Enzymatic Removal of Pharmaceuticals from Wastewater using Column-System

A Feasibility Study on Silica-Encapsulated Vermiculite matrix as Support for Enzymatic Quaternary Treatment at Kungsängsverket

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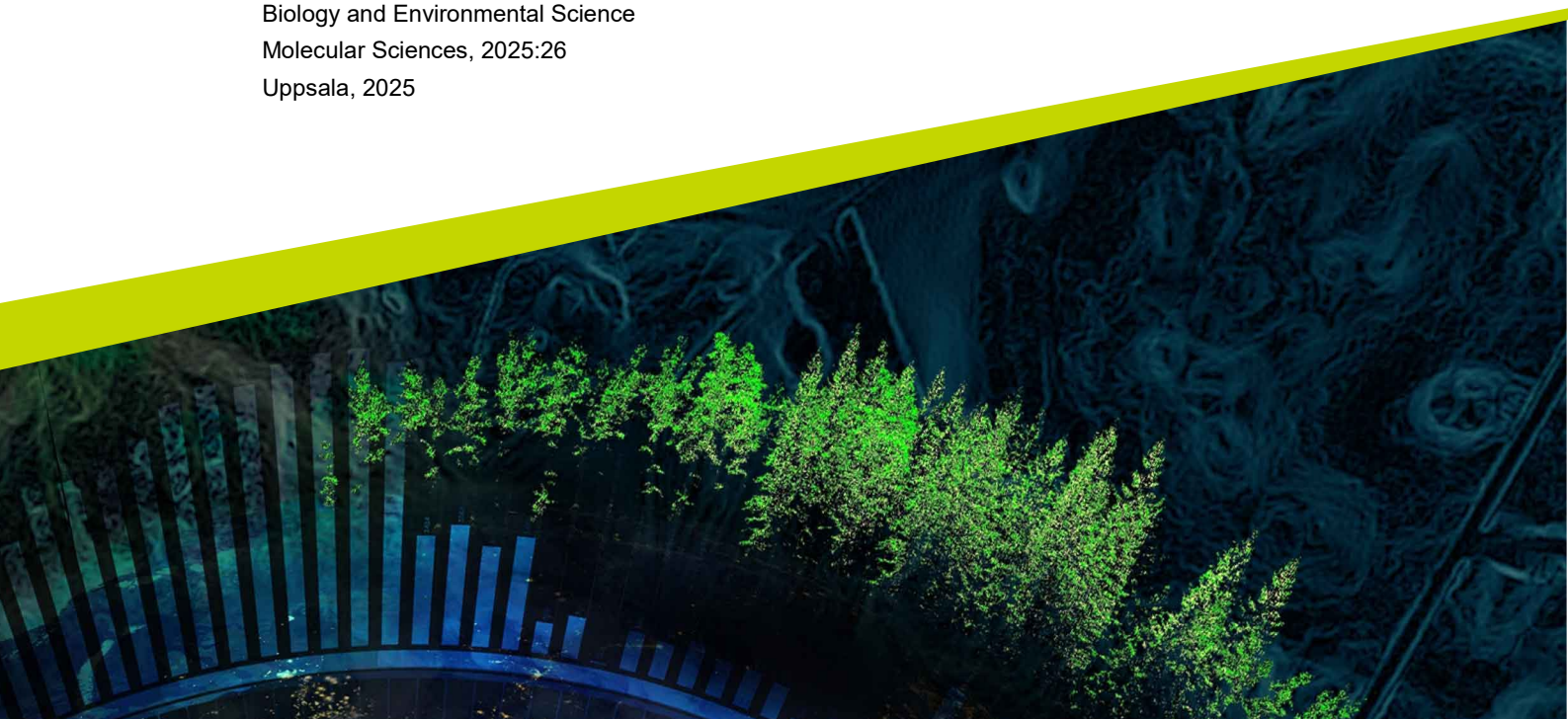

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Abstract

Pharmaceutical residues in municipal wastewater represent a persistent environmental challenge, as conventional treatment processes for these types of compounds are insufficient. This study investigates the feasibility and performance of a GOD-HRP cascade reaction, with silica encapsulated vermiculite matrix as support for degradation of pharmaceutical residues in conventionally treated wastewater. Enzyme activity assays confirmed successful immobilization of the enzymes on vermiculite and retained catalytic function following silica encapsulation. The immobilized enzymes composite was incorporated into a laboratory-scale column system and evaluated over three continuous sample periods, for a total time of 9 hours. High-performance liquid chromatography (HPLC) analysis of eluates demonstrated that 9 out of 16 targeted pharmaceuticals, including venlafaxine, metoprolol, clarithromycin, bisoprolol, and citalopram, were removed with efficiencies exceeding 95% throughout the whole experiment. Nuclear magnetic resonance (NMR) analysis further confirmed the transformation of organic compounds during enzymatic treatment and revealed excessive H2O2 production in later sampling periods, likely causing partial inactivation of HRP and reduced removal of irbesartan, fexofenadine, and oxazepam. Compounds, such as carbamazepine and lamotrigine, showed minimal removal, which highlights the need for further optimization through multi-enzyme systems or mediator-assisted strategies. Obtained results demonstrate the potential of enzyme immobilization on mineral supports for advanced wastewater treatment. Further long-term testing under realistic operational conditions, scale-up studies are needed to fully evaluate the potential of application of this method as a purification technology. This work contributes to ongoing pilot initiatives at Kungsängsverket and provides insights for the development of sustainable solutions to pharmaceutical pollution in urban water systems.

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1. Introduction

Water is a vital natural resource, and scarcity is already a major problem in many regions across the globe, while climate change, overexploitation, and rapid population growth are further stressing the freshwater resources (OECD, 2022). In addition, the remaining water resources are increasingly contaminated by pollutants, which poses significant threats to both ecosystems and human health (Daughton, 2024). Among the most concerning contaminants are pharmaceutical residues, which are persistent and biologically active micropollutants (Kümmerer, 2010; Gupta et al., 2024). Their widespread occurrence, even at trace concentrations, have proven to adversely affect the environment which reflects the growing pressure that anthropogenic activity places on aquatic environments and the resource itself (Brodin et al., 2022). This highlights the need for more careful pharmaceuticals- and freshwater management as well as implications of effective water purification technologies.

Pharmaceutical residues enter aquatic environments through multiple pathways, such as hospitals, pharmaceutical manufacturing, domestic sewage, and agricultural runoff (Zhang et al., 2023). Most of these compounds are excreted unchanged or as active metabolites, and conventional wastewater treatment plants (WWTPs) are not specifically designed to remove them (Bilal et al., 2018). Consequently, pharmaceutical residues, including antibiotics, hormones, and psychiatric drugs, are frequently detected in surface water, groundwater, and even drinking water sources (Brodin et al., 2022). The trace concentrations of these pollutants are enough to pose risks to aquatic ecosystems and may disrupt biological processes in non-target organisms (Hayes et al., 2014).

Among the most pressing concerns is the positive correlation between antibiotic contamination and the development of antimicrobial resistance (AMR). Continuous low-level exposure to antibiotics in aquatic environments have shown to promote antibiotic-resistant bacteria (ARB) and facilitate the spread of antibiotic resistance genes (ARGs) and thereby reducing the efficacy of critical

medicines (Daughton, 2024). The World Health Organization has declared AMR as an urgent global health crisis, projecting that it could cause more deaths worldwide than cancer by 2050. While pharmaceuticals, such as hormones and psychoactive drugs, have been shown to affect the reproductive and behavioural patterns of aquatic organisms (Hayes et al., 2014). In addition, diclofenac, a widely used anti-inflammatory drug, has caused kidney failure in vultures and led to a rapid and dramatic population decline in India (Science History Institute, 2017). Moreover, there is a growing concern regarding diffuse, chronic, and low-level exposure to mixtures of pharmaceutical residues through the consumption of drinking water and food products. Although only a limited number of studies have been done regarding the potential long-term, cumulative, or synergistic effects on human health (Daughton, 2024).

Despite increasing evidence of these risks over the past three decades, global regulatory frameworks have been slow to respond. However, the European Union has recently, through Directive 2024/3019, set a legal framework that strengthens requirements for the collection, treatment, and discharge of urban wastewater. This includes the implementation of quaternary treatment stages in WWTPs to target micropollutants more effectively. The directive mandates that large-scale treatment plants (≥150,000 population equivalent) and facilities in pollution-sensitive areas adopt advanced purification technologies. By year 2033, 20 % of the large WWTPS must be upgraded with a post-treatment step, by 2039, 60 %, and from 2045, all treatment plants required to have implemented an effective method. The directive defines 12 indicator pharmaceuticals for substances which expose risks even at low concentrations. The indicator compounds are divided into two categories based of how easy they can be treated, see table 1. From these 12 pharmaceuticals must at least six substances reach 80 % removal efficiencies, and the number of substances from category 1 shall be twice the number of substances category 2 (European Union, 2024).

Table 1: EU Directive 2024/3019 list of indicator pharmaceuticals and their respective category for quaternary treatment of discharges from urban WWTPs. Category 1 includes pharmaceuticals that's easier to treat in comparison to category 2.

Category 1 (easier to treat)	*Category 2 (difficult to treat)*
Amisulprid	Benzotriazole
Carbamazepine	Candesartan
Citalopram	Irbesartan
Clarithromycin	mixture of 4-methylbenzotriazole, 5-methylbenzotriazole
Diclofenac	
Hydrochlorothiazide	
Metoprolol	
Venlafaxine	

Among emerging treatment options, enzymatic purification has gained significant attention due to its efficiency, environmental compatibility, and operational simplicity (Bilal et al., 2018). Enzymes are highly specific biological catalysts capable of transforming complex and stable organic molecules. Compared to chemical or physical processes, enzymatic degradation offers advantages such as reduced energy input, minimal toxic byproduct formation, and adaptability to different pollutants (Mohamad et al., 2015). Laccases, peroxidases, and tyrosinases are among the most studied enzymes for this application, with promising results in degrading pharmaceuticals, dyes, phenolic compounds, and endocrine-disrupting chemicals (Veitch, 2004).

However, challenges remain, free enzymes in solution are often unstable and may lose activity due to temperature, pH fluctuations, or the presence of inhibitors They are also soluble in water, causing risk of washing out of treatment systems, which would require continuous dosing, thus limiting cost-efficiency and scalability (Guisan, 2013).

A promising solution to this challenge is enzyme immobilization, i.e. the process of anchoring enzymes onto support matrixes to enhance their stability, reusability, and resistance to environmental stressors (Mohamad et al., 2015). In this context,

the selection of appropriate support materials and immobilization strategies becomes crucial. The immobilization matrix influences enzyme kinetics, structural integrity, and long-term operational performance. Additionally, substrate should ideally be biocompatible, chemically stable, mechanically robust, and provide a high surface area for enzyme binding. Common strategies include mechanisms such as, adsorption, covalent bonding, entrapment, and encapsulation—each with different impacts on enzyme activity and stability (Guisan, 2013).

This study focuses on immobilizing enzymes onto vermiculite covered by silica-based shells, giving rise to distinct advantages. Vermiculite is a naturally occurring clay mineral with a 2:1 layered structure, consisting of two tetrahedral silica sheets sandwiching one octahedral sheet. To maintain electrical neutrality, the interlayer spaces contain exchangeable cations such as magnesium, calcium, and sodium (Sari et al., 2007). Additionally, upon treatment, vermiculite expands, increasing porosity and improving accessibility for enzyme immobilization. Chemically, vermiculite is a hydrated magnesium-aluminium-iron silicate, which offers a high surface area, ion-exchange capacity, and chemical stability (Sari et al., 2007). The low cost and availability make it an attractive candidate for large-scale applications. Moreover, studies have shown that vermiculite can improve enzyme reusability, thermal stability, and catalytic efficiency (Mohamad et al., 2015).

Silica encapsulation is widely used in nanomaterial-based immobilization due to its tuneable porosity, chemical inertness, and easy functionalization. Encapsulation within silica matrices has been shown to protect enzymes from denaturation to improve preservation of their catalytic activity (Vardanyan et al., 2024).

These materials could represent a feasible and scalable approach to stabilizing enzymes for quaternary wastewater treatment. Immobilization not only enhances enzyme resilience during storage and operation but also simplifies recovery and is reducing operational costs (Guisan, 2013). Importantly, immobilized systems can

be integrated into continuous flow reactors, making them compatible with existing WWTP infrastructure.

For this study, we developed an environmentally friendly dual-enzyme catalytic system, where horseradish peroxidase (HRP) and glucose oxidase (GOD) were immobilized separately onto the silica encapsulated vermiculite matrix (Li et al., 2013). Multienzyme systems plays a crucial role in many metabolic pathways in various biological system and has been highlighted as an efficient tool for green chemistry that can enhance substrate selectivity as well as enzymatic activity (Sheldon & Woodley, 2018; Turner, 2003).

Here, HRP is used as a target enzyme for the pharmaceuticals, while GOD produces hydrogen peroxide (H_2O_2), which is required for HRP to initiate the catalytic reaction. This can provide a controlled range of H_2O_2 concentration, which is crucial since HRP is known to be inactivated by high concentrations of H_2O_2, and thereby reducing the enzymatic removal efficiency of the pollutants (Baynton et al., 1994). Moreover, by producing H_2O_2 locally and in appropriate concentrations, the system reduces the need of external chemicals (Li et al., 2013).

HRP is a commercially available heme-containing oxidoreductase, derived from the root of horseradish (*Armoracia rusticana*), that has shown promising results to catalyse the oxidation of a broad range of electron-rich aromatic and phenolic substrates. For instance, pharmaceuticals, dyes, hormones and endocrine disruptors, phenolics, as well as some other organic pollutants (Veitch, 2004). The reaction relies on H_2O_2, which serves as an antioxidant that initiates the formation of an active state of HRP, allowing the binding of the substrate, which results in generation of more stable radical products and water (Veitch, 2004). The 3D structure of HRP with its active site is illustrated in figure 1, which was constructed using PyMOL based on PDB entry 1HCH (Gajhede et al., 1997).

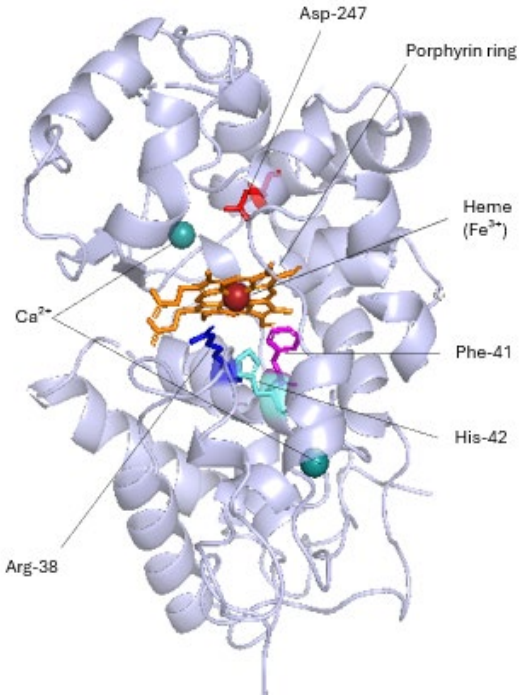

Figure 1: Structure of HRP including its active site with the heme group (Fe3+), coordination amino acid residues, and calcium ion (Ca2+). Based on PDB ID:1HCH, constructed in PyMOL (The PyMOL Molecular Graphics System, Version 2.5, Schrödinger LLC).

GOD is a widely used enzyme that catalyses the oxidation of β-D-glucose to gluconic acid by using oxygen as an electron acceptor, while simultaneously producing H_2O_2 as a byproduct. It is most derived from *Aspergillus niger* and has many different applications such as, biosensors, food preservation and biomedical applications. Its robustness, availability, and substrate specificity make it a strong candidate for use in multi-enzyme systems (Bankar et al., 2009; Wong et al., 2004).

The aim of this study was to evaluate the feasibility and performance of an immobilized dual-enzyme system with GOD-HRP, for the degradation of pharmaceutical residues in wastewater collected from Kungsängsverket, Uppsala's largest wastewater treatment plant. By testing the enzymatic activity, removal efficiency, and reusability of a column-based method, to evaluate the potential of this method as a quaternary purification method at WWTPs.

This thesis is conducted in collaboration with IVL Swedish Environmental Research Institute as part of a broader pilot study led by Uppsala Vatten och Avfall AB at Kungsängsverket. Their pilot study aims to evaluate advanced treatment technologies for the removal of pharmaceutical residues and PFAS. By contributing with insights into enzyme immobilization as a quaternary treatment method, this thesis complements ongoing efforts within this pilot project to develop an effective, full-scale post-treatment stage.

2. Materials and methods

2.1 Materials

The enzymes used in this study included: HRP, Sigma-Aldrich Sweden AB, CAS 9003-99-0, catalogue No. 8375-5, ≥257 U/mg; GOD, CAS No. 9001-37-0, Cat. no. 7141-50, ≥156 U/mg. The following chromogenic substrates were used for the enzyme activity assays: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) liquid peroxidase substrate, Sigma-Aldrich Sweden AB, Cat. No; 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS).

For preparation of support matrix: vermiculite, Impecta Fröhandel, Sweden; nitric acid, Sigma-Aldrich Sweden AB, CAS No. 7697-37-2, Cat. No. 438073, purity: 70%; tetraethoxysilane (TEOS), Sigma-Aldrich Sweden AB, CAS No. 78–10–4, Cat. No. 8.00658; ammonium fluoride Sigma-Aldrich Sweden AB, CAS No. 12125–01–8, Cat. No. 338869; ethanol, Sigma-Aldrich Sweden AB, CAS No 64-17-5, Cat. No. 2210, purity: 99,6%;

Additional chemicals and reagents used in this study: D-glucose, Sigma-Aldrich Sweden AB, CAS No. 55-99-7, Cat. No. 200-075-1, purity: ≥99.5%; monopotassium dihydrogen phosphate, Sigma-Aldrich Sweden AB, CAS No. 7778-77-0, Cat. No. 00662, purity: ≥99.0%; dipotassium hydrogen phosphate, Sigma-Aldrich Sweden AB, CAS No. 7758-11-4, Cat. No.1.05104; methanol, Sigma-Aldrich Sweden AB, CAS No. 67-56-1, Cat. No. 1.06007, 99,9%.

The following materials were used for the experiments and the analytic setup:

municipal treated wastewater from Kungsängsverket, Uppsala; The column unit was made at SLU workshop and it was constructed from PVC, a total volume of 144.3 cm^3, an internal dimensions of 3.5 cm in diameter and a height of 15 cm for the sorbent compartment; peristaltic pump (Pharmacia Fine Chemicals, Peristaltic Pump P-1); glass fiber filters (Whatman GF/F, Ø47 mm) Sigma-Aldrich Sweden AB, catalog no. 1825-047; Oasis HLB cartridges (200 mg, 6 mL, 30µm, Waters), Fisher Scientific, Sweden AB. Cat. No. 50-786-045.

2.2 Methods

2.2.1 Nitric acid treatment for vermiculite

To immobilize the enzymes on vermiculite, 500 ml of vermiculite was treated with 1 L of 0,1 M nitric acid and placed on a shaker (125 rpm) for four hours. After that, the vermiculite was separated and washed with distilled water and placed on a shaker (125 rpm) for 1 hour. This was done repeatedly until it reached a pH-value of 5-6 after which it was placed in an oven at 60 °C until completely dry. This procedure was done several times until an appropriate amount was produced. This is an important step to remove impurities, increase surface area and porosity, and enhance the interaction with the enzymes.

2.2.2 Scanning Electron Microscopy

To evaluate the effects of the nitric acid treatment on the vermiculite, scanning electron microscopy was utilized. It is a method commonly used to produce images of samples. By scanning the surfaces with a focused beam of electrons, various signals can be observed which provide information of the surface's topography and composition. Micrographs were acquired at various magnifications to assess changes in surface structure, porosity, and particle morphology resulting from the acid treatment. Images from untreated and treated samples were compared to determine and confirm desired modification of the support material.

The SEM analysis was carried out using a Hitachi (Tokyo, Japan) Flex-SEM 1000-II environmental scanning electron micro-scope at an acceleration voltage of 5 kV, a spot size of 20, with a working distance of 5 mm. The elemental surface analysis was done using energy-dispersion spectroscopy (EDS) and the electron microscope mentioned above, in combination with an AZtecOneXplore EDS detector by Oxford Instruments (UK). Five different areas were analysed for each sample, using an acceleration voltage of 20 kV, a spot size of 50, and a working distance of 10 mm.

Since vermiculite is a clay mineral, no fixation or hydration was necessary before the SEM analysis, instead were small fragments of vermiculite directly placed on the SEM stub using carbon tape to avoid being clamped and keep them fixed during the analysis.

2.2.3 Immobilization of Enzymes on Vermiculite

To immobilize the enzymes on the treated vermiculite, 94,08 mg of GOD (156,713 U/mg) and HRP (257,2 U/mg) was placed in separate solution with 1176 mL of 0,1 M phosphate buffer (pH = 7,04) with resulting concentrations of 20 U/ml and 12,56 U/mL, and magnetically stirred for 30 min. Consequently, 11,17 g of treated vermiculite was placed in 392 mL of the enzyme solution, three of these samples were made for each immobilized enzyme and then placed on the shaker (125 rpm) overnight. This ratio (1:9.33) between enzyme volume and vermiculite was considered too high, a volume ratio of 1:3 was used for the last batches instead.

2.2.4 Enzyme Activity Assay

The enzymatic activity of both free and immobilized samples was evaluated spectrophotometrically by monitoring the oxidation of ABTS, a chromogenic substrate. This was conducted in the presence of hydrogen peroxide, for the activity of GOD, it was produced from a 0,1 mg/mL ABTS solution by GOD, but for HRP was an 18 µM ABTS solution utilized, that contained hydrogen peroxide. All measurements were performed in duplicate by using Multiskan Sky High (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer with a 96-

well microplate at 25 °C, and absorbance was measured at a wavelength of 420 nm for a total duration of 10 minutes with a reading interval of 10 seconds.

A chromogenic stock solution was prepared by dissolving 10 mg of ABTS (2,2'-Azinobis [3-ethylbenzthiazoline-6-sulfonic acid]-diammonium salt) in 10 mL of phosphate buffer (0.1 M, pH 7.04. This solution was subsequently diluted tenfold by mixing 1 mL of the ABTS solution with 9 mL of phosphate buffer. To quantify the activity of GOD, a coupled reaction system with HRP was employed. Where HRP oxidized ABTS in the presence of hydrogen peroxide, which was generated as a byproduct of GOD's conversion of D-glucose to gluconic acid. Each batch contained 40 µL phosphate buffer, 20 µL of the enzyme solution (10× dilution), 20 µL of the ABTS-oxidizing enzyme (20 U/ml diluted 50 times), and 30 µL of a substrate mixture containing 35 mM glucose and 20 mM ABTS. The increase in absorbance, reflected by the oxidation of ABTS, was used to determine the activity of the enzyme which produced hydrogen peroxide.

To isolate the activity of HRP, a separate assay was performed using a ABTS solution which already contains hydrogen peroxide. The solution was prepared by mixing 1 mL of ABTS with 9 mL phosphate buffer and then diluting the mixture 10-fold. In each batch, 20 µL of buffer and 20 µL of the enzyme solution (diluted to a final concentration of 5 U/mL) were combined with 80 µL of the activated chromogenic substrate solution, bringing the final volume to 120 µL. The increase in absorbance at 420 nm was recorded under identical conditions.

After that, the enzymatic activity was calculated for the enzyme solution before and after immobilization onto the vermiculite matrix, to confirm successful adsorption of the enzymes and determine the retained activity per gram vermiculite, see appendix 1 for calculations.

2.2.5 Silica Shell Encapsulation

The vermiculite with immobilized enzymes was separately covered by silica and for each batch of vermiculite. First, a solution with 20 mL MQ water, 20 mL ethanol and 800 µL NH_4F was prepared and to synthesize the silica shell, 16 mL TEOS mixture with 20 ml ethanol, was added dropwise, 1 ml at the drop for a total time of 30 min, while whole solution was under constant stirring. After that, 11,17 g of the prepared vermiculite composite was added to the above-mentioned mixture and placed on a shaker (125 rpm) for 4 hours. Subsequently, the vermiculite was separated from silica suspension and washed with ethanol (99,6 %) and MQ water two times each, then dried with nitrous gas overnight, or until dry, keeping covered in parafilm and stored in 4 °C. This procedure was repeated for each vermiculite batch.

2.2.6 Enzymatic Activity and Reusability Evaluation

To check the activity of enzyme after the entrapment of vermiculite in silica shell, 69 mg of the vermiculite composite was placed in a falcon tube and mixed with 1 mL ABTS, 0,5 mL ABTS-oxidizing enzyme (20 U/mL was diluted 50 times with phosphate buffer) and lastly 1 mL of 35 mmol/L D-glucose. After 60 seconds, 0,2 mL of the solution were removed with a syringe and 0,1 mL transferred to a 96-well plate and assayed in the spectrophotometer at the wavelength of 420 nm to measure the oxidation of ABTS, which was used to determine the activity of the immobilized enzyme that produced H_2O_2. Subsequently, the immobilized enzyme was separated from the mixture and then placed in phosphate buffer and stored at 4 °C covered in aluminium foliage. This procedure was done repeatedly to evaluate the activity of the enzyme during time.

2.2.7 Column-based Wastewater Treatment

The enzymatic purification was carried out using a column system with a contact time of 60 minutes, see figure 2. The column used for treatment was a 210 mL cylindrical column, which was filled with the two different immobilized enzymes with a GOD:HRP mass ratio of 1,33:1. Prior to packing, immobilized enzymes

were carefully mixed to ensure even distribution. A peristaltic pump (Pharmacia Fine Chemicals, Peristaltic Pump P-1) was used to control the flow rate of the wastewater through the column at a constant rate of 3.5 mL/min, and the treatment was conducted at 25°C. Before collecting samples, the wastewater was prepared by spiking with D-glucose to a final concentration of 35 mmol/L and the column was flushed for 30 minutes and after this equilibration step, sample collection commenced. The experiment was performed in triplicate for a total of 9 hours, where one sample (~600 ml) was collected each 3 hours. A total volume of approximately 2 L of glucose-spiked wastewater was treated across the experiment. After that the samples were stored at –20 °C until further analysis. All collected wastewater samples were initially stored at 4°C for up to three weeks and then stored at –20 °C until the water treatment.

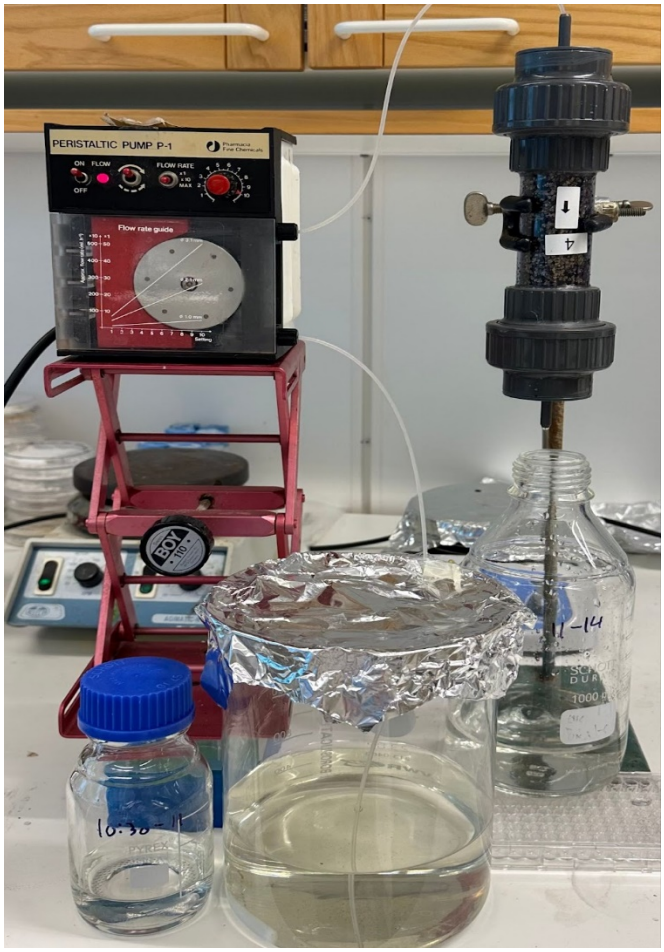

Figure 2: Schematic illustration of the column-based enzymatic wastewater treatment setup used in this experiment. The large beaker covered with aluminium foil (center) contains the untreated wastewater and the glass flask to the right of it collects the treated water after passage through the enzymatic column. The smaller container left of the big one, is already purified water. The column is connected to a peristaltic pump to control flow rate.

2.2.8 Sample Preparation and HPLC

2.2.8.1. Sample Preparation

Triplicate samples of 200 mL were prepared from stored samples, pre- and post-treatment, one matrix sample and two blanks (Milli-Q water and tap water) were included. All samples were transferred into polypropylene (PP) bottles, which had been thoroughly cleaned. Each empty bottle was labelled and weighed.

Glass fiber filters (Whatman GF/F, Ø47 mm, catalog no. 1825-047) were combusted at 400 °C overnight and all samples were gently shaken before the experiment. Each sample was then vacuum filtered and collected in the pre-weighed PP bottles. A volume of 150 mL was collected from each filtrate for SPE processing, and then were samples weighed again to determine the exact volume of the filtered sample. The samples (excluding the matrix sample) were spiked with 20 µL of internal standard (IS) solution containing each target compound at 1 µg/mL concentration. The IS solution was brought to room temperature prior to spiking and mixed.

2.2.8.2 Solid Phase Extraction

SPE was performed using Oasis HLB cartridges (200 mg, 6 mL, 30 µm). The cartridges were flushed with 6 mL of methanol followed by 6 mL of Milli-Q water through each cartridge by gravity flow. Each spiked sample was loaded onto the cartridge at a flow rate of approximately one drop per second using only mild vacuum. After sample loading, cartridges were rinsed with 6 mL of Milli-Q water and then dried under vacuum for 20 minutes. The procedure of the sample preparation and SPE is described in detail by Sörengård et al. (2019)

2.2.8.3 Elution and HPLC Preparation

Cleaned and labelled plastic tubes were placed under each cartridge. Elution was performed by using a solvent with 2 × 4 mL methanol, passed by gravity, to retain the content of interest for further HPLC analysis.

The samples were then evaporated under a nitrogen stream to ~0.5 mL and transferred to 1 mL amber HPLC vials and the plastic tubes were rinsed three times with ethanol. Subsequently, the respective vial content was evaporated again to 0.5 mL with nitrogen gas. The samples were then vortexed and stored at –20 °C. Before HPLC analysis, the samples were defrosted and 0.5 mL MQ water was added to each vial, resulting in final volume of 1 mL. Finally, they were vortexed for another 30 s.

2.2.8.4 HPLC analysis

To evaluate concentration and removal efficiency, of the pharmaceuticals in the wastewater treatment, HPLC was employed. This technique is widely used for the separation, identification, and quantification of specific components. High-pressure pumps create the flow of the sample through a mobile phase, while the compounds interact with a stationary phase in a column, resulting in different migration rates based on their unique interactions.

The samples were analysed using a DIONEX Ultimate 3000 ultraperformance liquid chromatograph (UPLC) system (Thermo Scientific, Waltham, MA, USA) coupled to a triple quadrupole mass spectrometer (MS/MS) (TSQ Quantiva, Thermo Fischer Scientific, Waltham, MA, USA). The data were evaluated with Tracefinder 4.1 (Thermo Fischer Scientific, MA, USA). Detailed information regarding instrument configuration and analysis is described by Rehrl et al. (2020)

During the SPE extraction was 20 µL of a 1 µg/mL stock solution of an internal standard (IS) added to each sample. This serves multiple purposes, such as

compensate for matrix effects, validation of calibration curves, calculations, compensating for sample variability and potential loss of analytes, and thereby enhances both accuracy and precision of the quantification. However, a total of 16 pharmaceuticals were identified and analysed, each with its corresponding internal standard and type presented in table 2. While most of the compounds utilized isotope-labelled standards, some employed structural analogues as their internal standards. The calculations of removal efficiencies, concentrations, LOQ, RSD is outlined in appendix 1.

Table 2: The internal standard, its type and their corresponding analytical pharmaceutical for the HPLC analysis.

Analytes	**Internal standards (IS)**	**Type of IS**
Trimethoprim	Sulfamethoxazole, D4	Isotope-labelled
Metoprolol	Atenolol-D7	Isotope-labelled
Bisoprolol	Atenolol-D7	Structural analogue
Venlafaxine	Venlafaxine-D6	Isotope-labelled
Carbamazepine	Fluoxetine_D5	Structural analogue
Fexofenadine	Venlafaxine-D6	Structural analogue
Propranolol	Atenolol-D7	Structural analogue
Citalopram	Venlafaxine-D6	Isotope-labelled
Oxazepam	Oxazepam-D5	Isotope-labelled
Irbesartan	Irbesartan-D7	Isotope-labelled
Amitriptyline	Fluoxetine_D5	Structural analogue
Clarithromycin	Lidocaine-(diethyl), D10	Structural analogue
Atenolol	Atenolol-D7	Isotope-labelled
Hydrochlorothiazide (HCTZ)	Hydrochlorothiazide-13C, D2	Isotope-labelled
Lamotrigine	Oxazepam-D5	Structural analogue
Sulfamethoxazole	Sulfamethoxazole, D4	Isotope-labelled

2.2.9 NMR analysis

To investigate the wastewater samples and confirm the results from HPLC analysis, Nuclear Magnetic Resonance (NMR) spectroscopy was utilized. NMR is a widely used method to determine the structure of organic molecules, by using nucleases within a constant magnetic field which are disturbed by a weak oscillating electromagnetic field, consequently producing signals that provide detailed information about the chemical structure and composition of the sample.

In this study, the NMR spectra was acquired using a Bruker Avance 600 MHz NMR spectrometer and the data was processed with TopSpin 4.3.0 software. The spectra were recorded over a chemical shift range of 0-12 ppm, which includes detection of aromatic (6-12 ppm) and aliphatic regions (0-4,5 ppm). Furthermore, multispectra were collected where the presence or absence of characteristic signals in different regions gave insight into what type of organic compounds were present, as well as the differences in their abundance. This increases the reliability and gives a deeper understanding of the composition of the wastewater samples and the effects of the enzymatic wastewater treatment.

3. Results and discussion

3.1 Characterization of the Treated Vermiculite

To confirm the morphological and structural changes of vermiculite after nitric acid treatment, SEM was used to generate images of the treated and untreated vermiculite. The SEM images were captured at a magnification 1000x with a scale bar of 50 μm and 100 μm respectively.

The results display clear difference of the morphological properties of the samples, as illustrated in figure 3, where the left images illustrate the untreated vermiculite and the right is the treated vermiculite. The untreated vermiculite

shows characteristics of intact and smooth surfaces structure, which is typical for natural vermiculite. In contrast, the acid-treated vermiculite appears to have undergone significant structural conformation by chemical erosion, with the increased porosity, fragmentation and reduced particle size. These are properties that have been proven to enhance the interactions with the enzyme, which can increase the retained activity as well as the stability of enzymes (Suquet et al., 1991; Stawiński et al., 2016). These findings suggests that nitric acid treatment is an effective method to improve the immobilization of the enzymes on vermiculite. While Fourier-transform infrared spectroscopy (FTIR) could be employed to further assess the removal of organic impurities, identify chemical bonds, and to confirm changes in the mineral structure (Stawiński et al., 2016). The SEM analysis was considered enough to demonstrate desired effects of the nitric acid treatment and supported the decision to proceed to next step: immobilization of the enzyme onto the vermiculite matrix.

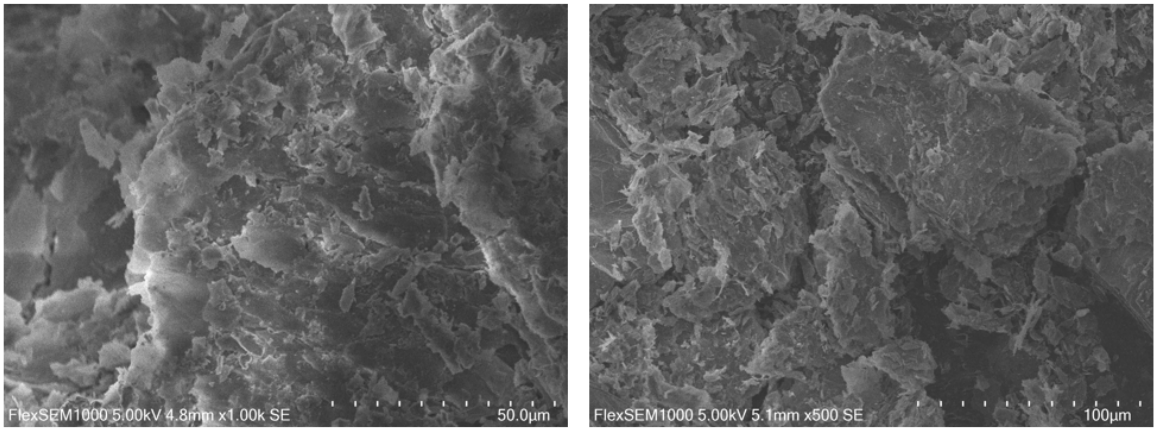

Figure 3: Images generated from SEM illustrating the morphological differences on the untreated (left) and nitric acid treated vermiculite (right).

3.2 Activity Assessment on Vermiculite Composite

To evaluate the immobilization on vermiculite and calculate the retained enzymatic activity of the support matrix, spectrophotometric analysis was

employed, where the enzyme solution before immobilization was compared with the solution after immobilization. The assays were conducted by monitoring the oxidation of ABTS, the chromogenic substrate, spectrophotometrically at 420 nm in the presence of hydrogen peroxide.

The results demonstrated a reduction in enzymatic activity after immobilization of both enzymes onto vermiculite. Where the rate of absorbance increase was higher for the free enzyme samples compared to the immobilized, which indicates a loss in catalytic efficiency after immobilization, illustrated in figure 4. This observation aligns with previous studies, where enzyme immobilization typically leads to decreased activity due to loss of accessibility for enzymes that get anchored onto the support and loss of some enzyme fraction during immobilization (Vardanyan et al. 2024). By calculations outlined in the appendix 1, the retained enzymatic activity per gram of vermiculite was determined to 390 U/g for GOD and 420 U/g for HRP.

These findings confirm that the enzymes were successfully immobilized onto the vermiculite surface. The differences in retained activity between the two enzymes may reflect differences in their structural stability or surface interaction behaviour and may be due to differences in their isoelectric points and molecular structures. At the working pH 7.0, HRP has a pI which is closer to neutrality and therefore may have adsorbed more effectively onto the vermiculite surface via electrostatic interactions (Lopes, et al., 2014).

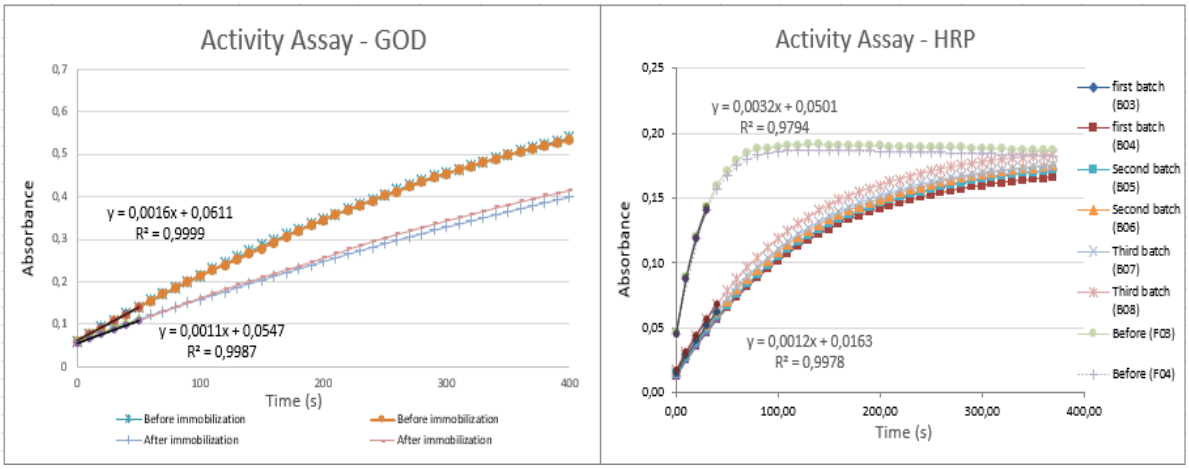

Figure 4: Enzyme activity of free and immobilized enzymes on vermiculite, on the left is the measurament for the H_2O_2 producing enzyme (GOD) and the right graph represents the H_2O_2-consuming enzyme (HRP). The intial measurments, marked in black, were used for the calculation of enzymatic activity and immobilization, together with their equation and R value. For the activity of HRP were duplicates assayed from each batch of the prepared vermiculite composite, while the activity assay for GOD was only one of the three batches assesed.

3.3 Silica Encapsulation and Enzyme Reusability

The reusability of the immobilized enzymes was assessed over ten cycles for approximately 30 days and were stored in phosphate buffer at 4 °C after each measurement. The test was carried out by monitoring the oxidation of ABTS spectrophotometrically at 420 nm in the presence of hydrogen peroxide.

The absorbance readings showed no significant decline in enzymatic activity across all ten cycles, indicating that the immobilized enzymes retained their catalytic function over repeated use, see figure 5. This finding suggests that the method used for immobilization on vermiculite provided sufficient enzyme-support stability to resist leaching or inactivation over time.
In comparison to Kim et al. (2012) study, where HRP was directly immobilized on different clay minerals such as, vermiculite, montmorillonite, and kaolinite, without silica encapsulation. Their result demonstrated up to 73% retained enzymatic activity over six cycles, followed by a rapid decline in later cycles. Although this present study only assessed 10 cycles, due to time limitation, the findings suggest that silica encapsulation in this experiment enhanced the reusability of the enzyme composites. Although, the different treatment of the clay minerals also affects the interaction with the enzymes, which may have influenced the results.
Findings from previous work supports this hypothesis, where Vardanyan et al. (2024;2025) demonstrated high retained catalytic activity for 20 cycles when HRP

and laccase were immobilized on silica encapsulated perlite- and leca material. While also showing enhanced resilience to both temperature and pH, compared to free enzymes. Similarly, high retained activity over 20 cycles was observed for HRP, lignin peroxidase, and urease immobilized on sol-gel covered magnetite- and iron oxide supports (Pylypchuk et al., 2020; Pogorilyi et al., 2017).
These results indicate the possibility to retain catalytic activity for more than 10 cycles, which further highlighting the potential of this method as a stable and reusable option. Moreover, both vermiculite and silica are considered as environmentally friendly materials in comparison to many synthetic alternatives (Ferronato et al., 2016; Maged et al., 2023).
However, it is important to acknowledge that the method used in this study is not completely accurate since slight variations in mixing, timing, and sample handling may limit precise quantification but the consistency across replicates highlights the robustness of the immobilized system. In addition, this experiment was not conducted with wastewater and the time for the project was limited.
Consequently, further studies under more representative conditions and additional cycles are required to get a more accurate evaluation of the practical reusability and long-term stability.

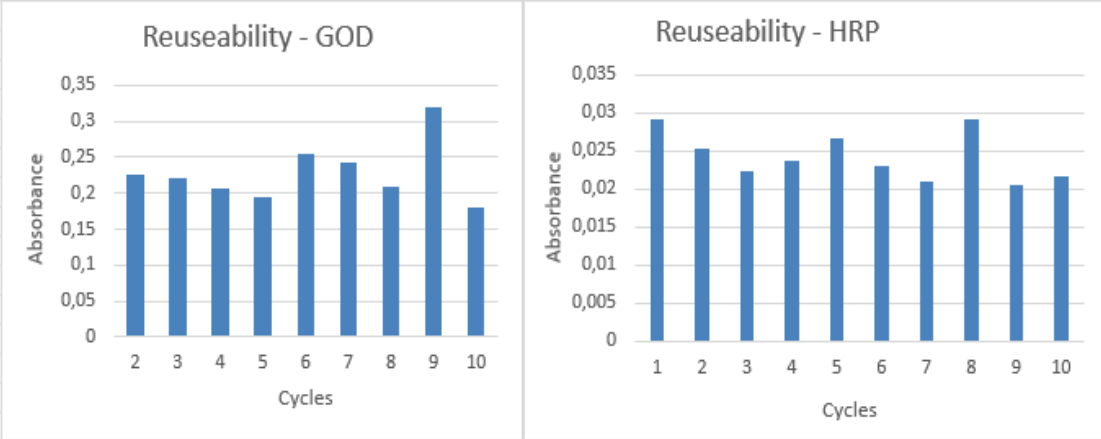

Figure 5: Enzyme activity over time of vermiculite composite, on the left is the measurament for GOD and the right graph represents the assays of HRP.

3.4 Column Treatment and Pharmaceutical Removal

3.4.1 Visual Comparison Pre- and Post-Treatment

The wastewater treatment was carried out using a column-based set up, containing two immobilized enzymes, at a constant flow rate at 3,5 mL/min, as outlined in the method section. A photographic comparison of water samples before and after enzymatic treatment clearly demonstrates a difference in colour intensity, the treated sample appears significantly less coloured and contains no visible particles, in contrast to the untreated sample, see figure 6. Even though visual comparison alone is not quantitative, it suggests cleaner water.

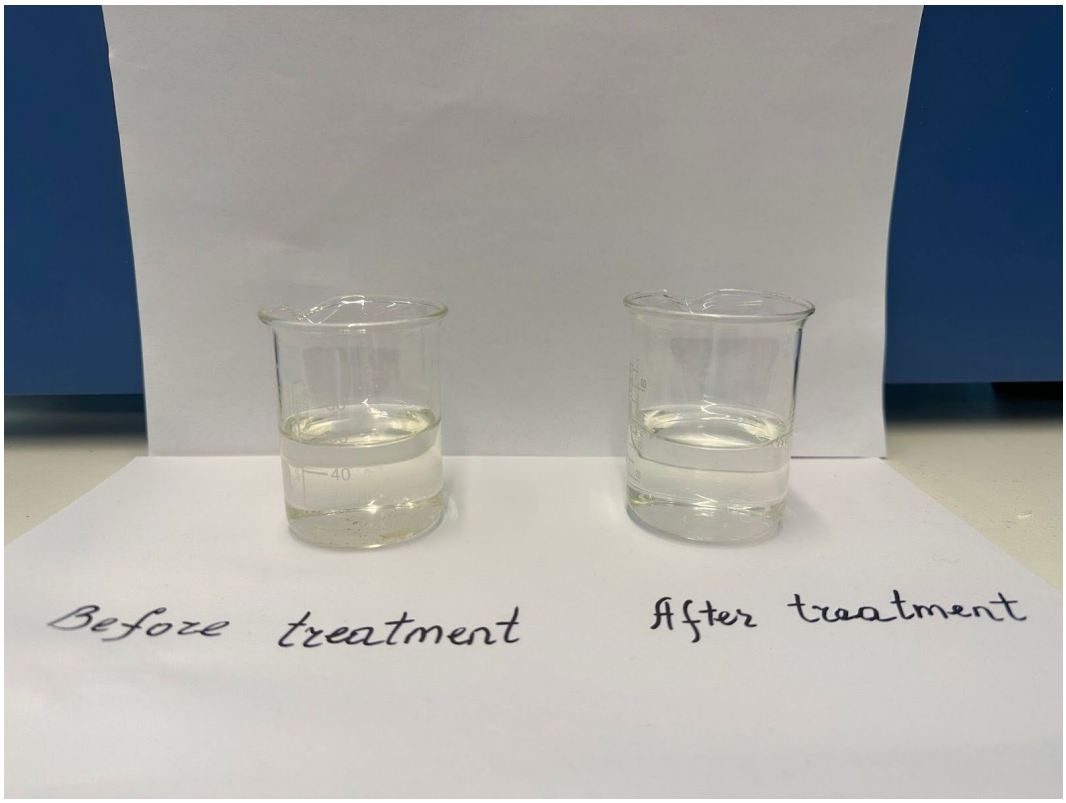

Figure 6: Visual comparison of the samples before (left) and after (right) enzymatic treatment, where the purified water shows reduced colour intensity.

3.4.2 HPLC

The enzymatic removal efficiency of 16 pharmaceutical compounds was evaluated across three consecutive sampling periods of three hours using an immobilized dual-enzyme column-system.

The system demonstrated very promising removal rates for nine compounds, showing near-complete degradation (>96% removal efficiency) throughout the whole treatment. While fexofenadine, oxazepam, and irbesartan showed high initial removal values that decreased in the following sample periods. In contrast, carbamazepine, lamotrigine, and HCTZ exhibited minimal removal throughout, as presented in table 3.

The initial concentrations of the pharmaceutical in the wastewater ranged from 18,9 ng/L to 35,9 ng/L. These findings align with concentrations that were reported at Kungängsverket from year 2021, by Swedish Geological Survey (2023), although the values slightly differ, they are within an expected range. This suggests that the occurrence and levels of pharmaceutical residues in the studied samples are representative of typical conditions at this WWTP.

Moreover, the HPLC method of use showed sufficient sensitivity, with lowest observed quantity (LOQ) values between 0,7-6,7 for most substances. The matrix effect (ME) of the compounds was generally close to 100 %, where most demonstrated ME values above 85%, suggesting limited signal suppression in the wastewater matrix.

Table 3: Enzymatic removal efficiency (%) for each sampling period (3 hours) of the experiment, concentration (ng/L) before enzymatic treatment, LOQ (ng/L), and ME (%), based on the results of the HPLC analysis. The cells marked in green indicate achieved required removal efficiency, yellow removal efficiencies close to the threshold, and red are far from passing the requirements of the EU directive.

Pharmaceutical	*Removal efficiency (%)*			C_{before} *(ng/L)*	*LOQ (ng/L)*	*ME (%)*
	0-3 h	*3-6 h*	*6-9 h*			
Trimethoprim	98.7	98.9	98.4	22.2	0.7	95.7
Metoprolol	99.9	99.8	99.6	34.0	3.3	90.4
Bisoprolol	99.6	99.6	99.5	24.3	6.7	95.4
Venlafaxine	99.8	99.7	99.7	31.4	0.7	85.9
Carbamazepine	0.0	0.0	0.0	28.3	0.7	89.8
Fexofenadine	96.8	69.8	42.6	35.3	6.7	65.1
Propranolol	99.9	99.4	99.4	22.4	6.7	97.2

Citalopram	99.5	99.7	99.7	26.8	3.3	93.1
Oxazepam	93.7	51.1	10.7	26.9	3.3	92.9
Irbesartan	84.7	73.0	45.6	23.5	3.3	95.7
Amitriptyline	97.4	98.5	98.5	19.9	0.7	98.2
Clarithromycin	99.5	99.7	99.8	23.8	0.7	96.8
Atenolol	-	99.7	99.6	27.9	3.3	91.0
HCTZ	27.9	4.1	5.8	34.1	6.7	83.2
Lamotrigine	7.3	0.0	0.0	35.9	3.3	46.2
Sulfamethoxazole	52.7	–288.8	–367.9	18.9	0.7	97.6

Compounds such as venlafaxine, metoprolol, clarithromycin, bisoprolol, and citalopram demonstrated removal efficiencies above 99%, consistently throughout the entire treatment. Similarly, propranolol, trimethoprim, and amitriptyline showed high and stable removal efficiencies, between 96–99% across all sampling periods. Atenolol lacked data from the first sample period; however, it demonstrated >99% efficiency during sampling period 2 and 3, as illustrated in figure 7.

Fexofenadine and irbesartan showed high initial removal rates (96.8% and 84.7%, respectively) but decreased in the following samples, decreased to 42.6% and 45.6% for the last 3 hours of the treatment. This may indicate enzyme inhibition or substrate competition in later sampling periods, but would need further investigation into the mechanism of action and potential enzyme saturation for confirmation.

Carbamazepine, lamotrigine, and HCTZ showed minimal to no removal values, with carbamazepine and lamotrigine demonstrated 0% throughout, while HCTZ only showed minor degradation (<28%) in the initial sampling period and declined thereafter.

Regarding sulfamethoxazole (SMX), the first three hours of treatment demonstrated a removal efficiency of 52.9%. But the following sampling periods showed a significant increase in concentrations, with values increasing by 286.8% and 363.9% compared to the untreated sample. These findings indicate that samples two and three contained higher levels of SMX than before the

purification process. Several factors may contribute to this, such as the chemical properties and the analytical methodology used.

SMX is primarily metabolized to N4-acetylsulfamethoxazole, a metabolite that closely resembles to the parent compound in both structure and polarity (Göbel et al., 2005; Joss et al., 2006). This similarity, which is clearer than for many of these pharmaceuticals, can pose challenges when using certain HPLC methods, as the two compounds often shows nearly identical retention times (Göbel et al., 2005; Gros et al., 2006). In this study, analysis was preformed using a UPLC system coupled with a triple quadruple mass spectrometer (UPLC-MS/MS), which is a highly sensitive and selective method that should be capable of distinguishing these compounds since it is based on specific mass-to-charge (m/z) transitions. To confirm these results, complementary methods such as, targeted LC-MS/MS (Triple Quad) and high-resolution mass spectrometry could be employed.

Another possible explanation is the adsorption of SMX, or its metabolites, onto the column material or surfaces and a delayed desorption in the following sample periods. Considering its polarity and amphipathic nature, SMX is prone to interact with certain materials, potentially resulting in carryover effects (Gros et al., 2006; Zhang et al., 2008). Moreover, potential errors during the SPE extraction and HPLC analysis could also affected the results, but further investigation is required to fully confirm the underlying cause. The potential adsorption of SMX could be further investigated by using FTIR or XPS to evaluate the surface of the material in the system. While extraction and analysis of equipment and adsorption media also could reveal any potential carryover effects. Furthermore, blank runs could be conducted after the collected sample to detect any potential desorption of SMX residues.

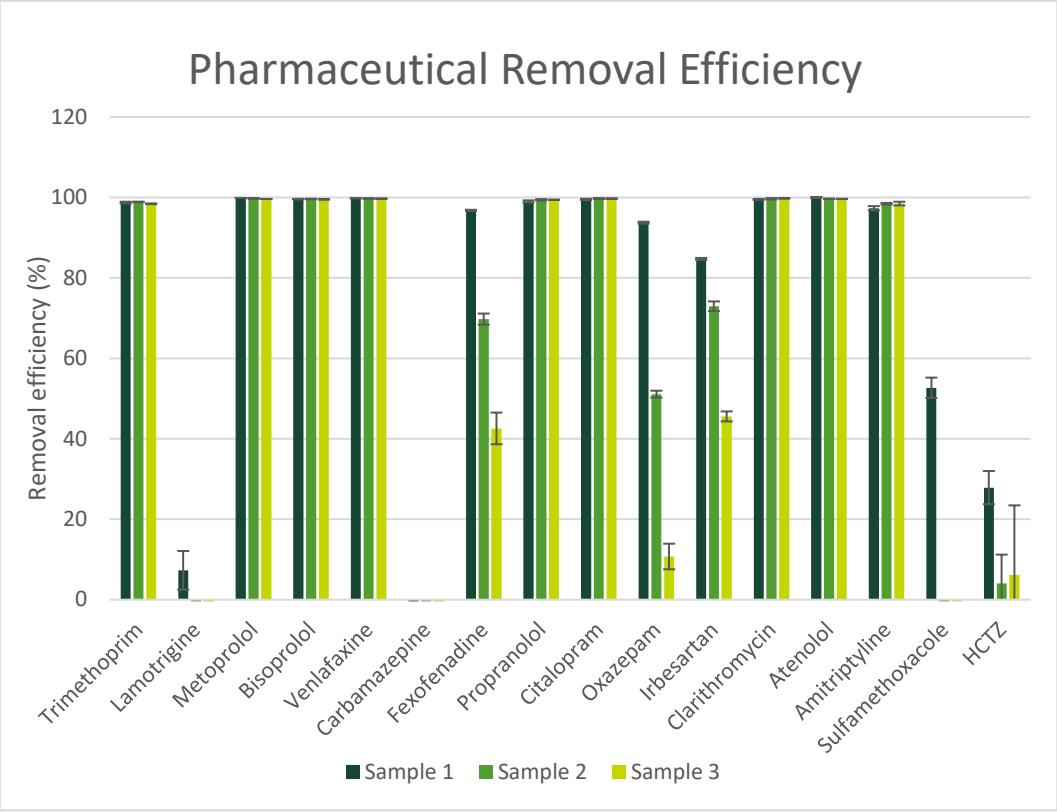

Figure 7: Enzymatic removal efficiency (%), from each treatment sample period with their respective standard deviation, based on the results from HPLC analysis.

3.4 NMR analysis

To confirm the removal of the pharmaceuticals and further investigate the wastewater samples, NMR analysis was employed. A multispectra were collected over a chemical shift range of 0–12 ppm to enable comparison between samples. Aromatic signals were determined within the 5.2–12 ppm range, while aliphatic signals were examined between 0–5.2 ppm (Claridge, 2016). In the presented spectra, the black curve represents the blank sample, green corresponds to the second sample (3-6 hours) of enzymatic treatment, red to the third sample (6-9 hours), and blue to the pre-treated sample.

The NMR spectra for aromatic signals (5.2–12 ppm) displayed clear differences between the treated and the untreated samples, as figure 8 illustrates. Notably, the

signals observed around 5.9–6.7 ppm which can be associated of electron-rich aromatic systems with extensive substitution or electron-donating groups, which aligns with the types of pharmaceutical residues that was identified by HPLC analysis (Claridge, 2016; Aranda et al., 2010).

The signals that were significantly reduced but still present in the last sample may be attributed to residues of compounds such as fexofenadine and irbesartan (Bilal et al., 2018), which showed high removal efficiencies during the initial sampling period (3 hours) that decreased in for the following samples.

Additionally, signals around 8.4 ppm were found for the treated samples exclusively and can be interpreted as products resulting from enzymatic degradation, for example, oxidation products of pharmaceutical residues (Aranda et al., 2010). These findings confirm the results from the HPLC analysis as well as the conclusion that the enzymatic purification was highly effective and that enzymatic transformation has occurred rather than any adsorption of the pharmaceutical residues, and that new, potentially, stable compounds have been formed.

Moreover, signals within the range of approximately 5-5.9 ppm were only observed in the treated samples. These signals are typical for D-glucose molecules, which was added before the treatment (Claridge, 2016). The presences of D-glucose in the treated samples suggests that GOD became saturated and was unable to convert all the added D-glucose. These findings are of importance since it may imply that excessive amounts of H_2O_2 were generated, which could have affected the activity of HRP. Since high concentrations of H_2O_2 have been shown to lead to irreversible inactivation of heme peroxidases, even with silica encapsulated supports, can it result in reduced removal efficiency of enzymatic treatment. This may have contributed to the decreased removal efficiency observed for pharmaceutical residues such as fexofenadine and irbesartan in treated samples two and three, as the HPLC analysis demonstrated.

To minimize these risks, future work should aim to optimize this step and adjust the amount of glucose added to match the enzyme's capacity, or alternatively, to use controlled dosing throughout the process to avoid saturation.

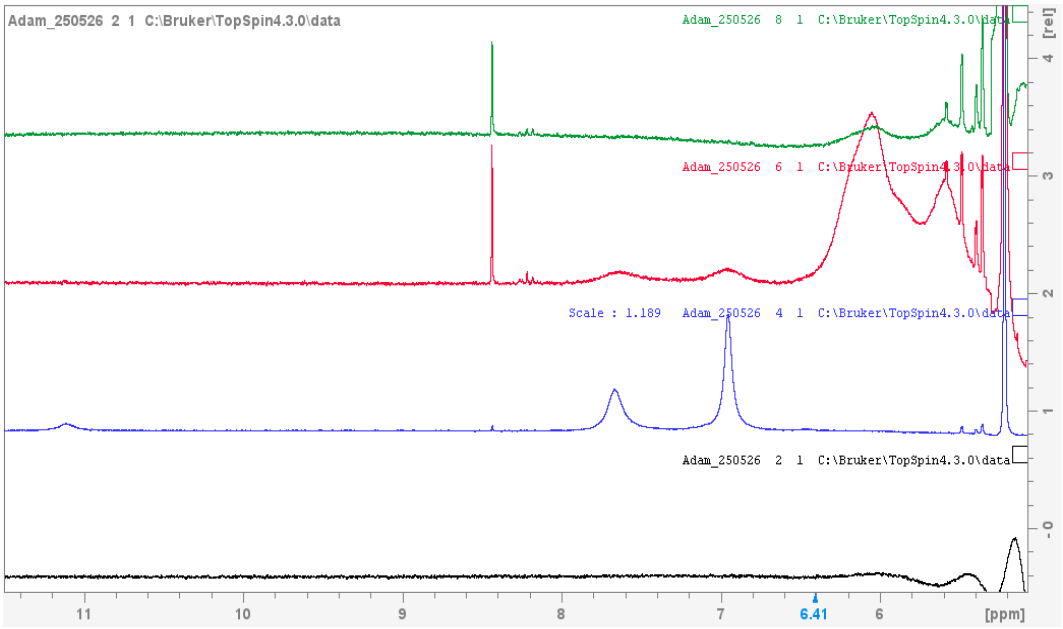

Figure 8: NMR multispectral showing the chemical shift range of 5,2- 11 ppm (aromatic region) for the before and after treatment samples. The blue curve represents the non-treated sample, green corresponds to the second cycle of enzymatic treatment, red to the third cycle, and black to the blank sample.

The Multispectra in the 3.2–5.2 ppm range, which was collected for the detection of aliphatic signals, also revealed distinct differences before and after treatment, see figure 9. First, small signals at ~4.02 and 4.12 ppm in non-treatment samples which likely originate from oxidized pharmaceutical residues containing ether or amine-linked methylene groups, such as atenolol, bisoprolol, and metoprolol (Claridge, 2016; Bahlmann et al., 2014). Despite the low intensity, this suggests some degradation products were already present before the treatment.

In addition, post-treatment samples showed increased intensity and new peaks between 3.8–4.3 ppm, indicating the formation of new degradation products from pharmaceutical residues or other organics pollutants (Bilal et al., 2018). These findings align with HPLC data showing high removal efficiency.

Signals just above 4.6 ppm was only observed in the post-treatment sample, which are consistent with the anomeric proton of β-D-glucopyranose (glucose in aqueous solution), which most likely is attributed to glucose residues of the added

D-glucose (Claridge, 2016). These results further support the conclusion that GOD was unable to convert all the added D-glucose, possibly due to saturation.

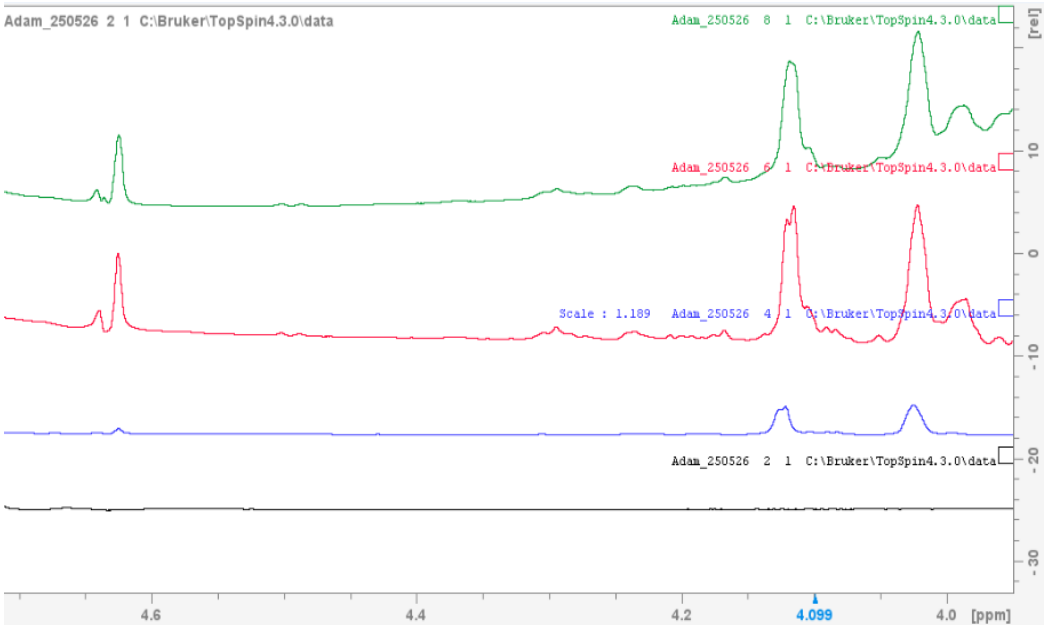

Figure 9: NMR multispectral displaying the chemical shift range of 3.5- 5 ppm (aliphatic region) for the before and after treatment samples. The blue curve represents the non-treated sample, green corresponds to the second cycle of enzymatic treatment, red to the third cycle, and black to the blank sample.

For the detection of aliphatic signals, a multispectral were collected from the chemical shift range of 0-3,2 ppm, see figure 10. A new signal at ~1.9 ppm likely represented aliphatic degradation products, possibly formed during enzymatic breakdown of the pharmaceutical residues. The signal at ~1.3 ppm, present only in the final treated sample, may be associated with small molecule such as late-stage breakdown products, other reagents used in the experiment, or contaminants.

Additionally, signals observed around 3,1 ppm, a region expected to find gluconic acid, the byproduct of D-glucose in this system, were exclusively detected in the post-treatment samples (Wilson & Turner, 2009). Which indicates that GOD was active throughout the whole experiment, supporting the hypothesis that it became saturated in the later stages due to excessive added amounts of D-glucose.

These investigations are not only of practical relevance but also important from an environmental perspective, since residual glucose and gluconic acid in wastewater can pose a concern. While glucose itself is not directly toxic, it serves as a nutrient source for microorganisms, which contribute to increased biological activity and potential eutrophication in receiving waters (Margot et al., 2013). However, an effective strategy would be to introduce a biofilter as a final step in the quaternary treatment process, where any remaining glucose and other organic compounds can be further degraded by microorganisms, ensuring that both glucose and other possible degradation products are addressed. This approach is supported by the fact that biofilters have been shown to effectively reduce soluble organic substances, including simple sugars, and can therefore provide a solution for maintaining acceptable levels of glucose (Margot et al., 2013).

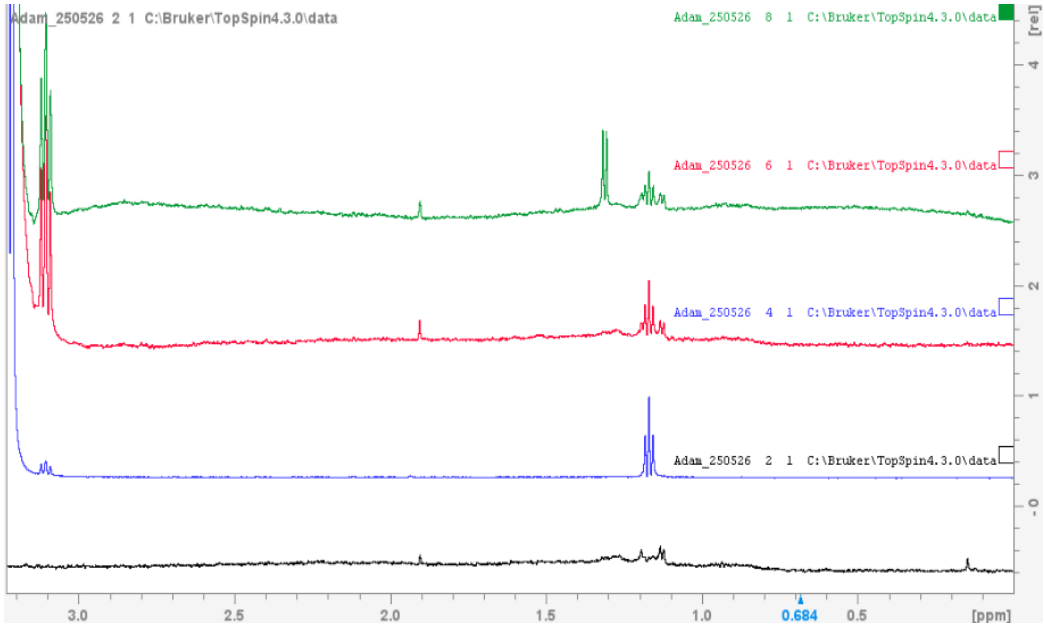

Figure 10: NMR multispectral for the chemical shift range of 0- 3,5 ppm (aliphatic region) for the before and after treatment samples. The blue curve represents the non-treated sample, green corresponds to the second cycle of enzymatic treatment, red to the third cycle, and black to the blank sample.

Overall, the NMR results together with HPLC results provide clear evidence of extensive pharmaceutical degradation and the formation of their metabolites, during enzymatic treatment, rather than any adsorption. While also highlighting

the presence of D-glucose and its metabolites in the later samples of the experiment, suggesting saturation of GOD, which may have affected the removal efficiencies due to high concentration of H_2O_2. Even though NMR provided valuable information on the overall changes in the composition changes, it is important to acknowledge that since this NMR method is less sensitive than HPLC, certain low-abundance compounds may remain undetected in the spectra, particularly at the trace concentrations typical for pharmaceutical residues in treated wastewater. For a more detailed NMR analysis, techniques such as correlation spectroscopy (COSY) or 13C-NMR could be employed. Which can provide complementary structural insight about the proton's positions and the carbon molecules (Claridge, 2016).

3.6 Persistent Pharmaceuticals and Optimization

The HPLC analysis revealed differences in the enzymatic removal efficiencies of the pharmaceuticals tested in this study. As the results indicate, 9 of the 16 compounds exhibited significant degradation, while carbamazepine, lamotrigine, and hydrochlorothiazide (HCTZ), showed little to no removal. These findings suggest that the enzyme used in this study either is unable to degrade these pharmaceuticals or possibly targets other, more reactive compounds present in the mixture instead. However, this can be attributed to several factors, but one likely cause may be linked to their chemical structures and the catalytic properties of the enzyme used. In this study, a heme-containing enzyme was employed, with H_2O_2 acting as the oxidant, which is known to efficiently oxidize electron-rich aromatic compounds in particularly those containing phenolic groups, due to the specific active site and its mechanisms. The general catalytic reaction for the enzymatic oxidation can be summarized as equation 1 shows.

$$2RH\ (\text{phenolic/aromatic}) + H_2O_2 \rightarrow 2R\cdot + 2H_2O \qquad (1)$$

Here, RH represents an electron-rich aromatic or phenolic substrate, which is oxidized to a radical species (R·) in the presence of H_2O_2. While this initial degradation produces radical intermediates, further oxidation will eventually result in complete mineralization of these organic compounds, which produces water and carbon dioxide.

A comparative analysis of the pharmaceutical structures reveals that compounds which are degraded typically possess electron-rich aromatic rings or functional groups, such as phenols, which enhances oxidation (Aranda et al., 2010; Margot et al., 2013). In contrast, carbamazepine and lamotrigine are relatively electron-poor molecules and lack such easily oxidizable groups (Bahlmann et al., 2014; Kovalova et al., 2012). Similarly, HCTZ is a polar molecule featuring sulphonamide and thiazide groups, which not only lack electron-rich character but may also hinder substrate binding or access to the enzyme's active site due to increased polarity (Kümmerer et al., 2010; Yang et al., 2017). This further reduces the likelihood of successful enzymatic oxidation due to the hem-group at the active site of the enzyme (Veitch, 2004). This aligns with previous studies which suggests that the limited enzymatic degradation values of these compounds are due to the absence of reactive sites compatible with the enzyme's catalytic mechanism (Aranda et al., 2010; Kovalova et al., 2012).

To address this, a suggested strategy for enhancing this method would be to effectively target these challenging pharmaceuticals. By focusing on expanding the range of enzymatic systems employed and use additional enzymes with complementary substrate specificities in the wastewater treatment. Instead of only using HRP as target enzyme for the pharmaceuticals, enzymes such as, laccases, tyrosinases, or other peroxidases could be utilized in the wastewater treatment as well. This could be achieved in the same way by simply using multiple enzyme types in tandem, either within the same column or in sequential columns.

In cases where multi-enzyme systems are insufficient to achieve the desired degradation, mediator-assisted enzymatic reactors could be a promising alternative for future work. This rapidly emerging field of research utilizes small, redox-active molecules known as mediators, which works as electron shuttles,

which enables enzymes to oxidize substrates that would otherwise be inaccessible (Asgher et al., 2014).

For instance, ABTS, a commonly used synthetic mediator, which has been proven to enhance the activity of oxidizing enzymes and even increase the spectrum of degradable compounds (Riva, 2006). However, ABTS can generate toxic byproducts such as sulfonated derivatives (Morozova et al., 2007). More sustainable options, such as syringaldehyde, offer a greener alternative (Peralta-Zamora et al., 2016). While syringaldehyde is less reactive than ABTS, it is also comparably less toxic and biofiltration can be employed to allow microorganisms degrade both mediators and other organic residues, while more persistent mediators have been proven to be purified by active carbon in case biofilters are insufficient (Peralta-Zamora et al., 2016).

In addition, previous studies have shown that laccase can degrade compounds such as carbamazepine, lamotrigine and sulfamethoxazole (Vardanyan et al. 2025; Pylypchuk et al. 2020). Which are compounds that demonstrated limited degradation in this experiment, using HRP as the target enzyme for the pharmaceutical residues.

To further investigate the potential of enzymatic degradation, a literature review was done focused on pharmaceuticals not included in this study. This was motivated by the limited number of analytes assessed in this study, and the fact that only one compound from category 2 of the EU Directive 2024/3019 was included. The findings from this review indicates that many pharmaceuticals have not yet been thoroughly studied in this context, and no literature were found regarding the category 2 substances not included in this study. For pharmaceuticals that have been investigated, the studies were conducted under different conditions in comparison to this present study. This includes conditions such as, contact time, matrix support, pH, and can thereby not be directly compared to the results of this study. Nonetheless, several studies showed promising removal efficiencies using laccases or HRP for a variety of pharmaceuticals that were not assessed in this study. A summary of relevant findings from previous literature, including what enzyme used, mediator, removal efficiencies is presented in table 4.

Moreover, tyrosinase, an enzyme that have been less studied in wastewater context, have shown promising results in multienzyme systems, for instance, together with laccases. Where the two enzymes operate synergistically, tyrosinase initiate the oxidation of a compound, such as diphenols or monophenols, and laccase further degrading the intermediate products (Durán et al., 2002).

Table 4: Summary of the results of previous studies on enzymatic degradation of pharmaceuticals which was not included in this study, presented together with the respective removal efficiency, reference, type of enzyme and mediator.

Pharmaceutical	**Enzymes**	**Mediator?**	**Removal efficiency**	**Reference**
Diclofenac	HRP, Laccase	No	>80%	Vardanyan et al. 2025; Pylypchuk et al., 2020
Ibuprofen	Laccase	Yes (HBT/ABTS)	50-90 %	Singh et al., 2024; Guardado et al., 2019
Paracetamol	HRP Laccase	No	>90%	Vardanyan et al. 2025; Pylypchuk et al., 2020
Naproxen	HRP, Laccase	Yes	70-80%	Singh et al., 2024;
Estradiol	HRP, Laccase	No	~100%	Singh et al., 2024; Chmelová et al., 2024
Ethinylestradiol	HRP, Laccase	Sometimes	80-90%	Singh et al., 2024; Chmelová et al., 2024
Sulfadiazine	HRP	No	60-90% depending on pH	Leng et al., 2016
Ketoprofen	Laccase	Yes	~70%	Singh et al., 2024
Chloramphenicol	HRP	Yes	70-100%	Dincă et al., 2022
Erythromycin	Laccase	Yes	~60%	Becker et al., 2016

Despite some promising results, these findings also implies that enzymatic purification of pharmaceuticals is highly dependent on the specific compound, enzyme system, and operating conditions. Where some pharmaceuticals require different enzymes and conditions. For instance, several of the studies was carried out using contact times of 24 hours, while the removal of sulfadiazine was depending on the pH value and required optimized condition to reach 90% removal efficiency. These settings are not feasible for this method and would require further tests under more realistic conditions for application as a quaternary treatment step. Besides this, the small number of existing studies and the limited evaluated analyte in this present work is further highlighting the need for continued research in this field.

To develop an optimal enzymatic treatment system, future efforts should focus on identifying which pharmaceuticals is degradable, which enzyme-mediator combinations are the most effective, and evaluate optimal operating conditions. This includes factors such as, contact time, pH, encapsulation and immobilisation method, and the effects of the wastewater matrix.

However, by integrating multi-enzyme systems with mediator reactors and sustainable management strategies, it might be possible to obtain a more versatile and sustainable approach for elimination of diverse types of pharmaceuticals in the wastewater. This study was carried out within the framework of an enzymatic project in a research group, which is developing and optimizing such approaches.

3.7 Requirements of EU Directive 2024/3019

According to EU Directive 2024/3019, 100 % of the large urban WWTPs must implement effective quaternary treatment technologies by 2045. The directive defines 12 indicator pharmaceuticals, that are divided into category 1 (easier to treat) and category 2 (difficult to treat). At least six of these substances must reach

≥80 % removal efficiency, while the number of pharmaceuticals from category 1 shall be twice of the number of pharmaceuticals from category 2.

To evaluate the extent to which the requirements of the directive were achieved, the removal efficiencies of the defined indicator substances were analysed. Of the 16 pharmaceutical residues assessed in this study were only seven included in the EU directive list of indicator substances, six were from category 1 and only one from category 2, see table 5. HPLC results showed high removal efficiencies across the whole experiment for four category 1 substances, including citalopram, clarithromycin, metoprolol, and venlafaxine. Irbesartan (category 2) exceeded 80 % removal during the initial 3 hours of the treatment but declined below the threshold following sampling periods. HCTZ and carbamazepine demonstrated limited removal efficiencies throughout, and indicator substances including diclofenac, candesartan, and benzotriazole, were not included in this study. Since only one category 2 substance (irbesartan) was assessed, is it not possible to determine whether the full requirements of the directive can be achieved.

Table 5: The defined indicator pharmaceuticals of EU Directive 2024/3019 with their respective category and the demonstrated removal efficiencies for each sampling period (3 hours) of the experiment. Removal values marked in green illustrates achieved requirements, yellow corresponds to removal efficiency close to the threshold, and red to values that is far from the EU directive requirements.

Pharmaceutical	*EU Cat*	*Removal Efficiency (%)*		
		0-3 h	*3-6 h*	*6-9 h*
Amisulpride	1	–	–	–
Carbamazepine	1	0	0	0
Citalopram	1	99.5	99.7	99.7
Clarithromycin	1	99.5	99.7	99.8
Diclofenac	1	–	–	–
Hydrochlorothiazide	1	27.9	4.1	5.8
Metoprolol	1	99.9	99.8	99.6
Venlafaxine	1	99.8	99.7	99.7
Benzotriazole	2	–	–	–
Candesartan	2	–	–	–
Irbesartan	2	84.7	73	45.6
4-/5-Methylbenzotriazole	2	–	–	–

3.7.1 Irbesartan

Irbesartan, the only substance from category 2 assessed in this study, demonstrated removal efficiencies exceeding the EU requirements during the initial sampling period (3 hours). For the next three hours, a slight decline was observed with removal efficiencies dropping just below the required values, which continued to decrease for the last three hours of the treatment. As previously mentioned, these findings may be attributed to the excessive amount of D-glucose added to the wastewater, which likely led to the generation of too high concentrations of H2O2 and potentially inactivating HRP. By optimizing the concentration of added D-glucose in combination with implementation of multienzyme systems with redox mediators, can this method be further enhanced and potentially maintain removal efficiency for irbesartan above the EU requirements during the whole treatment.

3.7.2 HCTZ

Regarding HCTZ, the results from the HPLC analysis showed a removal efficiency of 27.9 % for the initial 3 hours of the treatment which, similarly to irbesartan, declined for the following sampling periods of the experiment. This decline may also be attributed to the potential inactivation of HRP from the production of excessive amounts of H_2O_2 by GOD. HCTZ is a diuretic medication used for treatment such as hypertension, swelling due to fluid build-up, and diabetes insipidus. Chemically it is classified as a thiazide and is known to be a persistent compound, degrading at relatively slow rates in the environment (ChemSpider, 2025; Fernández-Perales et al., 2020; Zou et al., 2015). Even though the observed removal efficiencies were below the required values of the EU directive, the results demonstrate that HCTZ, to some extent, is degradable by HRP under the tested conditions. Which suggests that significantly higher removal efficiencies are potentially achievable with the presence of mediator reactors as well as optimized concentrations of D-glucose added.

3.7.3 Carbamazepine

The results demonstrated 0% enzymatic removal of carbamazepine throughout the whole treatment, indicating that HRP is not susceptible to these types of compounds, or that the catalytic reaction requires optimized conditions such as contact time, pH or redox mediators. However, previous literature demonstrated effective degradation of carbamazepine by laccase, without reactive mediator, but the experiment was conducted with a contact time of 24 hours (Pylypchuck et al., 2020). These findings highlight the need for further investigation under feasible conditions to evaluate the potential to reach 80 % removal even for this substance.

3.7.4 Amisulpride

Amisulpride is a drug used in the treatment of schizophrenia, acute psychotic episodes, depression, and is usually classed with atypical antipsychotics. Chemically it is a benzamide and contains structural features such as a methoxy-substituted aromatic ring, a sulphonamide group, and an aromatic amine (see figure 11), which are properties known to be susceptible to enzymatic oxidation under the right conditions. However, amisulpride have a relatively electron poor aromatic system with a high redox potential that may make the reaction very slow without the presence of any mediator reactors (ChemSpider 2025; Singh et al.,2024). Although specific literature on enzymatic degradation of amisulpride degradation is limited, the structure of amisulpride is similar to compounds like sulphonamides and aromatic amines, both of which have been effectively degraded using laccase or HRP in the presence of mediators. Importantly, these reactions required longer contact times, often exceeding 6-24 hours to reach significant degradation (Chmelová et al 2024.; Singh et al.,2024). Given the relatively short contact time used in this study, significant degradation of amisulpride in unlikely under the tested conditions. However, the lack of experimental evidence highlights the need for further investigation to determine under which conditions this purification method is effective and what removal efficiencies that can be achieved for amisulpride.

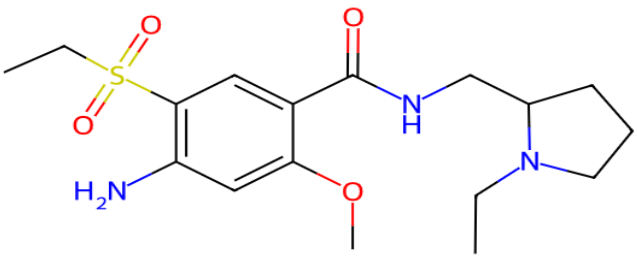

Figure 11: The molecular structure of amisulpride (ChemSpider, 2025).

3.7.5 Candersatan

Candesartan belongs to category 2 and, like irbesartan, is an angiotensin II receptor blocker used to treat high blood pressure (Koh et al., 2004; Abraham et al., 2015). Although these two substances share key features, such as biphenyl-tetrazole and a benzimidazole ring system can their compatibility with the active site of HRP differ significantly even due to small structural or chemical properties, see figure 12. Irbesartan contains a relatively accessible aromatic system, meanwhile candesartan possesses a carboxylic acid group and are often bulkier, which can increase its redox potential and may limit the access to potential oxidation sites for HRP (ChemSpider, 2025). These features, as previously noted, reduce the susceptibility for direct oxidation and suggest that candesartan is a less favourable substrate for HRP compared with irbesartan, even though further investigation is needed to determine. Again, the addition of redox mediators may bridge this potential gap and allow oxidation of regions such as biphenyl ring or the benzimidazole and potentially reach removal values observed with irbesartan. This would be an interesting work for future research, since if both candesartan and irbesartan can reach removal efficiencies of 80 % this method could potentially meet the EU requirements, at least under the tested conditions in this study. Even if the requirements would be achieved, further testing is needed under more realistic conditions, such as operational scale setups, and additional treatment cycles to fully evaluate this methods potential for application at Kungsängsverket.

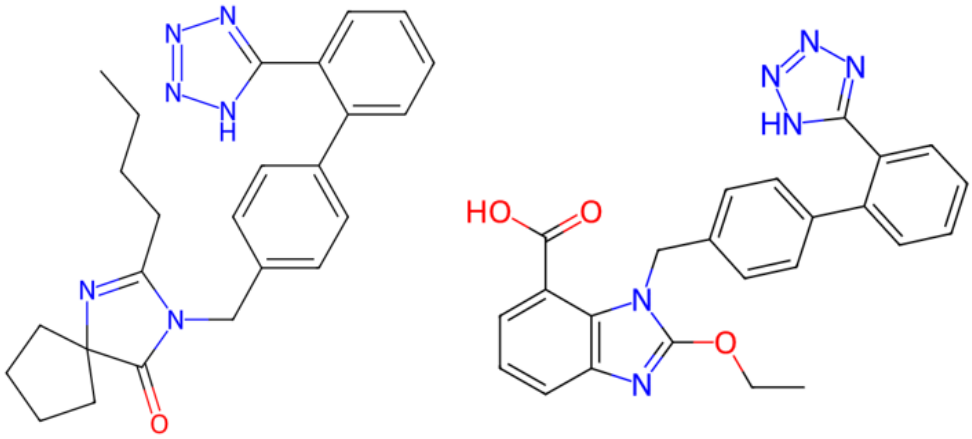

Figure 12: Illustration of the molecular structure of irbesartan (left) and candesartan (right) from ChemSpider, (2025).

3.7.6 Diclofenac

While diclofenac was not included in this study, previous studies demonstrated to effectively degrade diclofenac by HRP. Notably, these experiments were conducted with a contact time of 24 hours without any redox mediators, highlighting the need for further investigation under feasible contact times and possibly with the addition of mediator reactors (Pylypchuk et al., 2020).

3.8.7 Benzotriazole and 4-/5-Methylbenzotriazole

Benzotriazole (BTA) is a heterocyclic compound and can be viewed as a fusion of benzene and triazole rings. The molecule is aromatic and stable with three nitrogen atoms in the triazole ring (ChemSpider, 2025). Although BTA itself is not used as an approved drug, it plays an important role in pharmaceutical chemistry, for instance in the development of anticancer or antiviral compounds, as a protease and kinase inhibitor, and for metal ion chelation (Ifran et al., 2019). The structure of 4- and 5-methylbenzotriazole are identical to BTA, except with the addition of one methyl group on the carbon atom in position 4 or 5 in the benzene ring (ChemSpider, 2025), as illustrated in figure 13. Similarly to BTA, these compounds have other applications than pharmaceutical chemistry and is

often used in cooling systems, aircraft fluids, and industrial processes (Shafi'I et al., 2024).

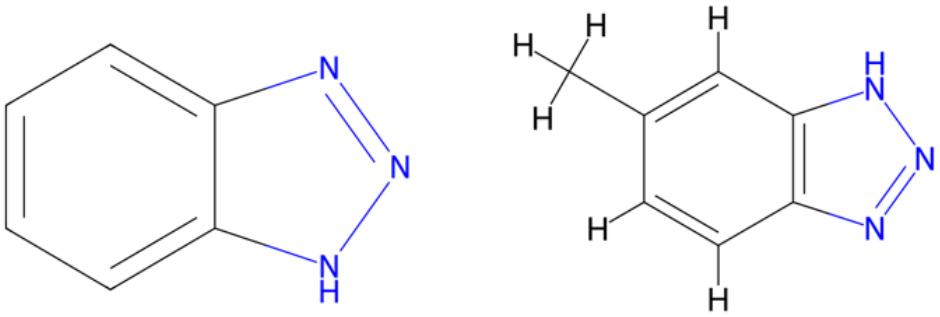

Figure 13: The structure of Benzotriazole and 5-Methylbenzotriazole (Chemspider, 2025).

These two substances are very stable due to their fully aromatic structure which provides high thermodynamic stability while also lacking oxidizable functional groups such as phenols or amines. Instead, they contain nitrogen-rich triazole rings that are chemically inert, resistant, and have a high redox potential, features which are considered unfavourable for enzymatic oxidizing by HRP (Ifran et al., 2019).

Furthermore, no studies have so far reported successful degradation of benzotriazole using HRP or laccase, supporting the idea that this compound is a difficult substrate for enzymatic oxidation due to structural features mentioned above. Research on compounds with similar structures, such as triazoles and aromatic systems like atrazine, has also shown limited degradation and even with redox mediators present. In these cases where degradation were often slow, partial or required optimal conditions (Stadlmair et al., 2018). Which suggesting challenges in using these enzymes for removal of this type of substances.

If BTA still proves to be difficult to degrade by enzymes, a potential solution is to use this method in combination with other water purification technologies. For instance, enzymes could be used as an initial or complementary treatment step, where the selective degradation can increase the availability of persistent compounds for following treatment steps. This may improve the effectiveness of

the follow-up processes such as, ozonation, GAC, AIX, or biological treatment. By increasing the selectivity and specificity, enzymatic treatment could also help minimizing the formation of toxic byproducts from technologies such as ozonation (Ullberg et al., 2021; Varga et al., 2019).

Finally, enzymatic purification is not only relevant for municipal wastewater treatment but also shows promising potential for targeted application. This includes removal of specific pharmaceutical residues in hospital and veterinary wastewater, or from in the effluents from the manufacturing of these substances. In such point sources could this method provide an effective, selective, biodegradable approach for substances known to be susceptible for enzymatic degradation.

3.8 Comparison with IVL Pilot Study Results

Both this lab-scale enzymatic treatment and IVLs pilot-scale advanced treatment trials at Kungsängsverket were designed to address the persistent issue of pharmaceutical residues in municipal wastewater. However, the two approaches differ fundamentally in technology and operational scale, and their results shows both promise and challenges for as a quaternary treatment step.

In this study, the system was tested in a 210 mL column with a contact time of 60 min over three continuous sampling periods of three hours. This type of method may supplement the current pilot study at Kungsängsverket, which evaluated a full-scale-relevant setup using granular activated carbon (GAC) filters, alone and in combination with ion exchange (AIX) filters. The pilot system was also operated with real wastewater, for 21 pharmaceuticals and 32 PFAS and other micropollutants, with particular focus on substances of local concern such as citalopram, diclofenac, oxazepam, metoprolol, and PFOS. Both approaches demonstrated high initial removal efficiencies for several pharmaceuticals, but

their long-term performance and robustness differed. The pilot GAC system was effective in the initial cycles but showed reduced removal rates for some pharmaceuticals due to filter saturation, especially oxazepam and PFOS. However, filter replacement and the use of ion exchange could restore or maintain high removal for certain compounds.

While the enzymatic treatment offers a potentially more environmentally friendly and operationally simpler alternative compared to GAC/AIX, it is still at a development stage, and several practical challenges remain before it can be ready for full-scale implementation. For instance, the contact time used in this experiment (60 minutes) was the maximum flow rate with the available peristaltic pump and may not be feasible at larger scales, which underscores the need for further investigations with shorter contact times. Another challenge, as previously highlighted, is the persistence of certain pharmaceuticals and the substrate specificity of the enzymes. Although many of the assessed compounds were effectively removed, substances such as carbamazepine, lamotrigine, and hydrochlorothiazide showed limited degradation. These finding indicate that HRP or lacasses are limited to structure and chemical properties of the compound and cannot degrade the whole spectrum of pharmaceutical residues, at least not under the tested conditions.

Moreover, the NMR results indicate that the system is sensitive to high concentrations of H_2O_2 which can cause irreversible inactivation of HRP and reduce the removal efficiencies. Future work should focus on optimization of the added D-glucose concentration minimize these risks. From a cost perspective, enzymatic treatment may sometimes be considered as a relatively expensive alternative. Since both HRP and GOD are still often associated with high costs, especially at the purity levels required for these applications. In addition, the continuous supply of D-glucose, use of TEOS for silica encapsulation, and potential need for frequent replacement of enzyme composite contributes to the overall cost of this method. This underscores the need to further studies on long-term reusability and contact time under realistic conditions to allow a more accurate cost estimation for implementation at Kungsängsverket.

Multi-enzyme systems with redox mediators in sequentially columns, potentially with adjusted pH environments, shows promising potential to broaden the range of degradable substrates. An additional option is to integrate this method with the GAC/AIX system, where the enzymatic treatment can be applied as a pre-treatment that breaks down the pharmaceutical residues in the incoming wastewater. The GAX step would then adsorb intermediate degradation products and remaining organic substances. After that, AIX can capture more polar compounds or transformation products. Studies have shown that such combination of methods can help to overcome the individual limitations of these technologies. First, enzymes degrade the molecules, rather than simply adsorbing them, reducing the load on GAC and AIX steps and thereby extending the lifespan of their media. In addition to improving the overall removal efficiency, this approach also lowers the risk of re-release of pharmaceutical residues to the environment during regeneration or disposal (Svenskt Vatten, 2017; Ullberg et al., 2021). This strategy would be an interesting work for future initiatives at Kungsängsverket.

One factor that may influence results and the comparison is the storage condition of the wastewater prior to treatment and analysis. In this study, the treated water was first stored at 4°C for approximately three weeks before being frozen at −20°C. Recent research shows that storage temperature and duration significantly affect how pharmaceuticals are preserved in wastewater. Storages at 4 °C were proven to still allow degradation of certain compounds under long-term storage, whereas −20°C provided the best stability for most of the analytes (Mijangos et al., 2019; Fedorova et al., 2014). Future experiments should aim to standardize the sample storage and shorten the time before freezing to preserve the integrity of the analytes.

4. Conclusion

This study demonstrates promising results of the performance and feasibility of using silica-encapsulated vermiculite as a support matrix for GOD and HRP as a quaternary treatment step for wastewater contaminated with pharmaceutical residues. Enzyme activity assays confirmed successful immobilization and retained over multiple cycles, highlighting the robustness and reusability of this enzyme system. The column treatment experiment demonstrated high removal efficiencies (>96%) for most pharmaceuticals tested, but some substances such as carbamazepine, lamotrigine, and hydrochlorothiazide, exhibited limited enzymatic degradation.

While these results highlight the potential of this method as a sustainable, and scalable solution with potential to meet future legal requirements for micropollutants in urban wastewater. Challenges remain, including the need of reduced contact time, assess a broader range of pharmaceuticals under realistic conditions, and determining optimal condition such as type of redox mediator, D-glucose concentration, and contact time.

Overall, this approach contributes valuable insights toward environmentally friendly technologies for improving urban water quality and protecting aquatic ecosystems and human health.

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Appendix 1. Calculations

1.1 Retained Enzyme Activity

The enzyme activity after the immobilization on the vermiculite matrix was determined by analysing the rate of change in absorbance over the initial linear portion of the absorbance-time curve, measured at 420 nm. According to the Beer–Lambert law (Equation 1), the absorbance is proportional to the concentration of the oxidized chromogenic substrate, allowing the reaction rate to be used as a measure of enzymatic activity:

$$A = \varepsilon l c \qquad (1)$$

To compare the activities of immobilized and free enzymes, the concentration was calculated as equation 2 shows, where $\Delta A/\Delta t_{free}$ is the rate of absorbance change for the immobilized enzyme, $\Delta A/\Delta t_{immobilized}$ is the rate of the free enzyme and $C_{initial}$ is initial concentration of the enzyme solution before immobilization (U/mL).

$$C_{rel} = \frac{\frac{\Delta A}{\Delta t}_{free} \times C_{initial}}{\frac{\Delta A}{\Delta t}_{immobilized}} \qquad (2)$$

To calculate the retained activity per gram vermiculite (U/g) the equation 3 was applied, where $C_{initial}$ is initial concentration of the enzyme solution before immobilization, V_{total} is the total volume of the enzyme solution, $m_{support}$ is the total mass of the vermiculite that was used for immobilization.

$$\frac{U}{g} = \frac{(C_{initial} - C_{rel}) \times V_{total}}{m_{support}} \qquad (3)$$

All calculations were based on duplicate measurements, and average values were used for determining final activities.

1.2 Calculation of RSD and LOQ

To calculate the concentration of the identified pharmaceuticals, calibration curves were constructed plotting the peak area to its corresponding IS, which represents the intensity of the detected signal and directly correlates with the compound concentration. The curves were constructed by using native standards with a concentration range of 0,1-1000 ng/mL.

Trendlines were generated from these calibration data, and the corresponding equations were used to solve for the concentration (x), where the y-value represents the mean peak area of the samples prior to treatment. These equations were used to calculate the concentration of each substance at the different concentration levels of the calibration curve. After that, RF values were calculated by applying equation 4, where $Area_A$ is the peak area from each analyte, $Area_{IS}$ is the area of the IS, RF is the response factor, while C_A and C_{IS} correspond to the concentration of the analyte and IS.

$$\frac{Area_A}{Area_{IS}} \times RF = \frac{C_A}{C_{IS}} \quad (4)$$

To assess the repeatability, the relative standard derivation (RSD) of the RF values across the replicates were calculated. Substances with an RSD higher than 3% were excluded, as this indicates insufficient consistency. The lowest observed quantification (LOQ) was determined as the lowest concentration level where RSD was below 3%. After that, new calibration curves were constructed based on the corrected dataset and a new equation generated which were used for the final calculations.

1.3 Removal Efficiency

For the calculation of enzymatic removal efficiency (%), the mean peak areas before and after treatment were used according to equation 5, where A_{before} represents the mean area of the signal from the pre-treatment sample, and A_{after} is mean area from the post-treatment one:

$$Removal\ efficiency\ (\%) = \frac{(A_{before} - A_{after})}{A_{before}} \times 100 \qquad (5)$$

1.4 Conversion and Initial Concentration

This value was then converted to ng/L according to equation 6, where $C_{extract}$ represent the concentration of the pharmaceutical, $V_{extract}$, the final volume used in the HPLC analysis and V_{water} is the total volume of the filtered wastewater sample of which the extract originated from.

$$\frac{ng}{L} = \frac{C_{extract} \times V_{extract}}{V_{water} \times 1000} \qquad (6)$$

1.5 Matrix Effect

Matrix effects were calculated to evulate any potential suppression or enhancement caused by the components of the wastewater matrix for which equation 7 was used.

$$1 - \frac{(A_m - A_w)}{(A_{cal} - A_{blanc})} \times 100 \qquad (7)$$

Where:

A_m = peak area from a spiked matrix sample

A_w= area from the untreated wastewater sample, only spiked with IS

A_{cal} = area calculated from the calibration curve equation at the same concentration as A_m

A_{blanc} = area from of the analyte in the blank samples (MQ- and Tap water)

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