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Treball Final de Grau

Degradation of diclofenac using Advanced Oxidation Processes and identification of its degradation products by LC-HRMS.

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En primer lloc, agrair al Dr. Jaume Giménez per la seva constant supervisió i recolzament que han sigut bàsics per tirar endavant aquest treball.

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Aquest treball ha estat realitzat conjuntament en al departament d'Enginyeria Química de la Universitat de Barcelona i en el laboratori d'Espectrometria de Masses i Contaminants Orgànics de l'Institut de Diagnosi Ambiental i Estudis de l'Aigua (IDAEA) del CSIC.

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SUMMARY

The anthropogenic pollution of water is becoming a severe problem because of the increasing wastewaters generated by the industry and the population day by day. In this context, pharmaceuticals are products of special concern. Indeed, pharmaceuticals are used to improve the quality of life of humans and animals, but the effect of their accumulation in the aquatic system can be dangerous. This study is focused on diclofenac, which is a non-steroidal anti-inflammatory drug (NSAID) and is one of the priority substances that, according to the European Union, must be monitored in order to determine the risk for the environment. The main concern of these pollutants is the persistence in the environment as they are not biodegradable and, therefore, they are not completely eliminated by classical wastewater treatments.

Advanced Oxidation Processes (AOPs) are a useful alternative to traditional water treatments. To perform the degradation of diclofenac, the most promising AOPs are direct photolysis with UV radiation and the combination with hydrogen peroxide. For this reason, in this work, the diclofenac was treated with UV/H_2O_2 and UV photolysis, achieving removals higher than 95% with UV/H_2O_2 and higher than 90% for UV. Experimental data fit well pseudo-first-order kinetics. Experiments were also done with only H_2O_2 and with solar light but the percentage of diclofenac removal in both cases was very low.

Another related problem is the low concentration of these compounds in the superficial waters, and for this reason are called micropollutants. Thus, analytical technologies with high sensitivity are needed to detect these compounds in the environment and to allow the identification of their degradation products. Therefore, liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) has been used, in this work, to identify the degradation products. Fourteen intermediates have been detected and degradation pathways have been tentatively proposed.

Finally, an estimation of the toxicity of the final products of the experiment with the computerized predictive system ECOSAR was carried out, because the transformation products can be more detrimental than the main product.

Keywords: Diclofenac, degradation products, Advanced Oxidation Processes, liquid chromatography coupled to high-resolution mass spectrometry, toxicity.

RESUM

La contaminació antropogènica de l'aigua esdevé cada cop un problema més greu degut a l'augment de les aigües residuals generades per la indústria i la població. En aquest context, els productes farmacèutics són compostos especialment preocupants. De fet, els fàrmacs s'utilitzen per millorar la qualitat de vida dels éssers humans i dels animals, però l'efecte en el sistema aquàtic degut a la seva acumulació, pot ser perjudicial. Aquest estudi es centra en el diclofenac, que és un fàrmac antiinflamatori no esteroïdal i és una de les substàncies prioritàries que, segons la Unió Europea, ha de ser monitoritzada per determinar-ne el risc per al medi ambient. La principal preocupació d'aquests contaminants és la persistència en el medi, ja que no són biodegradables i, per tant, no s'eliminen completament després dels tractaments clàssics de les aigües residuals.

Els processos d'oxidació avançada (POAs) són una bona alternativa als tractaments d'aigua tradicionals. Per dur a terme la degradació del diclofenac, els POAs més prometedors són la fotòlisi directa amb radiació UV i la combinació amb peròxid d'hidrogen. Per aquest motiu, en aquest treball s'ha tractat el diclofenac amb UV/H_2O_2 i amb fotòlisi UV, aconseguint una degradació superior al 95% amb UV/H_2O_2 i superior al 90% amb només UV. Les dades experimentals s'ajusten bé a la cinètica de pseudo-primer ordre. També s'han realitzat experiments amb només H_2O_2 i amb radiació solar, però en aquests dos casos pràcticament no hi ha hagut degradació.

Un altre problema relacionat és la baixa concentració d'aquests compostos a les aigües superficials, per la qual s'anomenen microcontaminants. Per tant, per detectar aquests compostos al medi ambient i permetre la identificació dels seus productes de degradació, es necessiten mètodes analítics d'alta sensibilitat. Per això, en aquest treball s'ha utilitzat la cromatografia líquida acoblada a l'espectrometria de masses d'alta resolució per identificar els productes de degradació. S'han detectat catorze intermedis i s'han proposat possibles vies de degradació.

Finalment, s'ha fet una estimació de la toxicitat dels productes finals de l'experiment amb el sistema predictiu computeritzat ECOSAR, ja que els productes de degradació poden ser més perjudicials que el producte principal.

Paraules clau: Diclofenac, productes de degradació, Processos d'Oxidació Avançada, cromatografia líquida acoblada a l'espectrometria de masses d'alta resolució, toxicitat.

1. INTRODUCTION

Water is essential for life and environment and plays an important role in the economy of the planet. Also, it is used in our daily life for alimentation, personal care, and cleaning tasks. Water is more than a resource and it must be preserved. The constant growth of the world population causes an increase in the demand for potable water while its anthropogenic contamination rises. To control this contamination, the European Parliament have been stablished the Water Framework Directive (WFD) 2000/60/EC.

There is a rising concern about the occurrence and persistence of emergent contaminants that come mostly from industrial activities and pharmaceutical and personal care products. Although these contaminants are often named ‘‘emerging’’, most of them have been present in the environment for many years but were not detected before due to the lack of proper and highly sensitive instruments. The development of the analytical methods such as electrospray ionisation mass spectrometry (ESI-MS) in the 1980s and the improvement in sensitivity of mass spectrometers are what have allowed to identify and measure these contaminants [1].

Pharmaceuticals are greatly used in modern life and their benefits for human and animal health, are widely known, but the adverse side effects that they can produce in the natural environment have not been enough studied. Although these contaminants are found in low concentrations, they can be damaging for aquatic organisms and for human health if they accumulate in the environment, the food chain or get into drinking water. For example, diclofenac is one of the pharmaceuticals used every day for millions of people in Europe.

Moreover, many of these contaminants can suffer a transformation in our organism by metabolization, and in the environment due to processes as microbial degradation, photolysis, and hydrolysis, and they can also react with disinfectants in drinking water or wastewaters to form disinfection subproducts [1]. Often, these transformation products are quite stable in the environment, but sometimes can be more toxic than the parent compounds.

The issues with these emerging contaminants are basically due to their bioaccumulation, persistence, and toxicity. They are not biodegradable and therefore the traditional water

treatment plants are not able to achieve the complete removal of them. However, Advanced Oxidation Processes (AOPs) are a useful alternative to these treatments.

1.1. **DICLOFENAC**

Is a nonsteroidal anti-inflammatory drug (NSAID) used to treat mild to moderate pain and inflammatory diseases such as gout, osteoarthritis or rheumatoid arthritis. It reduces swelling and stiffness in the joints, muscles and bones. Diclofenac comes as tablets, capsules and suppositories.

Like all medicines, diclofenac can cause side effects. The most common are abdominal pain, gastrointestinal bleeding, dizziness, nausea, swelling and headache.

Diclofenac was patented in 1965 and was started to use as a medicine in the United States in 1988. [2]

The presence of diclofenac in the aquatic environment has been described in different studies. For example, concentrations up to the μg/L have been found in surface water samples collected from canals and rivers in Berlin [3]. In the Baltic Sea region 54 ng/L of diclofenac have been measured as a maximum concentration in water samples [4]. Also, in the Llobregat River this compound exceeded 10 μg/L [5].

1.2. **LEGAL FRAMEWORK**

The Water Framework Directive 2000/60/EC recognize “water not as a commercial product like any other but, rather, a heritage which must be protected, defended and treated as such” [6]. The purpose of the directive is to achieve a good ecological and chemical quality of ground and surface waters. For that, it is important to prevent pollution, promote sustainable use, protect its environment, improve the state of ecosystems and relieve the effects of floods and drought episodes.

A surface water Watch List (WL) mechanism of the Water Framework Directive (WFD) was stablished in 2008 under the Priority Substances Directive 2008/105/EC (as amended by directive 2013/39/EU). The aim of this mechanism is to collect high-quality monitoring data to determine if the chemicals selected represent a significant risk to the river basins of the European Union [7].

This Watch List is thought to be regularly updated in order to better inform the determination of adequate measures to minimize the risk.

Diclofenac appears on the Watch List of pharmaceuticals described on the directive 2013/39/EU as a priority substance, and an annual average environmental quality standard (EQS) of 10 ng/L has been proposed for it [4]. The EQS is a maximum acceptable concentration limit of a substance in a body of water, necessary to maintain the quality of the environment.

1.3. ADVANCED OXIDATION PROCESSES

The anthropogenic pollutants are hardly eliminated by the microorganisms in biological treatment of wastewater produced in the industry, therefore there is an important necessity to study new technologies such as advanced oxidation processes. These treatments are used to complement the conventional physicochemical and biological treatments and accomplish the day-to-day more exigent limits fixed by environmental regulations [8].

AOPs use the powerful hydroxyl (OH·) radical as a major oxidizing agent. The hydroxyl radical has a high oxidation potential (E^o=2.80V). Its non-selective behaviour allows to attack almost any organic compound and is useful to degrade contaminants at very low concentration.

The generation of these radicals can be achieved from different chemical reactions. Depending on whether there is radiation or not, AOPs can be classified as photochemical processes or non-photochemical processes.

Table 1. AOPs classification. (Litter & Quici, 2010) [9]

Photochemical	Non-photochemical
UV/O_3	
UV/H_2O_2	Ozonation at alkaline pH (>8.5)
$O_3/UV/H_2O_2$	Ozone +catalyst
Photo-Fenton	Fenton
UV/TiO_2	Non-thermal plasma
Photolysis	Ultrasonic cavitation
$UV/H_2O_2/TiO_2$	

1.3.1. Photolysis combined with Hydrogen Peroxide (H_2O_2)

Ultraviolet (UV) light irradiation is widely used for disinfection of drinking water and is a promising process for wastewater purification.

The combination of UV with H_2O_2 can improve the removal of pharmaceuticals compared to UV alone. The hydroxyl radical is produced during the process as a result of photolysis of the peroxide. It is a strong oxidant and can attack a wide range of organic compounds causing degradation through hydroxylation and dehydrogenation.

Photodegradation reactions can occur directly and indirectly. In direct photodegradation, a compound absorbing radiation can become unstable and consequently decay, whereas the indirect way involves compounds that produce strong reactive species (such as hydroxyl radicals) that then react with organic compounds [10].

UVC monochromatic lamps emitting at 254 nm can provide enough energy to break a wide range of organic bonds. This cleavage is possible when the photonic energy absorbed by the compound surpass the bond energy. For example, UVC can break a carbon-chlorine bond of a chlorinated compound like diclofenac and lead to the formation of chlorine radical [11]. Also, at this wavelength, hydrogen peroxide has a high molar extinction coefficient so that a part of photons emitted by the lamp is used in its photolysis and therefore two hydroxyl radicals are created [12].

$$H_2O_2 + hv\ (254\ nm) \rightarrow 2HO\bullet \qquad \text{(Eq.1)}$$

1.4. ANALYTHICAL METHODS

The improvement in analytical methods is a great step in the detection of emerging contaminants. They allow to establish the presence of these chemicals in the environment, to estimate their concentration levels and to determine their degradation pathways.

In the environmental analysis, it is required a highly selective and wide range of analytical methods, because of the complexity of the matrices of water samples, and the large number of organic contaminants present in these samples. The low concentrations at which these pollutants are found in the environment and their different physicochemical properties demand high detection sensitivity. In addition, structural information is needed for the characterization of the main parent compound and its degradation products. Most of the studies are focused only

on the identification and degradation of the contaminant but sometimes the intermediates can be more persistent and toxic than the original. That's why it requires a thorough study [13] [14].

Due to the continuous contamination of water, an improvement in technology is required to provide sensitive, selective and specific methods able to obtain a rigorous identification, confirmation and quantification of these compounds. This breakthrough is possible as a result of the combination of high-resolution mass spectrometry (HRMS) and liquid chromatography (LC). While liquid chromatography provides the separation of multiple components, mass spectrometry allows to identify the structure of the individual components with high molecular specificity and detection sensitivity.

Liquid chromatography is a separation technique based on the interaction of the samples with the stationary and mobile phases and it is especially satisfying for the separation of low volatility polar compounds such as diclofenac.

The high-resolution mass spectrometers, such as TOF and Orbitrap, represent a promising option to identify non-targeted compounds in real samples. Its main characteristics are the high resolving power, the mass accuracy measurement and the high full-scan sensitivity.

The resolving power allows to detect two compounds with the same nominal but different exact mass. There is some controversy when it comes to defining this concept. According to the IUPAC, the resolving power (Δm) is the ability to discern between ions differing in the quotient mass/charge (*m/z*) by a small increment [15]. While the resolution is defined as m/Δm, where m is the nominal mass of the interest molecule and Δm is the width of the peak at fifty percent of the maximum peak height, sometimes named Full Width of the peak at Half its Maximum (FWHM).

The mass accuracy (eq.2) is evaluated in ppm units of error, and depends on diverse factors such as ionisation source, peak shape and calibration.

$$\text{ppm} = \frac{\text{Theoretical mass} - \text{Experimental mass}}{\text{Theoretical mass}} \cdot 10^6 \qquad \text{(Eq.2)}$$

In full-scan mode, the mass spectrum is continually acquired between two different *m/z* ratio in a certain period of time (mostly ≤1 s), and all the compounds between this ratio can be detected [16].

2. OBJECTIVES

The main objectives of this project are the degradation of diclofenac using Advanced Oxidation Processes (UV/H_2O_2 and UV photolysis) and the identification of the transformation products by liquid chromatography coupled to high-resolution mass spectrometry.

As specific objectives, kinetics will be studied and, with identified degradation products, possible reaction pathways will be proposed.

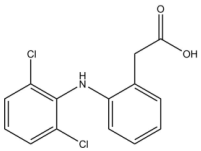
Finally, the toxicity of the final samples will be estimated to be able to determine if the subproducts formed are more detrimental than the main compound.

3. MATERIALS AND METHODS

3.1. REAGENTS

Diclofenac sodium salt, from Sigma-Aldrich, was the pharmaceutical used to carry out the research.

Table 2. Diclofenac properties [17].

Names and Identifiers	
IUPAC name	2-[2-(2,6-dichloranilino) phenyl]acetic acid
CAS Number	15307-86-5
Molecular formula	$C_{14}H_{11}Cl_2NO_2$
Molecular structure	
Chemical and physical properties	
Property	**Value**
Monoisotopic Mass [g/mol]	295.02
Solubility (at 25ºC) [mg/L]	2.37
pk_a	4.15
Melting point [ºC]	283-285
log K_{ow}	4.51

The following table shows the other reagents employed for the experiments.

Table 3. Information about the other reagents [17].

Name	**CAS No.**	**Formula**	**Company**	**Purity** [%]	**Used in/for**
Hydrogen peroxide	7722-84-1	H_2O_2	Merck	30 w/v	UV/H_2O_2
Acetonitrile	75-05-8	CH_3CN	LiChrosolv	99.9	LC-HRMS
Methanol	65-56-1	CH_3OH	SupraSolv	-	LC-HRMS/ SPE
Formic acid	64-18-6	HCOOH	EMSURE	98-100	LC-HRMS
Water	7732-18-5	H_2O	SupraSolv	-	LC-HRMS/ SPE

3.2. WATER MATRIX

For all experiments, it was used bottled commercial water of glass.

Table 4. Characteristics of water.

Composition	[mg/L]
Dry waste	203.0
Calcium	71.3
Sodium	0.8
Magnesium	1.9
Bicarbonates	198.0
Sulphates	15.7
Chlorides	1.9

This water has been selected as a blank of water matrix without the presence of pollutants. Also, its organic composition was previously characterised by the laboratory.

3.3. EXTRACTION METHOD

For some experiments, solid phase extraction (SPE) in reversed phase was employed to clean up the samples and pre-concentrate it prior to analysis. In this extraction process, the analyte of interest is retained in the stationary phase (sorbent) of the cartridge and then is eluted by an adequate solvent.

It was used an Oasis HLB cartridge, with a capacity of 6mL and 200 mg of sorbent, as its name suggests, it is a hydrophilic-lipophilic balance polymer, that remains wetted with water and can retain a wide spectrum of both polar and nonpolar compounds.

The sensitivity of the analysis of the degradation products rises doing the extraction.

3.4. ANALYTHICAL METHODS

3.4.1. LC-HRMS

The analysis of the samples was done by LC-HRMS in an Orbitrap mass spectrometer using electrospray ionisation.

The Orbitrap is a mass analyser composed of two outer electrodes and a central one (figure 1). Ions generated, by using an ion source, enter to the device and then are radially trapped around the central electrode, where after they oscillate. Finally, measuring the different oscillation frequencies (depending on the mass/charge ratio of the ions), the mass spectra of the ions are acquired using image current detection [14].

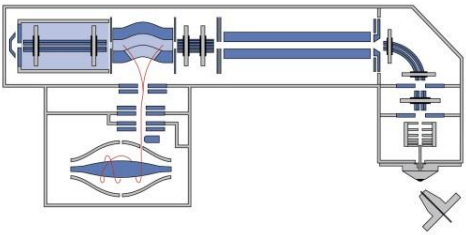


Figure 1. Orbitrap diagram.

The chromatographic separation was performed on a reversed phase Phenomenex Luna C18(2) column (150 mm x 2.0 mm, 5 µm) using as a mobile phase 0.1% of formic acid in water as a solvent A and 0.1% of formic acid in acetonitrile as a solvent B at a constant flow rate of 200 µL/min. The gradient started with 5% of B and increase to 90 % of B for 15 min and then, after 9 min, returned to initial conditions for re-equilibrate (5% of B). The total duration of the method was 34 min. The injection volume was 5 µL for the samples.

Mass spectrometry data were acquired in both positive and negative ionisation and in full-scan mode from 50 to 1000 *m/z*, with a resolution of 50,000 (at 200 *m/z*; 2 Hz). Also, an injection time of 250 ms and Automatic Gain Control (AGC) of $1x10^6$ were used.

3.4.2. Total Organic Carbon

Total organic carbon (TOC) is the total amount of carbon present in organic compounds and it is used as a parameter to assess the quality of the aqueous systems.

To analyse the TOC, it has used a Shimadzu 5055 TOC-VCSN analyser with an ASI-V Autosampler following the 5220D-standard method.

3.5. EXPERIMENTAL DEVICE

The AOPs experiments were done in a 2 L jacketed batch reactor made of Pyrex (figure 2), with a continuously cooling current at 25 ºC. It was 23 cm high and 11 cm of inside diameter. Three monochromatic UVC lamps (Philips TUV 8 W, G8T5) with a wavelength of 254 nm were located in the axis of the reactor and a magnetic stirrer was used to provide a uniform mixture of the solutions during the experiment.

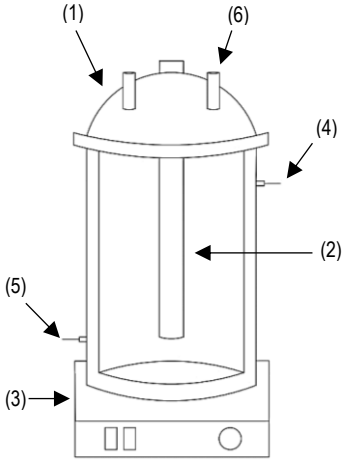


Figure 2. Schematic diagram of 2 L batch reactor: (1) reactor, (2) lamp, (3) stirrer, (4) cooling water input, (5) cooling water output, (6) sampling gap.

For one experiment was utilized a solarbox XENOTERM-1500RP, with a UV lamp (≈290-2000 nm) that simulates the solar radiation spectrum. In this case the photoreactor was 24.5 cm length and 2 cm of diameter and is connected to a supply tank. The recirculation rate is large enough and the reactor volume is small enough to be able to consider the same concentration in all the system in a specific moment. Figure 3 shows a schematic diagram.

After the degradation experiments, a SPE of the samples was done. The cartridges of the extraction were placed in a cube connected to a vacuum pump and to load the samples an adapter of 60 mL was put, as it can be seen in the figure 4. After this, the samples were concentrated at 500 µL using a nitrogen flowrate of 40ºC by TurboVap Cassic II with sensor endpoint detection and a maximum sample volume of 200 mL. To finish to concentrate at 250 µL was used a Pasvial.

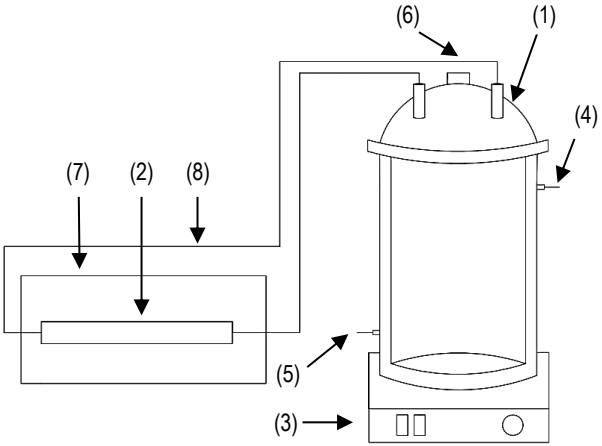


Figure 3. Schematic diagram of solarbox: (1) tank, (2) photoreactor, (3) stirrer, (4) cooling water input, (5) cooling water output, (6) sampling gap, (7) solarbox, (8) recirculation.

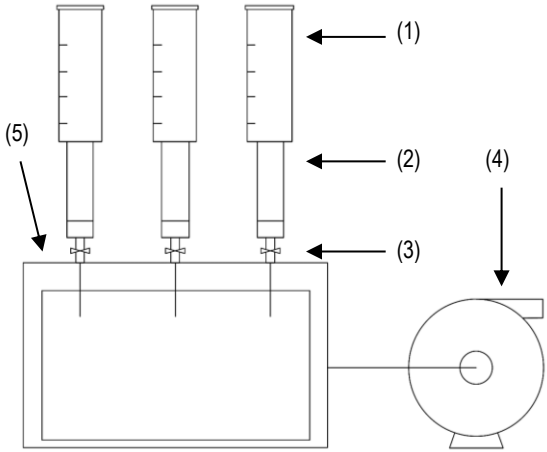


Figure 4. Schematic diagram of SPE: (1) adapters, (2) cartridges, (3) valves, (4) vacuum pump, (5) cube.

3.6. EXPERIMENTAL PROCEDURE

The diclofenac degradation was carried out during one hour using distinct concentrations of peroxide and types of light in several experiments shown in table 5.

Table 5. Completed experiments.

Experiment	H_2O_2 [mg/L]	Light
UV/H_2O_2	20	UVC
UV/H_2O_2	20	UVC
UV/H_2O_2	50	UVC
UV	-	UVC
H_2O_2	50	-
Solarbox	50	Sunlight simulator

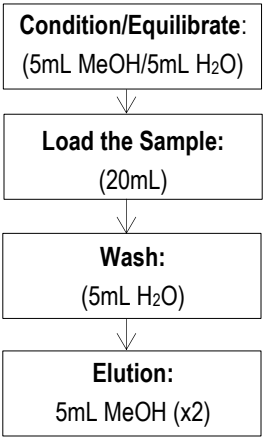
Steps followed for the realization of the experiments:

1. Preparation of the solution of diclofenac with mineral water. Weigh 100 mg of the pharmaceutical and dissolve in a 2 L volumetric flask.
2. Agitation to dissolve the contaminant properly for about 15 or 20 minutes.
3. Activation of the thermostatic bath to reach 25ºC and switch on the light to tempered it.
4. Introduction of the solution into the reactor with a stirring of 300 rpm and with the lamp off.
5. At zero time the first sample was taken before introducing the H_2O_2 with the solution.
6. Injection of the H_2O_2, at the same time, switch on the light and then the experiment begins.
7. 20 mL of sample were taken at minutes 0, 0.5, 2, 4, 5, 10, 20, 30, 45 and 60. At zero time and at the end of the experiment, 15 mL were also extracted for the TOC.
8. Disconnection of thermostatic bath turn off the light and stop the agitation.

One aliquot of 1 mL of the sample was reserved for the analysis by direct injection in the Orbitrap and the remaining was treated with SPE in order to pre-concentrated.

To conduct the extraction, the cartridges were first conditioned and equilibrate with 5 mL of MeOH and then 5 mL of water. The samples (≈20 mL) were loaded at a flow rate of 3-5 mL/min retaining the compost of interest into the cartridge, then 5 mL of water to eliminate interferences soluble on it. The diclofenac and the extracted degradation products were eluted with 5 mL of MeOH two times at a flow rate of 1-2 mL/min.

Extraction method diagram:



Before the analysis, the elution was concentrated at 250 µL and then was reconstituted with water until 1 mL to get an adequate proportion of MeOH/H_2O_2. Considering that the mobile phase of the LC starts with a proportion of aqueous phase of 95% regarding to organic phase, the more similar the proportion of the sample at the initial mobile phase, the better is to obtain a more defined peak.

Finally, the samples were stored at 6ºC in amber vials of 1 mL until the LC-HRMS analysis.

3.7. **IDENTIFICATION PROCEDURE**

A bibliographical search of the possible degradation products of diclofenac under different AOPs was done in order to make a database with the exact mass of both positive and negative ions from the molecular formulas of these degradation products. The ion exact mass were calculated using Xcalibur software.

After that, the following ruler based on accurate mass measurements was used to identify and describe the degradation of products. First, a visual inspection of the TIC (total ion current) chromatogram was done to detect the most intense peaks and then the mass spectrum of each chromatographic peak, which shows the experimental *m/z*, was evaluated.

For each *m/z*, the software generates various molecular formulas by delimiting the elements and the number of them based on the molecular formula of diclofenac and considering the degradation process by oxidation, therefore C, H, N, O, and Cl were the elements considered in the present study. Then the most adequate formula was selected taking into account:

- The Ring Double Bond (RDB) equivalent value, which provides us the information about how many unsaturations the molecule has.
- The mass error, which has to be less than 5 ppm.
- The isotopic pattern has to be compared with the theoretical.
- The bibliography: Validate if the metabolites are described by other authors.

Once the molecular formula of the signal was confirmed, it was verified that the retention time (t_R) was maintained in the different samples of the same experiment. It cannot vary more than 2.5%. Also, the isotopic pattern, the mass accuracy and the RDB of each sample were checked.

All this procedure was done for each peak detected in TIC both in positive and negative ionisation mode. Low abundance compounds maybe are not apparent visual peaks at first inspection, and intense peaks do not necessarily have to be associated with a single component or with organic contaminants of interest.

Additionally, to describe the structural formula of the degradation products, the bibliography was checked. If the molecular formula was not defined there, the structural formula could be established from the difference in mass with diclofenac and considering diverse possible mechanism of degradation, such as the loss of chlorine or group acid, the gain of hydroxyls or oxygen and dimerization.

Finally, the areas of the peaks of each sample were compiled to evaluate the evolution of the compound during the experiment and to graph it.

4. RESULTS AND DISCUSSION

Table 6 shows all the experiments done using distinct concentrations of hydrogen peroxide and types of light. It shows also the percentage of organic carbon mineralized and the percentage of diclofenac degradation.

Table 6. Percentage of diclofenac removal in the different experiments carried out and TOC analysis.

Experiments	H_2O_2 [mg/L]	Light	TOC [%]	Degradation [%] (SPE)	Degradation [%] (direct injection)
UV/H_2O_2	20	UVC	10.6	90.5	97.3
UV/H_2O_2	20	UVC	8.1	-	96.1
UV/H_2O_2	50	UVC	8.3	91.1	97.0
UV	-	UVC	9.8	81.4	91.8
H_2O_2	50	-	1.3	-	2.0
Solarbox	50	Sunlight simulator	0.6	-	0.8

In the first four experiments, TOC degradation is really low. However, diclofenac removal can achieve high percentages. In the experiment carried out only with H_2O_2, there is practically no degradation of TOC or diclofenac. The same can be said for the experiment done in a solar simulator with a lamp emitting radiation close to the solar one, which no degradation of TOC or diclofenac were observed due to the particular wavelength range of the used light, as it will be commented below.

The SPE has been employed to clean up the samples and concentrate them before the analysis. So, it is obtained a better response in the analysis of the degradation products.

4.1. DEGRADATION OF DICLOFENAC

Figure 5 correspond to all degradation processes carried out. As it can be observed, there is no degradation in the experiments done with a solar simulator or with only hydrogen peroxide without any type of light. Whereas with UV photolysis and UV/H_2O_2 there is practically a total degradation. The hydrogen peroxide itself does not affect diclofenac. However, H_2O_2 combined with UVC increase lightly the diclofenac removal, due to the ability of the hydroxyl radicals generated to attack organic compounds. The points of the figure show the experimental data

while the tiny discontinuous lines are drawn to be able to appreciate better the trend of the results.

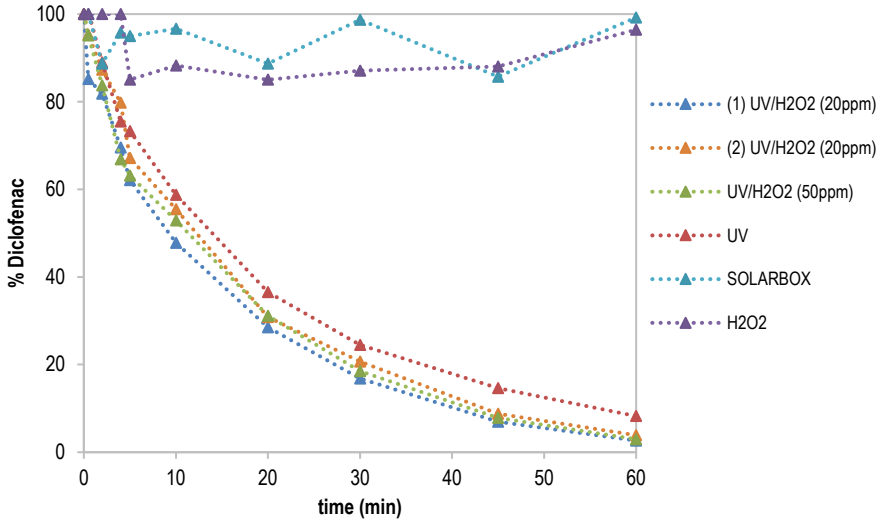


Figure 5. Degradation of diclofenac by different AOPs. Initial concentration of 50 ppm of diclofenac. Data from direct injection by LC-HRMS.

Diclofenac has a maximum absorption at 276 nm [11], but in the range of wavelengths of solar radiation, the part corresponding to 300 nm is small in comparison with all radiation (see figure 6). Therefore, the solar radiation is not able to directly degrade diclofenac and nor to generate hydroxyl radicals from hydrogen peroxide. Consequently, one hour of experimentation in the solarbox, with 50 ppm of hydrogen peroxide, produces no degradation of diclofenac.

As figure 5 shows, diclofenac is easily photodegradable by UV photolysis and the combination of UV/H_2O_2 does not mean a significant improvement. With just UV radiation, 92% of degradation was obtained and adding H_2O_2 it increases only 5%. According to other authors, there is no difference in increasing the hydrogen peroxide concentration [10] [18] [19] [20].

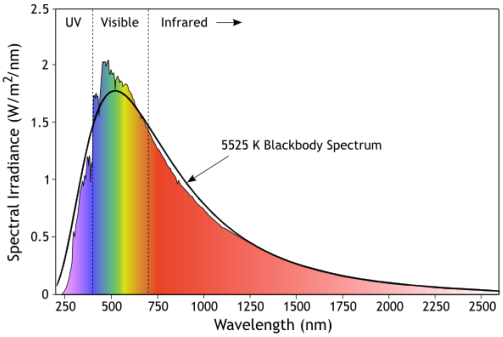


Figure 6. Solar radiation spectrum [21]

So, the hydroxyl groups formed practically do not step in the degradation of the compound. This might be due to the fact that the action of photolysis is faster than hydroxyls or the main target of these hydroxyls is another compound present in the sample. In any case, the predominant degradation mechanism in the experiments was the photolysis.

In this experiment, mineral water with a concentration of 198 mg/L of bicarbonate has been used. This bicarbonate has a reaction rate constant with hydroxyl radicals of 8.5×10^6 L/(mol·s) [22], whereas the reaction rate constant of diclofenac is 1.36×10^{10} L/(mol·s) [19]. Diclofenac clearly has a higher constant, but considering that there is almost four times more bicarbonate than diclofenac, it could be possible that bicarbonate will be degraded by the hydroxyls before.

4.2. **KINETICS OF DEGRADATION**

The degradation reaction was fitted to a pseudo-first-order kinetics using the following equation:

$$C = Co \cdot exp(-kt) \qquad \text{(Eq.3)}$$

Linearizing the equation, it remains in the form:

$$ln\left(\frac{C}{C_0}\right) = -k \cdot t \qquad \text{(Eq.4)}$$

Where *k* is the pseudo-first-order rate constant for direct or indirect photolysis, *Co* is the initial concentration of diclofenac, *C* the concentration at any time and *t* is the experimental time.

Thus, fitting the data of figure 5 to this equation drives to figure 7.

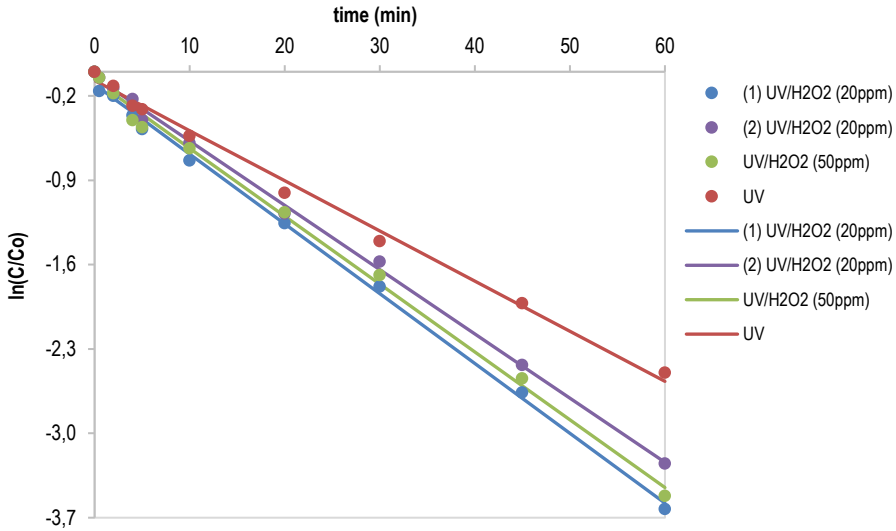


Figure 7. Kinetics linearization for the degradation experiments. Experimental values are shown with dots and the fitted values with the straight lines.

Figure 7 shows more clearly how the experiments with UV/H_2O_2 achieve higher degradation than UV photolysis, and it can be verified in table 7, where the kinetic constants obtained from linearization are shown. Effectively, the kinetic constants obtained when H_2O_2 was used are, as minimum as 20% higher than the kinetic constant obtained using only UV light, without H_2O_2. This fact corroborates what has already been said before about the fact that photolysis has a much more preponderant role in the diclofenac removal than hydroxyl radicals. In both cases, from figure 7 and table 7, it can be said that data fit well pseudo-first-order kinetics.

Table 7. Kinetic constants.

Experiments	k [min^{-1}]	R^2
(1) UV/H_2O_2 (20 ppm)	0.058	0.998
(2) UV/H_2O_2 (20 ppm)	0.053	0.998
UV/H_2O_2 (50 ppm)	0.056	0.996
UV	0.042	0.994

4.3. **FORMATION OF DEGRADATION PRODUCTS**

The degradation products were analysed in three different experiments: UV/H_2O_2 with 20 ppm and 50 ppm of hydrogen peroxide and UV photolysis.

The three graphs below (figures 8, 9 and 10) show the degradation of the diclofenac and the formation of its major transformation products. It can clearly be seen the formation of a main product (D1). Comparing the graphs, a higher degradation of D1 and the formation of more subproducts and more intense was observed with UV/H_2O_2 regarding to the UV photolysis. This can be due to D1 might be more affine to be degraded by hydroxyls than diclofenac.

These figures show the transformation products appearing after the D1, because probably they are formed from it. From the shape of the curves and from the time at which appeared the different intermediates, it seems clear that D1 comes from the direct degradation of diclofenac, because it appears when experiment just started. The other compounds appear later and can be the result of the degradation of D1 or diclofenac. All this will be discussed widely when the possible reaction pathways will be commented below.

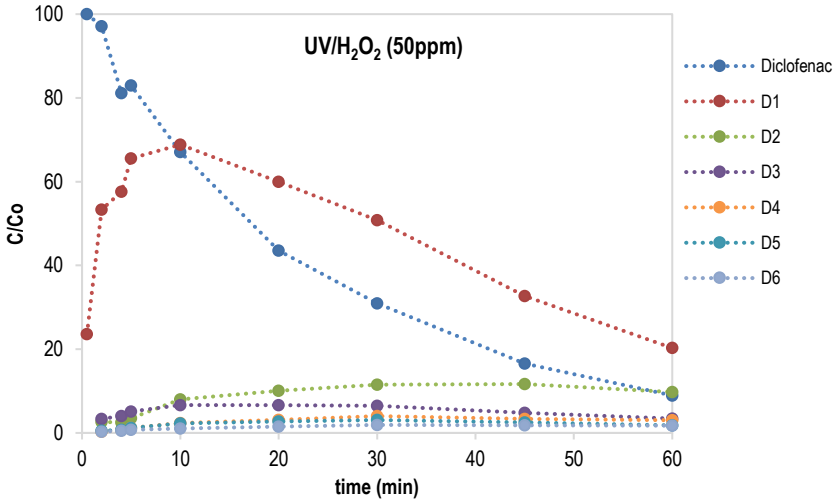


Figure 8. Evolution of main degradation products formed during degradation of diclofenac (50 ppm) by UV with 50 ppm of hydrogen peroxide.

Figure 8 and 9 are practically the same, therefore there is no difference in the degradation or the generation of subproducts when the H_2O_2 concentration is increased.

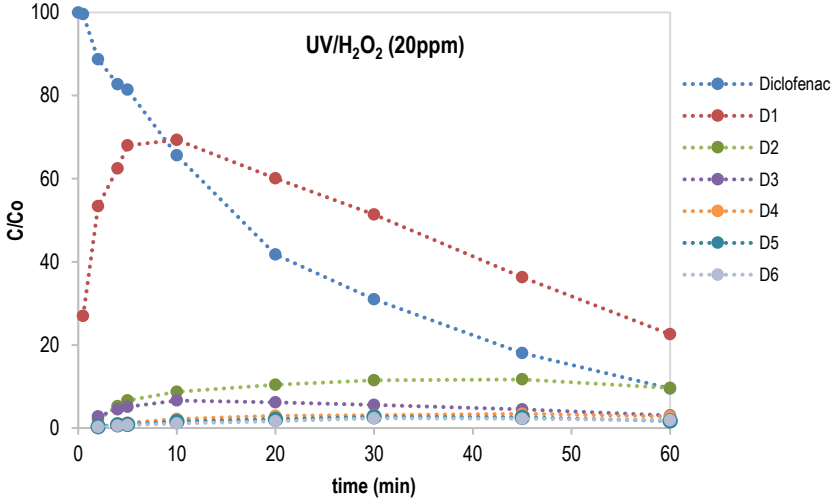


Figure 9. Evolution of main degradation products formed during degradation of diclofenac (50 ppm) by UV with 20 ppm of hydrogen peroxide.

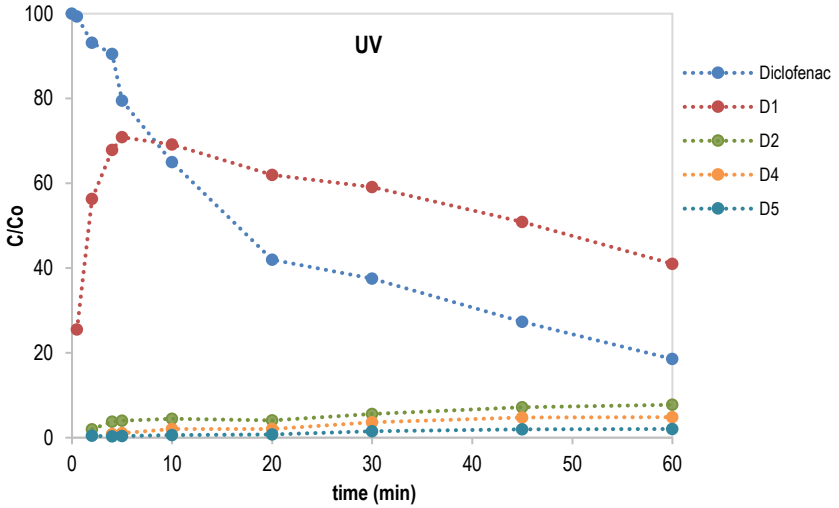


Figure 10. Evolution of main photolytic degradation products formed during degradation of diclofenac (50 ppm) by UV.

Figure 10, when only UV is used without H_2O_2, shows, as indicated above, a slowdown in the degradation of intermediate D1, which is also related to the lower amounts observed of the other transformation products.

4.4. IDENTIFICATION OF DICLOFENAC AND ITS DEGRADATION PRODUCTS

4.4.1. Diclofenac

In order to identify the diclofenac and its degradation products, the ruler based on the accurate mass measurements explained before has been followed. In figure 11, the experimental pattern isotopic of diclofenac in a specific sample, coincides with the theoretical as well as the RDB, and the mass accuracy is less than 5 ppm.

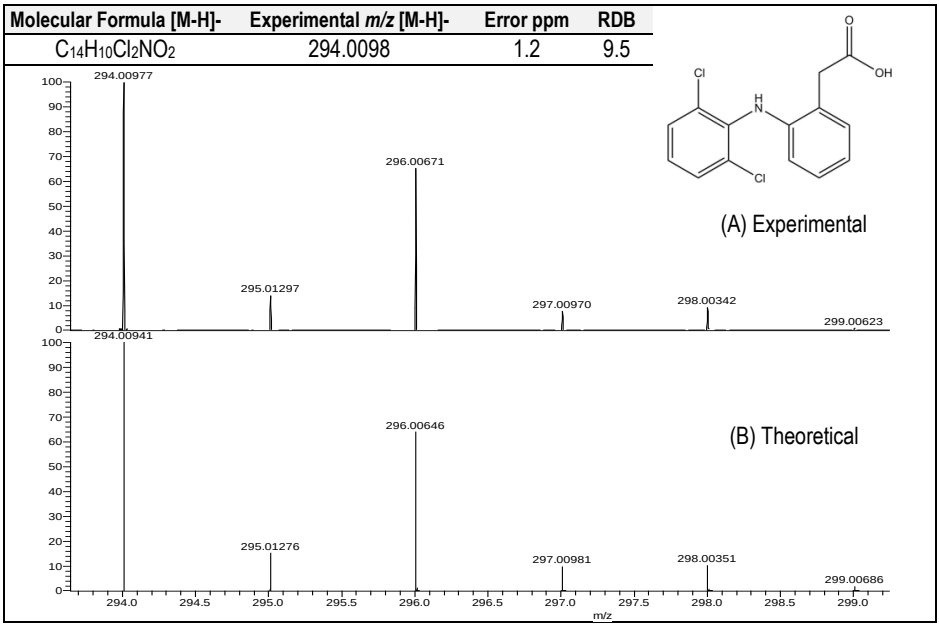


Figure 11. (A) Experimental LC-HRMS mass spectrum of diclofenac in the sample of 10 min of experimentation by UV/H_2O_2 (20ppm) and (B) theoretical mass spectrum of diclofenac simulated by Xcalibur software.

Diclofenac has a characteristic structure. The presence of two chlorines provides a characteristic pattern isotopic. The amino group and the acid group, that form it, allow to detect the compound at both positive and negative scan mode. The first group is more likely to be seen in positive and the second one in negative.

At figure 11, it is possible to see the characteristic pattern isotopic of chlorine which helps to identify diclofenac and the subproducts. The spectrum shows the molecular ion peaks M, M+2, and M+4 which appear due to the various combinations of chlorine isotopes that are possible. Each one represents, respectively, the possibility to have two ^{35}Cl, one ^{35}Cl and one ^{37}Cl, and two ^{37}Cl. The separation between them is of 2 *m/z* units and with peak heights in the ratio of 9:6:1.

If the compound has only one chlorine atom, the spectrum has to show the peaks of molecular ions M, M+2 with a ratio of 3:1 in the peak heights. It is a good way to know if the compound has one or two chlorines just by looking at the spectrum.

Diclofenac and most of its transformation products were detected in both positive and negative ionisation, but in practically all of them we get a more sensitive signal in negative. During the analysis by LC-HRMS, they have been fragmented due to the high voltage at the ionisation source. Therefore, two signals appear at the spectrum for each chromatographic peak.

As it can be observed in figure 12, we have one signal for diclofenac and the other for the fragment. The difference between both shows the loss of the acid group. At the qualitative representation of the results, the fragments were not considered because the ratio between the fragment and the compound remained during all the experiments.

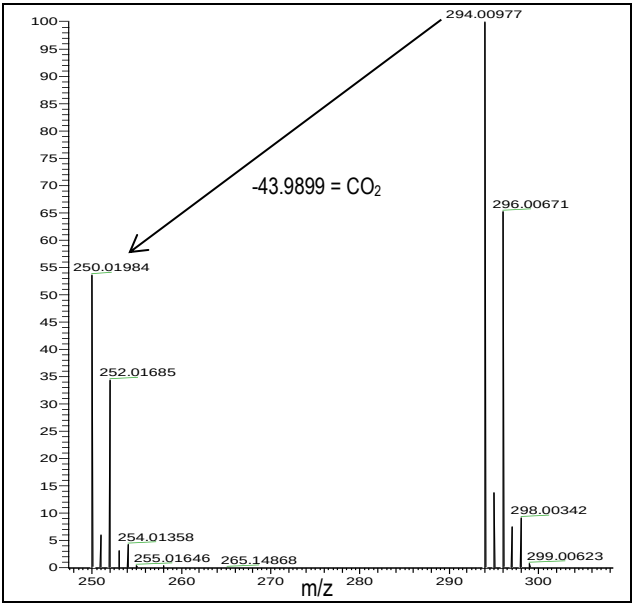


Figure 12. LC-HRMS mass spectrum of diclofenac and its fragmentation in the sample of 10 min of experimentation by UV/H_2O_2 (20ppm).

4.4.2. Degradation products

In the degradation of diclofenac, fourteen different degradation products have been identified and described, and some of them have been reported for the first time.

Diclofenac transformation products were eluted through the chromatographic column at different retention times based on their polarity. All of them appeared at a lower t_R than diclofenac as shown in figure 13, suggesting that they are more polar.

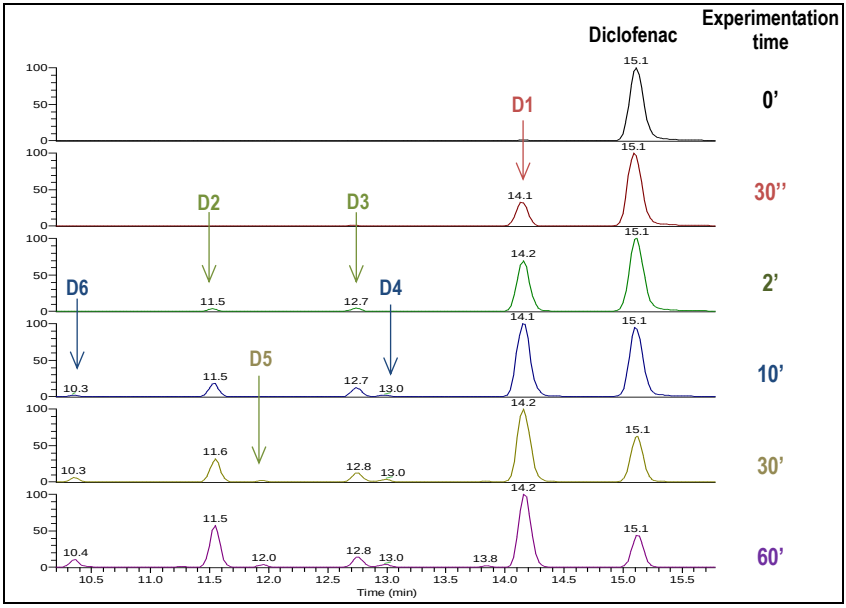
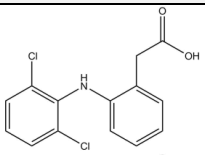
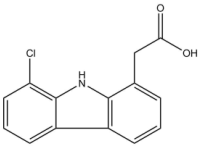
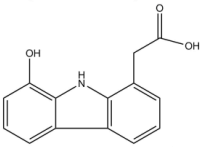
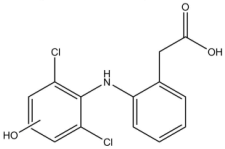
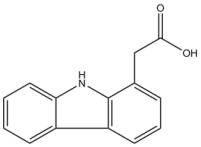
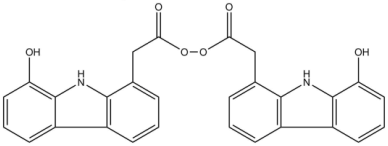
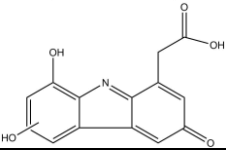


Figure 13. LC-HRMS chromatogram of the experiment with UV/H_2O_2 (50 ppm). Evolution of diclofenac and its degradation products.

The previous chromatogram shows the evolution of the diclofenac and the degradation products during an experiment. It is possible to see the increasing trend of D1 during just the first 10 minutes, whereas the others increase progressively and diclofenac decrease until the end.

Hereunder, from all the identified compounds, the most significant in the experiments performed with UV/H_2O_2 has been described below in table 8, that is, those with a major peak abundance.

Table 8. Diclofenac degradation products produced under UV/H_2O_2 and identified by LC-HRMS.

Peak abundance [1]	Neutral Structural Formula	ESI	Molecular Formula $[M-H]^-$ /$[M+H]^+$	Experimental *m/z* $[M-H]^-$ /$[M+H]^+$	Error ppm	RDB	t_R
Diclofenac 100%		NEG	$C_{14}H_{10}Cl_2NO_2$	294.0098	1.2	9.5	15.1
D1 70%		NEG	$C_{14}H_9ClNO_2$	258.0330	1.2	10.5	14.2
D2 12%		NEG	$C_{14}H_{10}NO_3$	240.0669	1.3	10.5	11.5
D3 7%		NEG	$C_{14}H_{10}Cl_2NO_3$	310.0048	1.4	9.5	12.7
D4 3%		NEG	$C_{14}H_{10}NO_2$	224.0721	1.7	10.5	12.9
D5 3%		POS	$C_{28}H_{21}N_2O_6$	481.1396	0.4	19.5	12.0
D6 3%		NEG	$C_{14}H_8N0_5$	270.0412	1.5	11.5	10.3

(1) Relative abundance regarding diclofenac initial amount.

The data included in this table refers to results from sample of 10 min of experimentation.

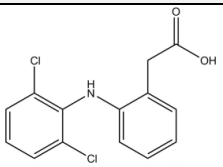
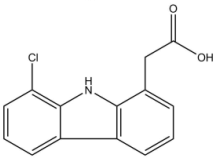
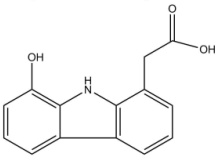
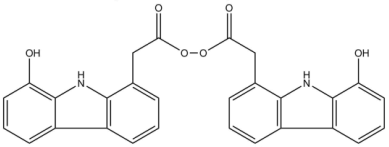
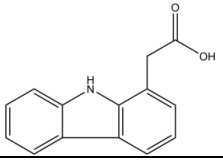
The structure of D5 and D6 has been tentatively assigned according to similar formulas found in the bibliography.

NEG: negative ESI, $[M-H]^-$

POS: positive ESI, $[M+H]^+$

The next table (table 9) shows the transformation products identified in the experiment with UV photolysis. As it can be observed, the subproducts described are the same as the other experiments, but with less abundance and without the presence of D3 and D6 that are formed by the presence of H_2O_2.

Table 9. Diclofenac degradation products produced under UV photolysis treatment and identified by LC-HRMS.

Peak abundance [1]	Neutral Structural Formula	ESI	Molecular Formula $[M-H]^-$ /$[M+H]^+$	Experimental *m/z* $[M-H]^-$ /$[M+H]^+$	Error ppm	RDB	t_R
Diclofenac 100%		NEG	$C_{14}H_{10}Cl_2NO_2$	294.0090	-1.4	9.5	15.1
D1 70%		NEG	$C_{14}H_9ClNO_2$	258.0323	-1.8	10.5	14.1
D2 8%		NEG	$C_{14}H_{10}NO_3$	240.0662	-1.9	10.5	11.5
D5 5%		POS	$C_{28}H_{21}N_2O_6$	481.1392	-0.5	19.5	12.0
D4 3%		NEG	$C_{14}H_{10}NO_2$	224.0714	-1.2	10.5	12.9

(1) Relative abundance regarding diclofenac initial amount.
The data included in this table refers to results from sample of 10 min of experimentation.
The structure of D5 has been tentatively assigned according to similar formulas found in the bibliography.
NEG: negative ESI, $[M-H]^-$
POS: positive ESI, $[M+H]^+$

With the identified and described compounds, an attempt has been made to trace a possible pathway of degradation.

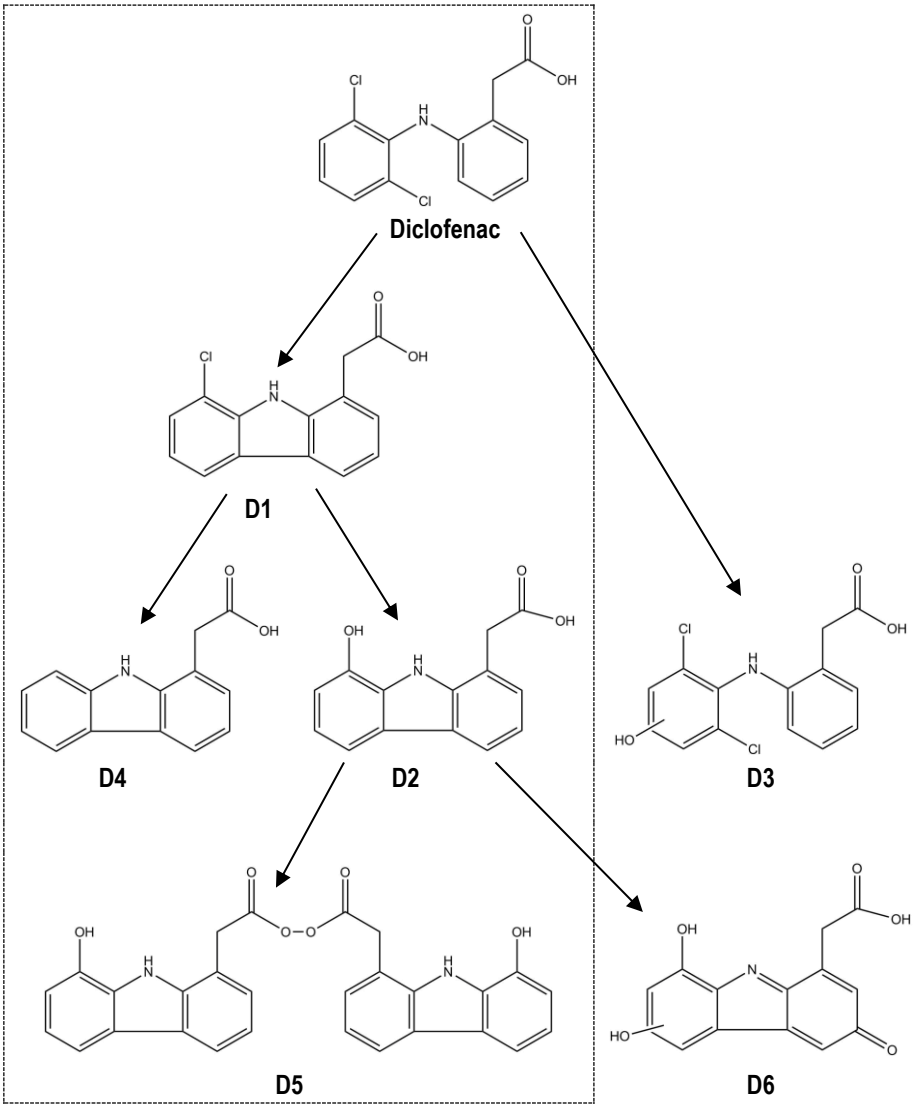


Figure 14. Tentative degradation pathway for diclofenac under UV/H_2O_2 treatment. Within the rectangle: direct photolytic degradation products.

As figure 14 shows, the UV light leads to sequential elimination of the chlorine substituents of diclofenac structure, followed by ring closure to form carbazole-1-acetic acid (D4), which is considered as the elemental photolytic degradation product [10] .

With the loss of the first chlorine, it is formed the most abundant product, D1, which reached its highest concentration after 10 min of exposure and represents 70% of the initial 50 mg/L of diclofenac. The second product of direct photolysis, D2, is formed by the substitution of the chlorine atom from D1 with the hydroxyl group, the product can be considered as a stable. Also, a dimer formation, such as D5, has been detected during the experiment, which could be formed by coupling two molecules of D2. The mechanism of dimer formation has previously been studied [11]. The authors report that singlet oxygen formed during the experiment can participate in reactions with unsaturated bonds and probably improve the formation of radicals that result in dimerization.

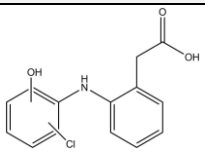
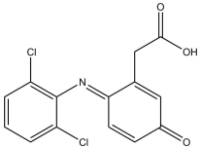
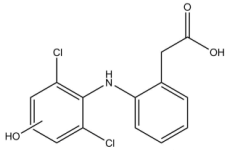
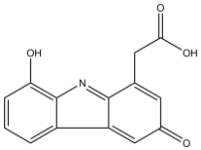
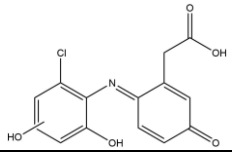
In this current analysis, two products have been identified resulting from radical transformation in the presence of hydrogen peroxide, i.e., product D3 and D6. D3 had a similar trend as D1, after 10 min both dramatically decrease which could indicate that this degradation product was also produced directly from diclofenac whereas D6 might be formed from the gain of one oxygen and one hydroxyl of D2.

Other products have been identified, but with an intensity less than 2% of the initial amount of diclofenac, so they have not been included in the degradation pathway, their data are described at table 10. Signals under 1% have been discarded.

In some cases, two or three different molecules can be associated with the same *m/z*, which could be attributed to isomeric compounds. D7, D8 and D9 are isomeric structures differing in the position of the hydroxyl group and the chlorine. Also, D11 can be an isomer of D3, but the first one is found in less abundance than the second one, suggesting that one structure is more probable.

With current methods, it is possible to determine the elements forming a molecule but it is not possible to define the exact position of each element.

Table 10. Other diclofenac degradation products produced under UV photolysis or UV/H_2O_2 treatments identified by LC-HRMS with a peak abundance regarding diclofenac lower than 2%.

Treatments	Neutral Structural Formula	ESI	Molecular Formula $[M-H]^-$ /$[M+H]^+$	Experimental *m/z* $[M-H]^-$ /$[M+H]^+$	Error ppm	RDB	t_R	Name
UV/H_2O_2 and UV		NEG		276.0432	-0.4	9.5	11.3	**D7**
		NEG	$C_{14}H_{11}ClNO_3$	276.0437	1.4	9.5	11.9	**D8**
		NEG		276.0436	1.1	9.5	13.0	**D9**
UV/H_2O_2 and UV		POS	$C_{14}H_{10}Cl_2NO_3$	310.0035	-2.6	9.5	12.6	**D10**
UV		NEG	$C_{14}H_{10}Cl_2NO_3$	310.0047	1.2	9.5	13.8	**D11**
UV		NEG	$C_{14}H_8NO_4$	254.0457	-0.6	11.5	10.4	**D12**
UV/H_2O_2	-	NEG	$C_{28}H_{17}N_2O_8$	509.1000	2.0	21.5	11.4	**D13**
UV/H_2O_2		NEG	$C_{14}H_9ClNO_5$	306.0179	1.4	10.5	11.4	**D14**

The data included in this table refers to results from sample of 30 min of experimentation.
The structure of D14 has been tentatively assigned according to similar formulas found in the bibliography. It was no possible to propose a reliable structure for D13.
NEG: negative ESI, $[M-H]^-$
POS: positive ESI, $[M+H]^+$

4.5. **TOXICITY**

Finally, the aquatic toxicity of the major identified transformation products has been estimated using ECOSAR (Ecological Structure Activity Relationships), which is a computerized predictive system that predicts the potential toxicity of industrial chemicals to aquatic organisms. It is developed by EPA (United States Environmental Protection Agency). The program contains a library of measured data of Quantitative Structure Activity Relationships (QSARs) based on the chemical class to predict the toxicity. QSARs provides acute (short-term) and chronic (long-term) toxicity values in mg/L for fish, aquatic invertebrates (Daphnia), and aquatic plants (green algae), which are species that represent the aquatic food net [23].

In this study the values used were lethal concentrations (LC_{50}) that is the concentration of a substance required to kill half of the population of study after a specific duration.

Figure 15 shows lethal concentrations by different organisms. So, the higher the concentration, the less toxic the compound, because it is needed a larger concentration to kill half of the population.

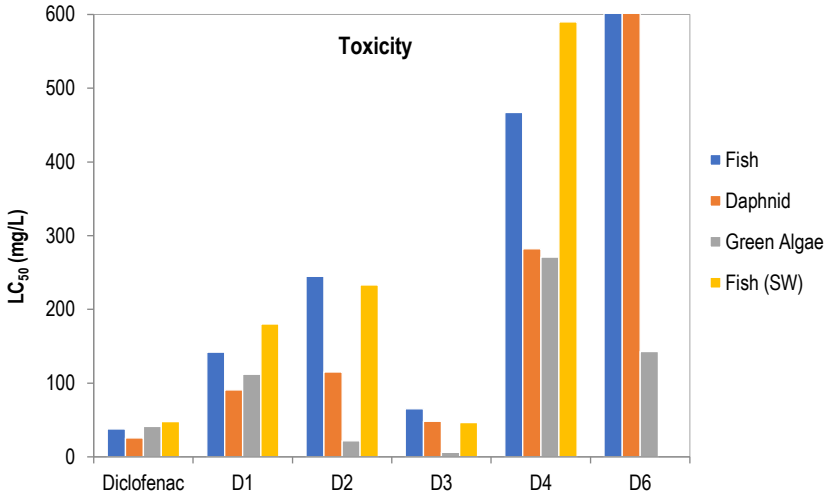


Figure 15. Toxicity of the identified compounds.

In general the toxicity decrease with the degradation of the main compound. The only compound that might be more toxic than diclofenac is D3, which is also the most detrimental for the green algae.

The least toxic compound for daphnid and fish is D6 which exceeds 600 mg/L. However, for green algae, the presence of D6 it could be damaging and for the seawater fish, it was not possible to estimate the toxicity.

The most significant compound to determine the toxicity is D1 because is the most abundant at the end of the experimentation and is considerably detrimental regarding the others. With direct photolysis, the final concentration of D1 is twice as the UV/H_2O_2 treatment (see figure 16). This means that the presence of H_2O_2 decreases the toxicity of the sample, because can remove a large amount of the one of the most toxic degradation compounds.

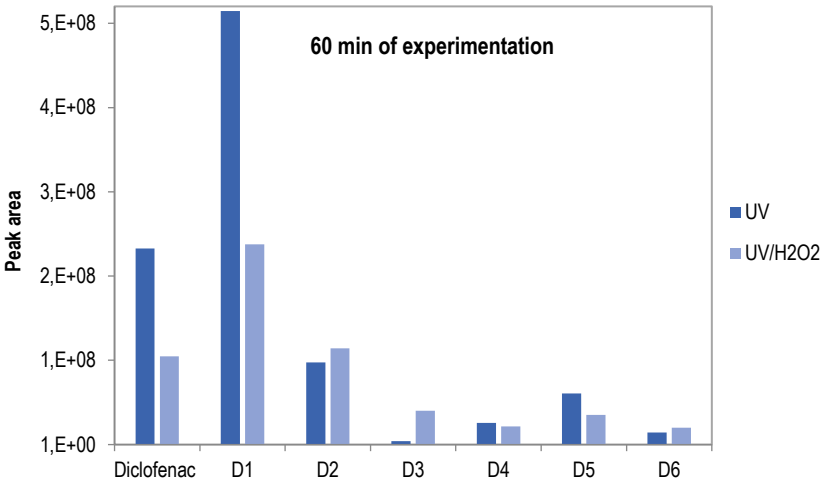


Figure 16. The final amount of the identified compounds after the degradation by UV and UV/H_2O_2.

Looking at the degradation pathway (figure 14) it can be deduced that, as expected, the loss of chlorines entails the decrease of the toxicity.

5. CONCLUSIONS

The conclusions deduced from the work carried out are the following:

- Direct photolysis with UV and the combination of UV with hydrogen peroxide are effective methods for diclofenac removal.
- The presence of hydrogen peroxide does not represent a significant improvement in the rate of diclofenac degradation.
- The degradation process follows a pseudo-first-order kinetics.
- Experiments done with only H_2O_2 or with solar light show a very low diclofenac removal.
- Using liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS), the transformation products were identified following the ruler based on accurate mass measurements, and a tentative degradation pathway was proposed.
- The toxicity of the final products was estimated by ECOSAR and it was observed that the loss of chlorines during the degradation results in a decrease of the toxicity.
- The use of hydrogen peroxide in the degradation process (UV/H_2O_2) implies a decrease in toxicity.

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ACRONYMS

AGC	Automatic Gain Control
AOPs	Advanced Oxidation Processes
ECOSAR	Ecological Structure Activity Relationships
EPA	Environmental Protection Agency
EQS	Environmental quality standard
ESI-MS	Electrospray ionisation mass spectrometry
FWHM	Full Width of the peak at Half its Maximum
HLB	Hydrophilic-lipophilic balance
IUPAC	International Union of Pure and Applied Chemistry
LC_{50}	Lethal concentrations
LC-HRMS	Liquid chromatography coupled to high-resolution mass spectrometry
NSAID	Non-steroidal anti-inflammatory drug
QSARs	Quantitative Structure Activity Relationships
RDB	Ring Double Bond
SPE	solid phase extraction
TIC	Total ion current
TOC	Total organic carbon
TOF	Time-of-flight
t_R	Retention time
UV	Ultraviolet
UVC	Ultraviolet C
WFD	Water Framework Directive

WL	Watch List