 3.5. The oxidation of ABTS to its cation radical was monitored at 420 nm using a Tecan Infinite 200 Pro spectrophotometer (Tecan, Zürich, Switzerland) in 25 cycles of 10 s each at 30 °C using 96-well microtiter plates (Greiner 96 Flat Bottom Transparent Polystyrene). The activity was calculated in unit (U/mL) in which one unit of enzymatic activity is defined as the amount of enzyme that oxidizes 1 µmol of ABTS per minute under the assay conditions. The assay was carried out at various pH-values to determine the activity optimum.

2.4. Laccase Activity in Organic Solvents

The relative activity of laccase in organic solvents was determined by modifying the above protocol (Section 2.3). 5, 10 and 20 (*v*/*v*) of a homogenous mixture of acetone and isobutyl alcohol (1:1 volume ratio) were mixed together with the aqueous buffer solution. The same aqueous buffer/organic solvents mixture was then used as blank for the laccase activity assay. The relative activity was then calculated according to the Formula (1).

$$Relative\ activity\ (\%) = \frac{Activity\ in\ organic\ solvents\ (\frac{U}{mL})}{Activity\ in\ acquous\ buffer\ (\frac{U}{mL})} \times 100 \quad (1)$$

2.5. Laccase Catalysed Oxidation of Trazodone Hydrochloride, Impurity H and Compound F

The oxidation of Trazodone hydrochloride, impurity H, and compound F was carried out with 5 µM of ThL, increasing amounts of the mediator HBT (1-hydroxybenzotriazole) ranging from 0 to 15 mM (0, 5, 10 and 15 mM), and different amounts of acetone and isobutyl alcohol in a 1:1 volumetric ratio, varying from 0 to 20% (0, 5, 10, 20% *v*/*v*) volume of organic solvents with respect to the total volume of the reaction. 5 mg of each API were weighted using an analytical scale. The 25 mM HBT solution was prepared by dissolving the mediator in 250 mL of 100 mM succinic acid buffer at pH 3.5 for 3 h. The oxidation reactions were carried out in 4 mL glass vials with a final volume of 1 mL for 72 h, using an orbital shaker set at 37 °C and 200 rpm. All the reactions were run in duplicates and a full set of blanks were included for each tested condition. The samples were then frozen at −20 °C before further analytical analyses were carried out.

2.6. High-Performance Liquid Chromatography (HPLC-DAD) Analysis

The samples were diluted using MQ-H_2O and ice-cold methanol, therefore facilitating the enzyme precipitation and removal. Samples were then centrifuged (Centrifuge 5427 R, Eppendorf AG, Hamburg, Germany) at 12700 rpm at 4 °C for 15 min and filtered through 0.20 µm PTFE filters (GVS, Indianapolis, IN, USA). The analytes were separated by high-performance liquid chromatography (Agilent Technologies, 1260 Infinity, Palo Alto, CA, USA) using a reversed-phase column C18 (Poroshell 120 EC, C18 2.7 µm 3.0 × 150 mm) equipped with a photodiode array detector (Agilent Technologies, 1290 Infinity II, Vienna, Austria) set at the wavelength of 245 nm to detect the released products. Analyses were run using methanol (MeOH, phase A) and formic acid (HCOOH, phase B) gradient. The flow rate was set to 0.35 mL min^{-1} at a constant temperature of 40 °C. The injection volume was 10 µL. Calibration lines of the compounds of interest (0.01 to 1.0 mg/mL,

Figure S3 in ESI) were used for the quantitative determination of the conversion of the compounds after the laccase-mediated treatment.

The theoretical concentration of APIs in the reaction was calculated based on the sample weight and their conversion was calculated according to Formula (2).

$$API\ Conversion(\%) = 100 - \left(\frac{Concentration\ measured\ by\ HPLC\ \left(\frac{mg}{mL}\right)}{Theoretical\ concentration\ \left(\frac{mg}{mL}\right)} \times 100 \right) \quad (2)$$

2.7. LC-HRMS Measurements

A previously described LC-HRMS method [32] was adopted for the identification of trazodone hydrochloride, impurity H, compound F and the related oxidation products. A Vanquish high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Bremen, Germany) was equipped with a C18 RP column (3.5 µm; 2.1 × 150 mm; XBridge®; Waters; Milford, MA, USA). Eluent A consisted of H_2O + 0.1% FA (*v*/*v*), while eluent B consisted of MeOH + 0.1% FA (*v*/*v*). Gradient elution was carried out after injection of 2 µL sample aliquots with a constant flow rate of 0.25 mL/min and column temperature of 25 °C, starting with 10% eluent B (hold time 1 min), increasing eluent B to 100% in 9 min. After a hold time of 3 min, the system was re-equilibrated for 7 min at 10% B, resulting in a total runtime of 20 min. The HPLC was coupled to a Q Exactive HF system (Thermo Fisher Scientific; Bremen; Germany) via a heated electrospray ionization (ESI) interface. Both positively and negatively charged ions were generated in fast polarity switching mode. Mass spectra were recorded in full scan mode with a resolution setting of 120,000 [full width at half maximum (FWHM) @*m/z* 200] and a mass range from *m/z* 50 to 750. Mass spectra were recorded in the profile mode. For positive ionization, the spray voltage was set to 3600 V, and for the negative mode, to 3400 V. Sheath gas was set to 55 and aux gas to 5 (both arbitrary units).

The sequence was set up in a block design in randomized order including biological samples, solvent blanks, reference standards, and a quality control (QC) sample (QC standard mix), consisting of a mixture of standards in pure solvent. Measurement performance was monitored by regular injection and evaluation of a QC standard mix, consisting of 25 reference standards in MeOH/H_2O/FA (1:1:0.01, *v*/*v*/*v*).

3. Results and Discussion

3.1. Laccase Activity in Organic Solvents

The activity of *Trametes hirsuta* laccase (ThL) in solvents was investigated using increasing amounts of acetone and isobutyl alcohol. Interestingly, the relative activity of ThL increased by 32%, 38% and 11% with 5, 10 and 20% (*v*/*v*) of the organic solvent mixture, respectively (Figure S2 in ESI). These results are in agreement with the work of Wu et al. [33], where various fungal laccases were tested for their tolerance to common water-miscible solvents such as acetone, methanol, ethanol, DMSO and DMF. It was found that pre-incubation with 30% of acetone (*v*/*v*) enhanced the activity of the tested fungal laccases in an effective, reversible and non-substrate-specific way, with up to 3-fold of enhanced activity. Interestingly, it was also reported that the DLac enzyme (laccase from *Cerrena* sp. RSD1) maintained an unchanged secondary structure up to 50% (*v*/*v*) of acetone concentration. It was suggested that organic solvents might enhance the enzymatic turnover rate by causing microenvironmental changes around the flexible regions forming the substrate-binding pocket, without altering the catalytic site. Nonetheless, it has to be considered that enzyme activity and stability in co-solvent are a complex phenomenon affected by several factors, such as, among others, solvent polarity, enzyme surface hydrophobicity and its intrinsic structural rigidity. In fact, it is known that tertiary structures play a crucial role in enzyme stability towards both polar and apolar solvents [34,35]. However, despite enhanced activity, beyond certain thresholds, solvents may disrupt hydrogen bonding, strip essential water layers from the enzyme surface, or even destabilize the overall structure, thus leading to activity loss [36].

On the other hand, the released chloride derived from the treatment of trazodone hydrochloride and its by-products could also represent a potential inhibition factor for the enzyme, despite previous studies having shown good tolerance for *Trametes hirsuta* laccase in a chloride-rich environment [37]. Based on these considerations, and on the need to balance ThL enhanced activity with its stability, it was decided to perform the oxidation experiments with an organic solvent concentration up to 20% (*v*/*v*).

3.2. Oxidation of Trazodone Hydrochloride and By-Products by Laccase-Mediator Systems (LMS)

The oxidation of trazodone hydrochloride, impurity H and compound F was carried out using ThL in combination with different concentrations of the mediator HBT (0, 5, 10 and 15 mM) and increasing volume of the organic solvents up to 5, 10 and 20% (*v*/*v*). Moreover, to ensure proper oxygen supply to the laccase throughout the oxidations, glass vials with a volume of 4 mL were selected to carry out the reactions. All APIs showed good solubility in the reaction buffer, with complete dissolution confirmed once the organic solvents were combined. The results of the oxidation of trazodone hydrochloride are shown in Figure 1.

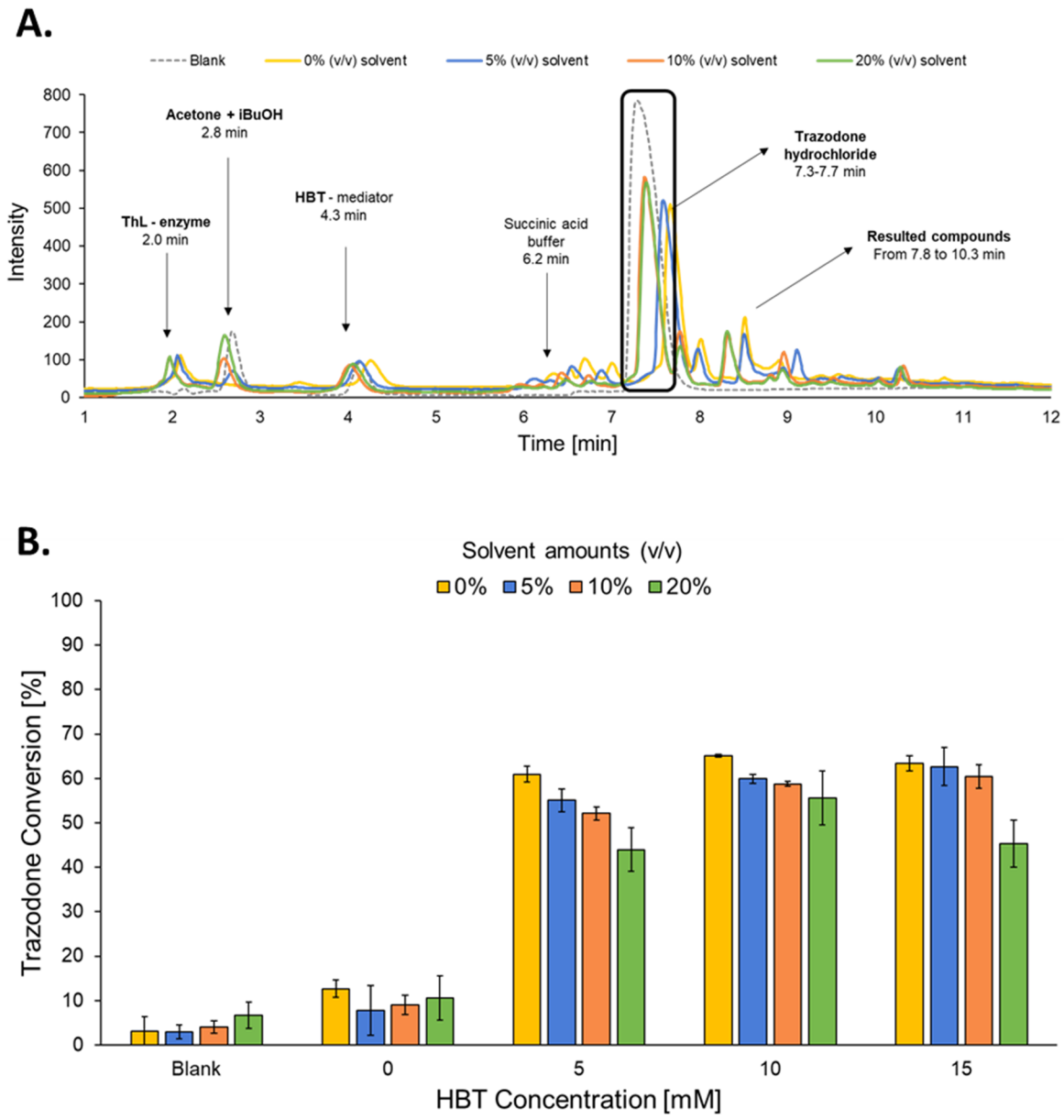

Figure 1. Oxidation of trazodone hydrochloride by a laccase from *Trametes hirsuta* in the presence of various concentrations of the mediator HBT and the solvents acetone and isobutyl alcohol (in a 1:1 ratio). (**A**) HPLC chromatogram and (**B**) Conversion. The figure shows the mean ± SD.

As can be seen from Figure 1B, trazodone was oxidized by the laccase-mediator system at all tested solvent concentrations. A conversion of 65% was seen at 10 mM of HBT in the absence of organic solvents. Similar conversions of 61 and 63% were obtained with 5 and 15 mM of HBT respectively, indicating that a higher concentration of HBT does not necessarily result in higher oxidative activity. In this regard, Mizuni et al. (2009) [38] observed improved oxidation of iso-butylparaben and n-butylparaben up to 2.0 mM HBT, beyond which no further improvement was achieved, while Ashe et al. (2016) [39] reported similar removal efficiencies of oxybenzone,

atrazine and naproxen at both 0.5 and 1.0 mM HBT, indicating no significant advantages at higher concentrations, thus confirming our observation.

Regarding the solvent stability, increasing the proportion of co-solvents to 5% *v*/*v* did not appear to negatively affect the laccase activity, since a conversion of 55%, 59% and 62% was achieved with 5, 10 and 15 mM HBT, respectively. On the other hand, the extent of conversion decreased slightly when 10% of acetone/isobutyl alcohol (*v*/*v*) was added. The increase to 20% *v*/*v* solvent resulted in further reduction, but the API was still converted (44%, 55%, and 45% conversion in the presence of 5, 10 and 15 mM HBT, respectively). As expected, only low conversions were observed by the laccase in the absence of HBT, with results ranging from 8 to 12%. These results could be explained by the fact that despite ThL belonging to the group of high redox potential laccases (E°= 790 mV) [40], trazodone hydrochloride possesses a higher oxidative potential at pH 7 in water calculated between 730 and 1000 mV [41] requiring the presence of a mediator in addition to the enzyme. HBT was reported to possess a redox potential of around 1080 mV, therefore resulting in full capability of oxidizing trazodone in combination with ThL via the radical hydrogen atom transfer mechanism [42].

Similar results were obtained for impurity H and compound F, common side products from the synthesis of trazodone. For impurity H (Figure S5 in ESI), the highest conversion of 73% was obtained with 15 mM HBT and 5% solvent, while all reactions at 0 and 5% solvent concentrations led to similar conversions (66 to 72%) regardless of the amount of HBT utilized. A general decrease in the conversion of impurity H was observed with increasing solvent concentration, yet maintaining a notable conversion up to 50% with the 20% (*v*/*v*) organic mixture. In contrast to trazodone, a conversion of around 20% was observed when the ThL laccase was applied in the absence of the mediator HBT, suggesting a possible lower redox potential of impurity H, which would allow partial oxidation by the sole laccase.

Regarding compound F, the highest conversion of 62% was obtained once again with no solvent and 15 mM of HBT (Figure S4 in ESI). Likewise, in this set of reactions, a slight decrease in compound F mediated oxidation was observed with an increased proportion of organic solvent. Except for the outlier value of 61% conversion obtained with 15 mM HBT at 10% solvent concentration, all other values fitted well with the trends already observed for the trazodone and impurity H oxidation. For example, a very low conversion of 17% was seen when only the laccase was applied without HBT in the oxidation of compound F, confirming once more the importance of the mediator.

3.3. Analysis of the Oxidation Products by Using LC-HRMS

The oxidation of trazodone hydrochloride, impurity H and impurity F was confirmed and assessed by HPLC-Orbitrap-HRMS. In Figure 2, the HPLC-Orbitrap HRMS chromatogram and mass spectrum for the detection of trazodone hydrochloride are shown. The dominant signal for trazodone was the $[M + H]^+$-ion at *m/z* 372.1582 (mass deviation of −1.07 ppm to theoretical *m/z*). The isotope fine structure (Figure 2B) confirms the presence of Cl in trazodone (signal at *m/z* 374.1552 with a *m/z*-difference of *m/z* 1.997 corresponding to the natural abundance of the ^{35}Cl and ^{37}Cl isotopes) and also the isotopic composition.

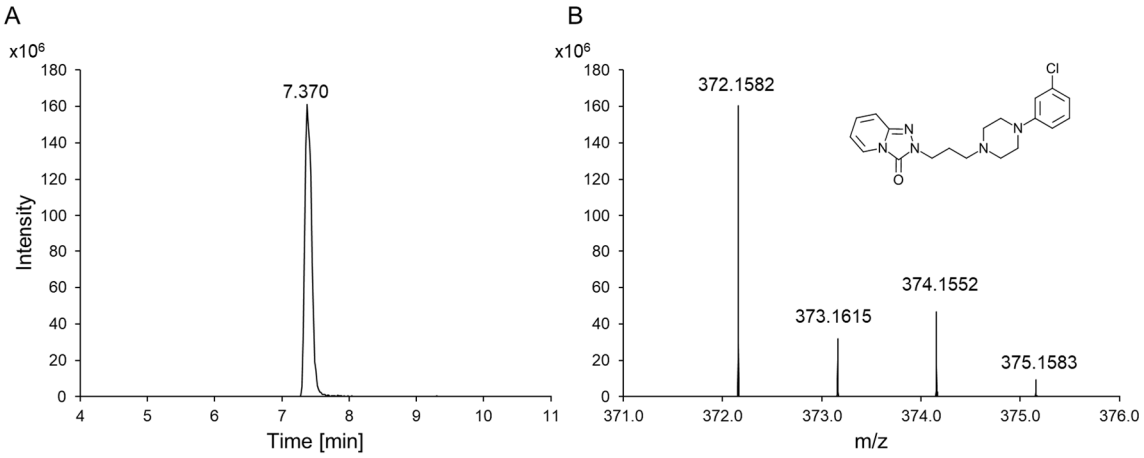

Figure 2. LC-Orbitrap mass spectrometry of trazodone. The chromatogram (**A**) and the isotope fine structure of the $[M + H]^+$-ion (**B**) are presented as intensity plotted against time [min] or *m/z*.

Based on the available literature [43–45], it was possible to identify various oxidation products upon incubation with the laccase-mediator system (Figure 3, Table 1.)

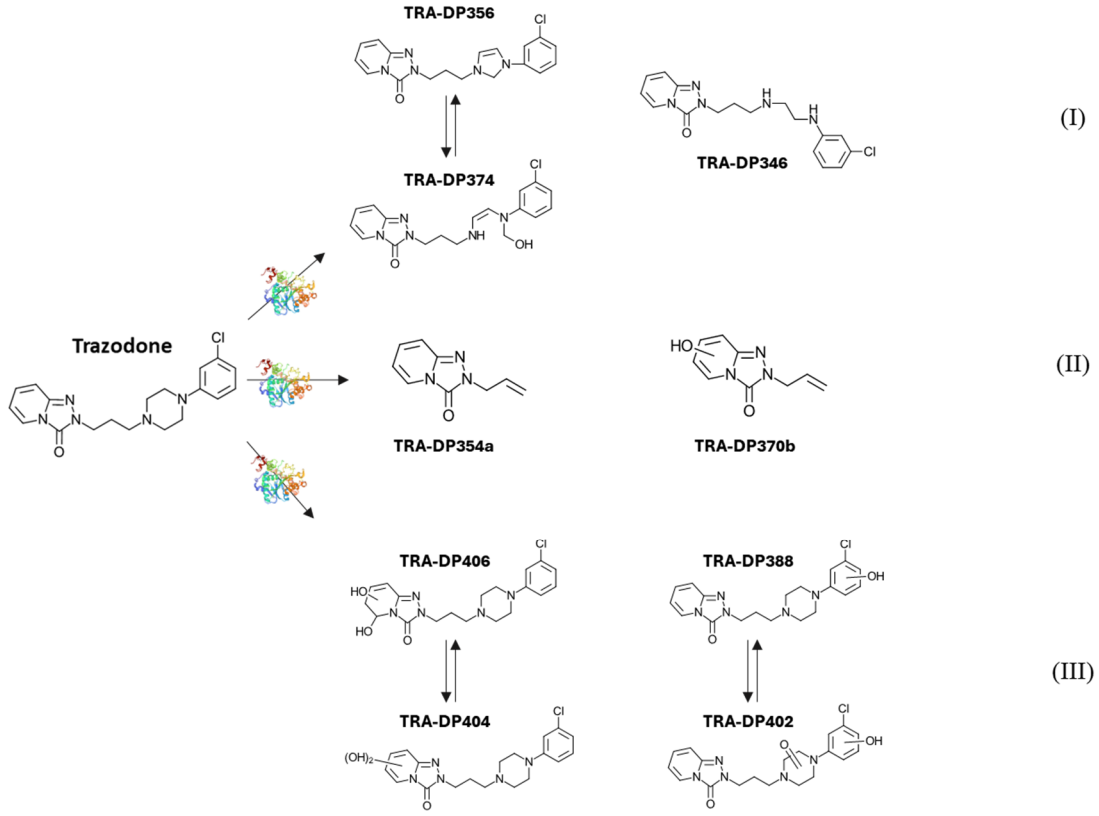

Figure 3. Oxidation pathway of the Trazodone incubated with laccase from *Trametes hirsuta* in the presence of the mediator HBT as analyzed by using LC-HRMS.

Table 1. High-resolution mass spectrometry data corresponding to the elemental composition of trazodone and its degraded products (DP) after the laccase-mediated treatment. All the compounds were detected as $[M + H]^+$.

Description	Molecular Formula	Observed *m/z*	Calculated *m/z*	Error [ppm]	Retention Time [min]
Trazodone	$C_{19}H_{22}ClN_5O$	372.1582	372.1586	−1.07	7.37
TRA-DP346	$C_{17}H_{20}ClN_5O$	346.1432	346.1429	0.87	7.93
TRA-DP354a	$C_9H_9N_3O_1$	176.0820	176.0818	1.14	3.42
TRA-DP356	$C_{18}H_{18}ClN_5O$	356.1276	356.1273	0.84	6.90
TRA-DP370b	$C_{19}H_{23}N_5O_3$	192.0770	192.0768	1.04	6.06
TRA-DP374	$C_{18}H_{20}ClN_5O_2$	374.1381	374.1378	0.80	7.06
TRA-DP388	$C_{19}H_{22}ClN_5O_2$	388.1537	388.1535	0.51	8.15
TRA-DP402	$C_{19}H_{20}ClN_5O_3$	402.1332	402.1327	1.24	8.42, 8.45 *
TRA-DP404	$C_{19}H_{22}ClN_5O_3$	404.1489	404.1484	1.24	8.70
TRA-DP406	$C_{19}H_{24}ClN_5O_3$	406.1645	406.1640	1.23	7.23

* Multiple peaks due to isomers.

As reported by Osawa et al. (2020), trazodone was photo-catalytically transformed into TRA DP-346, DP-374, DP-388, DP-406, and TRA DP-404 through different molecular mechanisms, including hydroxylation, dehydrogenation, and ring opening. In particular, the modification of the piperazine group (Figure 3, I) would lead first to TRA-DP-374 with the partial opening of the functional group and then to TRA-DP-356 by dihydroxylation, forming the dihydroimidazole. Moreover, TRA-DP-346 was generated by the complete opening of the piperazine group from trazodone. A similar oxidase pathway was reported in the study by Mathur et al. (2024), where a combination of crude laccase extracts degraded five fluoroquinolone antibiotics, predominantly targeting the piperazine functional moiety, leading to hydroxylation and ring opening mechanisms [46]. Further oxidation products (Figure 3, II) by the laccase-mediator system were identified in our study in TRA-DP354a and TRA-DP370b, DPs already reported in the photo-catalytical work by Osawa et al. (2019), which would correspond to half of the trazodone molecule, suggesting a possible carbon bond cleavage. However, a molecular mechanism for

these two DPs was not elucidated, despite confirmation of their presence in all laccase-oxidized trazodone samples in our study. As for TRA-DP404 (Figure 3, III), it was suggested that it could be obtained by a double hydroxylation of trazodone, possibly occurring in the pyridine group, leading to the linkage of two hydroxyl groups to the aromatic ring.

A similar degradation pattern involving mainly the piperazine functional group was also observed for compound F and impurity H, resulting in an overall 5 and 7 confirmed oxidation products, respectively (ESI Figures S8–S9, Tables S2–S3). Notably, for impurity H, since the molecule was symmetric—it consists of two identical 4-(3-chlorophenyl)piperazine moieties connected through their nitrogen atoms via a linear propane-1,3-diyl linker—it was possible to detect the single oxidation of one functional group as well as the oxidation of both groups, resulting in the partial opening of the ring group or the formation of dihydroimidazole. However, for both compound F and impurity H, it was not possible to observe further non-specific oxidations as observed for trazodone. Overall, a preferential oxidation of the piperazine group was observed for all three compounds.

On the other hand, it is also essential to consider the overall toxicity and biodegradability of the enzymatically obtained DPs. In this regard, Osawa et al. (2020) [44], performed an in silico assessment of the mutagenic and ecotoxic potential of several trazodone DPs, revealing that most of the compounds were predicted to show mutagenicity, with the exceptions of TRA-DP346 and TRA-DP374. Additionally, the same DPs were found to be potentially toxic to aquatic organisms and not ready to be biodegraded. However, as the same authors emphasized, experimental validation through in vivo assays must be carried out in the future to confirm the predicted toxicity and assess the real environmental persistence of these compounds.

4. Conclusions

High concentrations (5000 mg/L) of trazodone hydrochloride and its synthesis by-products, namely impurity H and compound F, were enzymatically oxidized using *Trametes hirsuta* laccase in combination with the mediator 1-hydroxybenzotriazole (HBT), in organic solvent conditions mimicking industrial waste streams (acetone/isobutyl alcohol 1:1 ratio, up to 20% *v*/*v*). Incubation with the laccase-mediator system led to notable conversions of 62%, 73% and 62% of trazodone hydrochloride, impurity H and impurity F, respectively, while the laccase alone did not lead to significant oxidation. These results make our work one of the few studies demonstrating the feasibility of enzymatic oxidation of high-concentration pharmaceutical residues in an organic solvent-rich environment, simulating industrial manufacturing and waste stream conditions. On the other side, the activity of ThL remained largely stable in solvent concentrations up to 20%, and increasing HBT concentrations above 5 mM did not significantly enhance conversion yields, supporting the feasibility of low-mediator usage. Given the enzyme's tolerance to organic solvents and high pollutant loads, integration into scalable systems such as packed-bed or membrane reactors could be envisioned for industrial applications, especially when combined with enzyme immobilization strategies to enhance stability and reusability.

High-resolution mass spectrometry (HRMS) revealed a preferential oxidation of the piperazine group for all three molecules, offering mechanistic insight into their enzymatic degradation. Further tests must be carried out in the future to assess the real ecotoxicity of the obtained degraded products.

These results shed light on the potential role that enzymes and in particular oxidoreductases could have on the remediation of pharmaceutical residues or impurities resulting during their production, not only in wastewater but also in the presence of organic solvents (such as the production flows of many APIs). Therefore, the importance of biocatalysis in the transition for a greener pharmaceutical industry is highlighted.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/2508071710348804/RC-1296-Supplemenatry-Materials.pdf. Figure S1: Chemical structures of trazodone and the synthesis side products. Table S1: Amounts of mediator and solvents used for laccase-catalysed oxidation of trazodone, impurity H and compound F. Figure S2: Relative activity of *Trametes hirsuta* laccase (ThL). Figure S3: Standard curves for quantification of trazodone, impurity H and compound F by using HPLC-UV. Figure S4: Oxidation of the trazodone synthesis side products compound F. Figure S5: Oxidation of the trazodone synthesis side products impurity H. Figure S6: LC-HRMS of impurity H. Figure S7: LC-HRMS of compound F. Figure S8: Oxidation pathway of the Trazodone synthesis side product compound F. Figure S9. Oxidation pathway of the Trazodone synthesis side product impurity H. Table S2. High-resolution mass data corresponding to the elemental composition of impurity H and its degraded products (DP). Table S3. High-resolution mass data corresponding to the elemental composition of compound F and its degraded products (DP).

Author Contributions

F.F.: Conceptualization, investigation, data curation, visualisation, writing—original draft, writing—review and editing. C.M.W.: Investigation, data curation, writing—review and editing. P.C.: Investigation, Data curation. D.R.: Investigation, data curation. M.D.: Methodology, data curation, writing—review and editing. A.P.: Supervision, writing—review and editing. G.M.G.: Resources, Funding acquisition, conceptualization, supervision, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Not applicable.

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Not applicable.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request.

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Conflicts of Interest

The authors declare no conflict of interest.

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