Master in Chemical Engineering

Degradation of anticancer drugs in urban effluents by ozonation

A Master's dissertation

of

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Abstract

Anticancer drugs have been found in surface waters in Portugal, proving that conventional treatments used in wastewater treatment plants are unable to degrade them. The risks these drugs pose to humans and to aquatic biota are severe, and include aquatic toxicity, genotoxicity and endocrine disruption. The search for an effective method of degradation is of paramount importance. This dissertation explored the use of ozonation for the degradation of these drugs.

Four anticancer drugs were studied, based on the risk they posed for aquatic biota: bicalutamide (Bica), cyclophosphamide (Cyc), mycophenolic acid (MPA) and mycophenolate mofetil (MMF). The degradation of these compounds was investigated in ultrapure water, at typical wastewater treatment conditions (pH = 7, T = 30 ºC), and for various concentrations of spiked ozone (0.5, 1 and 5 $mg \cdot L^{-1}$). The kinetics of Bica and Cyc were also studied, under the same conditions. Lastly, the degradation of these two drugs was analysed for different experimental conditions, namely by altering the pH (experiments were performed for pH = 2.9 and pH = 10.9).

The experimental results showed that MPA and MMF are easily degradable, having achieved full removal, using any of the tested O_3 concentrations, at pH = 7.1. Bica and Cyc proved to be more recalcitrant, and their full removal was not achieved under any experimental conditions tested. Nevertheless, it was shown that their degradation is more effective under alkaline pH values, achieving removal rates of 54.5% for Bica, and 74.1% for Cyc, at pH = 10.9 after 30 minutes. However, hydrolysis may have played a part in the degradation rates obtained. Kinetic constants could not be calculated for any of the anticancer drugs, since ozone was not monitored continuously during the reactions, and ozonation follows a second-order kinetics that depends on ozone's concentration as well as on the concentration of the anticancer drugs. However, a qualitative analysis of Bica and Cyc's kinetics allows the conclusion that ozonation in the presence of $^{\bullet}OH$ presents higher rates of degradation, which shows that this is, in fact, the predominant path for the removal of both anticancer drugs.

Ozonation proved to be an efficient process to degrade MPA and MMF. For Bica and Cyc, however, this process alone is not effective. In fact, the addition of a substance that increases the presence of $^{\bullet}OH$ radicals, such as H_2O_2, or the incidence of UV radiation, should be considered, thus enhancing the elimination of the anticancer drugs.

Keywords (Theme): anticancer drugs, degradation, ozonation.

Resumo

Medicamentos anticancerígenos têm sido encontrados em águas superficiais em Portugal, demonstrando que os tratamentos convencionais utilizados em estações de tratamento de águas residuais são incapazes de degradar estes compostos. Os riscos que estes medicamentos representam, tanto para os seres humanos como para os ecossistemas aquáticos, são graves e incluem toxicidade aquática, genotoxicidade e desregulação endócrina. A procura de um método eficaz de degradação é, assim, de suma importância. Esta dissertação analisou a implementação da ozonização como método de degradação dos anticancerígenos.

Quatro anticancerígenos foram selecionados, com base no risco que representam para os ecossistemas aquáticos: bicalutamida (Bica), ciclofosfamida (Cyc), ácido micofenólico (MPA) e micofenolato de mofetil (MMF). Inicialmente, a degradação destes medicamentos foi testada em água ultrapura, para condições típicas de tratamento de águas residuais (pH = 7, T = 30 ºC) e para várias concentrações de ozono (0.5, 1 e 5 $mg \cdot L^{-1}$). As cinéticas de Bica e Cyc também foram estudadas, nas mesmas condições. Por último, a degradação destes dois anticancerígenos foi testada em diferentes condições experimentais, variando o pH (pH = 2.9 e pH = 10.9).

Os resultados experimentais mostraram que o MPA e o MMF são facilmente degradáveis, sendo completamente removidos, seja qual for a concentração de O_3 utilizada, para pH = 7.1. Bica e Cyc provaram ser mais recalcitrantes, e a sua remoção completa não foi alcançada sob quaisquer condições experimentais. No entanto, foi demonstrado que a sua taxa de degradação é superior em pH alcalino, tendo sido obtidos valores de 54.5% para Bica e 74.1% para Cyc, para pH = 11, após 30 minutos de reação, apesar da hidrólise poder ter contribuído para as taxas de remoção que foram obtidas. Não foi possível calcular as constantes cinéticas para nenhum dos compostos, dado que estas reações seguem uma cinética de segunda ordem em relação aos compostos e ao ozono, e não se procedeu à monitorização contínua da concentração de ozono durante as experiências. No entanto, é possível efetuar uma análise qualitativa destas reações, e observa-se que, de facto, a reação de ozonização na presença de iões hidroxilo apresenta taxas de degradação superiores, demonstrando que este é o método de degradação predominante.

Foi demonstrado que a ozonização é um processo eficiente para degradar MPA e MMF. No entanto, para a Bica e Cyc, este processo, por si só, não é eficaz. De facto, aconselha-se a adição, por exemplo, de H_2O_2, ou a incidência de radiação UV, de modo a aumentar a presença de radicais OH e, consequentemente, a eliminação dos anticancerígenos.

Palavras Chave (Tema): **anticancerígenos, degradação, ozonização.**

Declaration

I hereby declare, on my word of honour, that this work is original and that all non-original contributions were properly referenced with source identification.

Porto, July 1st, 2019

Index

List of Figures

List of Tables

Notation and Glossary

$[O_3]$	Concentration of ozone throughout the experiments	$mg \cdot L^{-1}$
$[O_3]_0$	Initial concentration of ozone	$mg \cdot L^{-1}$
k	Kinetic constant for complete ozonation reaction	$M^{-1} \cdot s^{-1}$
$k(O_3)$	Kinetic constant for the direct ozonation reaction	$M^{-1} \cdot s^{-1}$
$k(OH)$	Kinetic constant for the indirect ozonation reaction	$M^{-1} \cdot s^{-1}$
R_{ct}	Ratio between concentration of ozone and OH radicals	
T	Temperature	ºC
V_T	Total volume of the indigo-ozone solution	mL
ΔAbs	Variation of absorbance between blank solution and ozonated solutions	
V_A	Volume of ozonated water added to the indigo solution	mL

Greek letters

ε_b	Mass absorptivity	$L \cdot (mg \cdot cm)^{-1}$

Indexes

0	t=0
t	time

List of Acronyms

Abs	Absorbance
AOPs	Advanced Oxidation Processes
AOTs	Advanced Oxidation Technologies
ATC	Anatomical Therapeutic Chemical
Bica	Bicalutamide
Cyc	Cyclophosphamide
DNA	Deoxyribonucleic Acid
LC-MS	Liquid Chromatography-tandem Mass Spectrometry system
MB	Micro-bubbles
MMF	Mycophenolate Mofetil
MPA	Mycophenolic Acid
UV	Ultraviolet
WWTP	Wastewater Treatment Plant

1 Introduction

1.1 Framing and presentation of the work

Cancer, malignant tumours or neoplasms are identical designations for the mutation and abnormal growth of malignant cells, that invade the body's tissues and organs. It is the second leading cause of death worldwide and its prevalence is increasing in first world countries, due to sedentarism and obesity, amongst other factors (World Health Organization, 2019). In fact, by 2030,it is expected that the number of new cancer cases will have risen to 23.6 million (National Institutes of Health, 2018).

Although there isn't yet a cure for cancer, most cases are treated with chemotherapy, that uses pharmaceuticals designated as anticancer drugs. These drugs have the property of inhibiting cell division and growth, which causes the death of cancerous cells. Healthy cells can also be affected by anticancer drugs, but in a less pronounced way, since their growth is slower (Cancer Society of Finland, 2019).

The chemotherapy drugs are classified according to the ATC – Anatomical Therapeutic Chemical by the World Health Organisation, in the category L, denominated as "Antineoplastic and Immunomodulating agents" (Norwegian Institute of Public Health - WHO Collaborating Centre for Drug Statistics Methodology, 2019). In turn, this group has four subsequent subcategories, that were divided based on how each drug attacks the cancerous cells.

L01: Antineoplastic agents

These drugs usually act by disrupting the cell's replication process, either by impeding the synthesis of DNA, or by damaging it in an irreversible way. This leads, inevitably, to the cell's death. These agents are cell-cycle-nonspecific, which means that they can damage the cell's DNA and prevent its replication at any point of this process (Canadian Institutes of Health Research; The Metabolomics Innovation Centre (TMIC); Alberta Innovates - Health Solutions, 2018).

- Cyclophosphamide (Cyc) belongs to this category (L01AA01) (Norwegian Institute of Public Health - WHO Collaborating Centre for Drug Statistics Methodology, 2019).

L02: Hormone therapy

Some types of cancer can be hormone sensitive or dependant, which means that they use hormones to accelerate their growth, like prostate cancer. Endocrine therapy is based on administering synthetic hormones to cancer patients, in order to supress the production of

natural hormones that may be enhancing the cancer's growth, and, consequently, slow its proliferation (National Institutes of Health).

- Bicalutamide (Bica) belongs to this category (L02BB03) (Norwegian Institute of Public Health - WHO Collaborating Centre for Drug Statistics Methodology, 2019).

L03: Immunostimulants

Immunostimulants, generally, help the body resist viral infections and cancers. Regarding cancer treatment, these drugs have been used in the creation of vaccines or antibodies that boost the body's immune system and directly attack cancer cells, either impeding their replication or provoking their apoptosis (Pranchevicius and Vieira, 2013).

L04: Immunosuppressants

These compounds can completely, or partly, suppress one or more factors in the immune system. For instance, tumour cells have an increased need for protein synthesis that noncancerous resting cells do not require, so immunosuppressants act by inhibiting these processes, thus suppressing the cell's growth (Nazanin, et al., 2014).

- Mycophenolic acid (MPA) and Mycophenolate mofetil (MMF) belong to this category (L04AA06) (Norwegian Institute of Public Health - WHO Collaborating Centre for Drug Statistics Methodology, 2019).

When administered to a patient, part of the anticancer drugs are metabolised and excreted in the urine and faeces, meaning that they end up reaching wastewater treatment plants (WWTPs), mostly via hospital wastewater (Ikehata, et al., 2006). For instance, cyclophosphamide has an excretion rate of 10-20%, according to Garcia-Ac, et al. (2010).

Research has shown that the conventional treatments currently implemented at WWTPs are unable to successfully degrade most pharmaceuticals, including anticancer drugs. Therefore, these hazardous drugs may reach the surface and, even, drinking water, posing the aquatic biota, the environment and the humans at serious risks (Ikehata, et al., 2006). The risks caused by exposure to these drugs include aquatic toxicity, resistant development in pathogenic bacteria, genotoxicity (which is the damage caused to the genetic information, and that, in severe cases, can lead to the appearance of cancer), endocrine disruption and potential chronic health effects (provoked by long-term ingestion). For all above mentioned reasons, the search for alternative processes aiming at the effective treatment of wastewaters contaminated with these hazardous drugs is of utmost importance and has become a priority (Ikehata, et al., 2006).

This constitutes the main objective of the present dissertation, in which the exploitation of ozonation as an alternative and effective treatment process is envisaged.

In Portugal, Santos, et al. (2017) performed a study to estimate the presence of anticancer drugs in portuguese surface water and the associated risks for aquatic biota. The values obtained varied between regions, but anticancer drugs were generally found in surface water at concentrations ranging between 0.15 and 137.08 $ng \cdot L^{-1}$. Santos, et al. (2017) also concluded that, out of the analysed drugs, mycophenolic acid (MPA) and mycophenolate mofetil (MMF) may represent a high risk to aquatic biota. Cyclophosphamide (Cyc), which is, by far, the most studied anticancer drug, and bicalutamide (Bica), show moderate risk for aquatic organisms.

Ozonation is an advanced oxidation process (AOP) that exploits ozone's high reactivity to remove compounds from wastewater. It is highly used in disinfection processes, since, similarly to chlorine, ozone is unstable in water, and reacts with some water matrix components. These reactions originate OH radicals (•OH), which are the strongest oxidants that are soluble in water (von Gunten, 2003). This decomposition results in two different oxidant species that will act in different ways regarding each compound: molecular ozone and •OH. Equations (1) to (5) represent the reactions that ozone undergoes when it is dissolved in water (Elovitz, et al., 2000). The remaining equations are depicted in Annex I.

$$O_3 + OH^- \rightarrow HO_2^- + O_2 \quad (1)$$

$$O_3 + HO_2^- \rightarrow O_3^- \cdot + HO_2^- \quad (2)$$

$$O_3 + O_2^- \cdot \rightarrow O_3^- \cdot + O_2 \quad (3)$$

$$O_3^- \cdot + H^+ = HO_3 \cdot \quad (4)$$

$$HO_3 \cdot \rightarrow \cdot OH + O_2 \quad (5)$$

Oxidation through molecular ozone is often named as direct ozonation and it occurs when O_3 selectively attacks the nucleophilic positions of the organic molecules. In this case, the reactivity is highly dependent on the molecular structure of the anticancer drug (von Gunten, 2003). In fact, von Gunten (2003) states that molecular ozone is selective to double bonds, aromatic systems and non-protonated amines. Also, all electron-donating groups favour the oxidation by molecular ozone, unlike electron-withdrawing groups.

On the other hand, indirect ozonation takes place when O_3 is allowed to decompose by reacting with water, generating •OH, that is highly reactive with any compounds, since their reaction is nearly diffusion-controlled, but will be present in lower concentrations than O_3 (von Gunten, 2003). The ratio between dissolved molecular ozone and hydroxyl radicals varies with the pH of the solution. In fact, when pH values are alkaline, the reaction that originates •OH is favoured, due to the OH^- ions that are naturally present in the water, and in higher

concentrations for these pH values. If, on the contrary, the pH is acidic, then there will be fewer ions and, therefore, fewer hydroxyl radicals. When these facts are taken into consideration, it becomes clear that direct ozonation is favoured by low pH values, while indirect ozonation is predominant when pH is alkaline.

This dissertation proposes to study the performance of the ozonation process in the degradation of four anticancer drugs of concern: bicalutamide, cyclophosphamide, mycophenolic acid and mycophenolate mofetil. The best conditions to maximise the removal of these compounds will be investigated, aiming at their complete degradation. In addition, the kinetics of the degradation of each compound by ozonation will also be analysed, hopefully allowing the identification of the predominant way of oxidation (direct vs. indirect), correlating it with the compound's molecular structure.

1.2 Contributions of the Work

The innovative aspects of this dissertation are related to the compounds analysed, considering that, to the best of my knowledge, this is the first time that the degradation of mycophenolic acid (MPA) and mycophenolate mofetil (MMF) is investigated using ozonation, under any conditions. In fact, I have found no studies that use any AOP to oxidise these compounds, either separately or combined.

It is also the first time that the kinetics of ozonation for these drugs is studied.

1.3 Organisation of the thesis

Chapter 2 of this dissertation is dedicated to the context and state of the art for the process, describing what research has been made so far, analysing its results and comparing each project based on their final outcomes.

On chapter 3, there will be a brief description of the materials and methods employed for the purpose of this dissertation, alongside the explanation of the procedure for each experiment. This chapter shall be divided between the reagents and the analytical methods that were employed, and the ozonation experiments that were performed.

Chapter 4 is reserved to the presentation and analysis of the results that were obtained, beginning with the results for the quantification method that was employed for the analysis of the anticancer drugs. Then, the results for the degradation experiments are presented in a chronological order, to justify all the decisions that were made and to allow for future reproducibility of this work.

The conclusions that were drawn from this dissertation are depicted on chapter 5, alongside an analysis of the process's advantages and disadvantages, and a reflexion on whether it could be applied for the purpose it was aimed.

Finally, chapter 6 provides an assessment of the work that was carried out, namely, whether the objectives that were proposed in chapter 1 were achieved, and also mentioning further work that was carried out and wasn't initially planned. In this chapter, the limitations of the process, and of the dissertation are also dissected, and complemented with suggestions for future work and future approaches, alongside an overall assessment of the project.

2 Context and State of the Art

The degradation of anticancer drugs is a relatively unexplored field of study. Most studies focus on the use of advanced oxidation processes (AOPs) and technologies (AOTs), such as ozonation, oxidation with Fenton's reagent, or UV-based oxidation approaches. The effectiveness of these processes is very dependent on the type of drug, since the structure of the molecule influences its stability and, consequently, its resistance to oxidation. When analysing the existing studies, it is easy to conclude that, for most compounds, ozonation is, by far, the most effective process, whether by itself or in combination with others. Therefore, this dissertation is focused exclusively on the exploitation of ozonation, justifying the fact that this section includes only the scientific knowledge gathered so far in the degradation of target anticancer drugs by ozone-based processes. Not all anticancer drugs have been analysed to ascertain its degradation through these processes. In fact, most research focuses on a very restricted group of compounds, due to its frequent use in treatments and its presence in wastewater and drinking water.

Table 1 summarises all relevant research that has been performed on the topic, and the most significant findings of each study. It includes the conditions in which the studies occurred, more specifically, concentrations of drug and ozone and other conditions, and the results obtained, whether by showing the percentage of anticancer drug degraded or by presenting the kinetics of the reaction.

Table 1: Published studies related with the degradation of target anticancer drugs by ozone and ozone-based processes.

Process applied	Anticancer drug	Water matrix	Conditions	Significant findings	References
	Bicalutamide (Bica)	Deionised water	T = 20 ºC $[O_3]$ = 6.5 mg·L^{-1} $Q(O_3)$ = 0.3 L·min^{-1} [Bica] = 5x10^{-4} mg·L^{-1}	Removal rates achieved were 42.9%, for regular ozonation. Reaction rate (assuming first-order kinetics) was k=0.037 min^{-1}.	(Azuma, et al., 2019)
	Cyclophosphamide (Cyc)	Deionised water	$[O_3]_0$ = 0.192 mg·L^{-1} [Cyc] = 1.04 mg·L^{-1}	Ozone cannot remove cyclophosphamide ($k(O_3)$ = 143 M^{-1}·s^{-1}).	(Chen, et al., 2008)
	Cyclophosphamide	Drinking water and effluent from a drinking water treatment plant	pH = 8.10 T = 20 ºC $[O_3]_0$ = 10 mg·L^{-1} [Cyc] = 2.0x10^{-4} mg·L^{-1}	Cyclophosphamide degradation rate with O_3 was low without significant natural water matrix effects ($k(O_3)$ = 3.3 vs $k(O_3)$ = 2.9 M^{-1}·s^{-1}). The removal rate was 87%.	(Garcia-Ac, et al., 2010)
	Cyclophosphamide	Deionised water	$[O_3]_0$ = 0.1-1 mg·L^{-1}	The degradation rate increased with the initial O_3 concentration. However, the reaction of cyclophosphamide with O_3 is negligible (k_{app} = 0.008 min^{-1}; $k(O_3$ *in excess)* = 2.5 M^{-1}·s^{-1}).	(Y. Lester, et al., 2011)

Process applied	Anticancer drug	Water matrix	Conditions	Significant findings	References
	Cyclophosphamide	Deionised water	pH = 7.6 [Cyc] = 1 $mg \cdot L^{-1}$; $[O_3]_0$ = 5 $mg \cdot L^{-1}$	Cyc removal decreases with increasing alkalinity (92% vs 58%) and concentration of model compounds (alginic acid, peptone and natural organic matter). This is more pronounced for peptone and less pronounced for alginic acid, most likely due to the increase in the scavenging effect of $HO^{\bullet}$.	(Lester, et al., 2013)
	Cyclophosphamide	MBR effluent from a pilot hospital wastewater treatment plant	[Cyc] = $1.85x10^{-4}$ $mg \cdot L^{-1}$; $[O_3]_0$ = 0.64 $mgO_3 \cdot mg_{DOC}^{-1}$; $[O_3]_0$ = 0.89 $mgO_3 \cdot mg_{DOC}^{-1}$; $[O_3]_0$ = 1.08 $mgO_3 \cdot mg_{DOC}^{-1}$	Cyc elimination increased from 33 % to 57% with the increase of O_3 concentration (0.64 to 1.08 $mgO_3 \cdot mg_{DOC}^{-1}$).	(Kovalova, et al., 2013)
	Cyclophosphamide	Deionised water	$[O_3]$ = 0-6 $mg \cdot L^{-1}$ (pH= 11-5.6) [Cyc] = 5 $mg \cdot L^{-1}$;	At pH=11, there was a full degradation after 5 minutes. At lower pH, removal rate decreases to 61.2 %.	(Lin, et al., 2015)
	Cyclophosphamide	effluent from WWTP	[DOC] = 5 $mg \cdot L^{-1}$; [Cyc] = $5x10^{-3}$ $mg \cdot L^{-1}$; $[O_3]_0$ = 0-1 $mgO_3 \cdot mg_{DOC}^{-1}$	Cyc was moderately degraded (≈70%) using the highest O_3 dose (1 $mg \cdot mg^{-1}$).	(Li, et al., 2016)

Process applied	Anticancer drug	Water matrix	Conditions	Significant findings	References
	Cyclophosphamide	hospital wastewater	pH = 8.89 T = 20 ºC $[O_3]$ = 45, 55, 70 mg·L^{-1}	The elimination yield for Cyc was 97% for $[O_3]_0$ = 45 and 99% for $[O_3]_0$ = 55 mg·L^{-1} in relatively short reaction times. Removal was complete for highest O_3 concentration. The degradation rate coefficient for direct ozonation is in the order of 34.7 M^{-1}·s^{-1}.	(Ferre-Aracil, et al., 2016)
	Cyclophosphamide	Deionised water	T = 20 ºC $[O_3]$ = 6.5 mg·L^{-1} $Q(O_3)$ = 0.3 L·min^{-1} [Cyc] = 5x10^{-4} mg·L^{-1}	Cyc's removal rates were 21.5%, for regular ozonation, and reaction rate (assuming first-order kinetics) was 0.020 min^{-1}.	(Azuma, et al., 2019)
O_3/H_2O_2	Bicalutamide	Deionised water	T = 20 ºC $[O_3]$ = 6.5 mg·L^{-1} [Bica] = 5x10^{-4} mg·L^{-1} $[H_2O_2]$ = 5 mg·L^{-1}	Removal rate decreased when compared to O_3 alone, from 42.9% to 39.2%. Reaction rate also decreased, to 0.019 min^{-1}.	(Azuma, et al., 2019)
	Cyclophosphamide	Deionised water	$[O_3]_0$ = 0.5 mg·L^{-1}; $[H_2O_2]$ = 0-0.35 mg·L^{-1}	The degradation rate increased with the initial H_2O_2 concentration. The process O_3/H_2O_2 showed the highest degradation rate for Cyc amongst different processes (UV, O_3, UV/O_3, UV/H_2O_2/O_3, O_3/H_2O_2).	(Y. Lester, et al., 2011)

Process applied	Anticancer drug	Water matrix	Conditions	Significant findings	References
	Cyclophosphamide	Deionised water	T = 20 ºC $[O_3]$ = 6.5 mg·L^{-1} $Q(O_3)$ = 0.3 L·min^{-1} [Cyc] = 5x10^{-4} mg·L^{-1} $[H_2O_2]$ = 5 mg·L^{-1}	The degradation rate decreased when compared to regular ozonation, from 21.5% to 13.9%. The reaction rate is also lower, from 0.020 min^{-1} to 0.014 min^{-1}.	(Azuma, et al., 2019)
UV/O_3	Bicalutamide	Deionised water	T = 20 ºC $[O_3]$ = 6.5 mg·L^{-1} $Q(O_3)$ = 0.3 L·min^{-1} [Bica] = 5x10^{-4} mg·L^{-1}	UV/O_3 > O_3 > H_2O_2/O_3. The combination of ozone with UV radiation achieved removal rates of 47.6%. Reaction rate is the highest for the O_3-based processes studied, being 0.109 min^{-1}.	(Azuma, et al., 2019)
	Cyclophosphamide	Deionised water	$[O_3]_0$ = 0.5 mg·L^{-1}; $[H_2O_2]$ = 2 mg·L^{-1}	Degradation rate decreases as follows: UV/H_2O_2/O_3 > UV/O_3 > UV.	(Y. Lester, et al., 2011)
	Cyclophosphamide	Deionised water	T = 20 ºC $[O_3]$ = 6.5 mg·L^{-1} $Q(O_3)$ = 0.3 L·min^{-1} [Cyc] = 5x10^{-4} mg·L^{-1}	O_3 > H_2O_2/O_3 > UV/O_3. The combination of ozone with UV radiation could not degrade cyclophosphamide, at all.	(Azuma, et al., 2019)
UV/H_2O_2/O_3	Bicalutamide	Deionised water	T = 20 ºC $[O_3]$ = 6.5 mg·L^{-1} $Q(O_3)$ = 0.3 L·min^{-1} [Bica] = 5x10^{-4} mg·L^{-1} $[H_2O_2]$ = 5 mg·L^{-1}	This process proved to be the least effective, achieving a removal rate of 15.4%. The reaction rate presents its lowest value, k = 0.015 min^{-1}.	(Azuma, et al., 2019)

Process applied	Anticancer drug	Water matrix	Conditions	Significant findings	References
	Cyclophosphamide	Deionised water	$[O_3]_0$ = 0.5 mg·L^{-1}; $[H_2O_2]$ = 2 mg·L^{-1}	Degradation rate decreases as follows: $UV/H_2O_2/O_3 > UV/O_3 > UV$.	(Y. Lester, et al., 2011)
	Cyclophosphamide	Deionised water	T = 20 ºC $[O_3]$ = 6.5 mg·L^{-1} $Q(O_3)$ = 0.3 L·min^{-1} [Cyc] = 5x10^{-4} mg·L^{-1} $[H_2O_2]$ = 5 mg·L^{-1}	Removal rate was 12%, which is higher than for UV/O_3, but lower than for the other processes. This reaction rate was the highest for cyc, k = 0.048 min^{-1}.	(Azuma, et al., 2019)
O_3-based processes in microbubbles (MB)	Bicalutamide	Deionised water	T = 20 ºC $[O_3]$ = 6.5 mg·L^{-1} $Q(O_3)$ = 1 L·min^{-1} [Bica] = 5x10^{-4} mg·L^{-1} $[H_2O_2]$ = 5 mg·L^{-1}	The use of micro-bubbles only proved effective for $UV/H_2O_2/O_3$, achieving almost full degradation of Bica (98.9%).	(Azuma, et al., 2019)
	Cyclophosphamide	Deionised water	T = 20 ºC $[O_3]$ = 6.5 mg·L^{-1} [Cyc] = 5x10^{-4} mg·L^{-1} $[H_2O_2]$ = 5 mg·L^{-1}	The addition of micro-bubbles increased removal rates. However, the highest removal achieved was 32%, for $UV/H_2O_2/O_3$.	(Azuma, et al., 2019)

Analysing the table, it is clear that all studies concluded that both bicalutamide and cyclophosphamide are recalcitrant drugs, difficult to degrade by ozone and related processes.

For instance, Chen, et al. (2008) studied Cyc's kinetics and concluded that $k(O_3)$ = 143 $M^{-1}\cdot s^{-1}$. $k(O_3)$ defines the kinetic constant for the direct ozonation reaction, that includes only molecular ozone. Garcia-Ac, et al. (2010) obtained even lower constants ($k(O_3)$ ~ 3 $M^{-1}\cdot s^{-1}$). This study also obtained a removal rate of 87%, for a Cyc concentration of $2.0x10^{-4}$ $mg\cdot L^{-1}$ and for a spike of 10 $mg\cdot L^{-1}$ of ozone, which is quite high (most studies presented don't exceed spikes of 5 $mg\cdot L^{-1}$). The concentration of Cyc is also low, which can help justify this percentage of removal.

Garcia-Ac et al. (2010) also studied the influence of the water matrix on the ozonation process, testing Cyc's degradation both in drinking water and in an effluent from a drinking water treatment plant, and concluded that $k(O_3)$ remained practically constant (2.9 $M^{-1}\cdot s^{-1}$ vs 3.3 $M^{-1}\cdot s^{-1}$).These constants were corroborated by Lester, et al. (2011), that obtained $k(O_3)$ = 1.5 $M^{-1}\cdot s^{-1}$. This study also concluded that, logically, cyclophosphamide's degradation increases with the initial ozone concentration.

In Lester, et al. (2013), the goal was to study the degradation of Cyc in deionised water that contained model compounds, such as alginic acid, peptone and natural organic matter. It led to conclude that these compounds acted as scavengers for •OH, impeding their reaction with cyclophosphamide. Furthermore, it states that the increase of pH decreases the removal of Cyc (92% vs 58%), possibly because the scavenging effect becomes more pronounced. However, studies like Lin, et al. (2015) verified the opposite. This concludes that the relationship between cyclophosphamide's degradation and pH is very dependent on the water matrix in which it is analysed.

Again, Kovalova, et al. (2013) proved that the increase of the ozone dose potentiates the degradation. In this case, removal rates were of 33% and 57%, using O_3 doses of 0.64 and 1.08 $mgO_3\cdot mg_{DOC}^{-1}$, respectively. However, cyclophosphamide's concentration was very low ($1.85x10^{-4}$ $mg\cdot L^{-1}$).

In Lin, et al. (2015), the effects of pH on the removal were analysed. pH values ranged from 5.6 to 11, and the corresponding ozone flow was between 6 and 0 $mg\cdot L^{-1}$. The concentration of cyclophosphamide is very high (5 $mg\cdot L^{-1}$), when compared to other studies. These researchers concluded that, when pH increased, the degradation of Cyc was favoured. Therefore, for pH = 11, full removal was achieved. While the O_3 dose is acceptable, the pH of a typical wastewater is 7, which means that the removal in those conditions would not be complete. At the lowest pH value, the removal rate was 61.2%, which, for Cyc, is a good result. The fact that ozone was fed continuously instead of spiked can help justify these positive results.

Li, et al. (2016) obtained a 70% removal rate, using an ozone dose of 1 $mgO_3 \cdot mg_{DOC}^{-1}$, which is the highest recommended dose. In the case of this study, it corresponds to 5 $mg \cdot L^{-1}$ O_3.

Ferre-Aracil, et al. (2016), achieved almost complete removal of cyclophosphamide for the studied conditions. The ozone concentrations used were 45, 55 and 70 $mg \cdot L^{-1}$, in continuous flow. These concentrations are very high, and, being continuously fed to the reactor, explain the achieved removal rates of 97%, 99% and 100%, respectively. The kinetics of Cyc's removal were also studied, with a calculated $k(O_3)$ = 34.7 $M^{-1} \cdot s^{-1}$, which is one order of magnitude above the kinetic constants obtained by Garcia-Ac, et al. (2010) and Lester, et al. (2011), and one order of magnitude below the value obtained by Chen, et al. (2008).

Azuma, et al. (2019) analysed the efficiency of ozone in the removal of $5x10^{-4}$ $mg \cdot L^{-1}$ of both bicalutamide and cyclophosphamide.

Using only O_3, in continuous feed, at a rate of 1.0 $mg \cdot (min \cdot L)^{-1}$, removal rates were 42.9% for Bica, and 21.5% for Cyc, which is normal, due to their recalcitrance. This study also calculated reaction rates for these compounds, assuming a first-order kinetics, and obtained $k(Bica)$ = 0.037 min^{-1} and $k(Cyc)$ = 0.020 min^{-1}, which are extremely low values, indicating that the reactions are practically inexistent. Also, it is very uncommon to assume a first-order kinetics for ozonation. In fact, all of the aforementioned studies that calculate reaction rates use a second-order kinetics for the calculations. However, since O_3 is in continuous feed, the approximation is possible, since the concentration of ozone throughout the experiment remains constant.

Table 1 also includes ozone-based processes, that combine O_3 with H_2O_2 and/or UV.

Garcia-Ac, et al. (2010) and Lester, et al. (2011), for instance, analysed the process of O_3 combined with H_2O_2. Both studies concluded that the addition of hydrogen peroxide favours the removal of Cyc.

The reaction constant for the ozonation reaction including both the direct and indirect pathways is much higher than $k(O_3)$ (k = $2.0x10^9$ $M^{-1} \cdot s^{-1}$ vs $k(O_3)$ = 3.3 $M^{-1} \cdot s^{-1}$) (Garcia-Ac, et al., 2010).

This study also states that, to obtain a full removal of cyclophosphamide, even with the addition of H_2O_2, a high oxidant concentration and a prolonged contact time are fundamental. More specifically, 45 $mg \cdot (min \cdot L)^{-1}$ of ozone only achieved a removal of 96%.

Lester, et al. (2011) also studied the combination of O_3 with UV and UV/H_2O_2. The results are mostly qualitative, and state that the addition of just hydrogen peroxide provides the highest removal rates, followed by UV/H_2O_2 and, lastly, just UV. All these combinations, however, are more effective in removing Cyc than just O_3.

Azuma, et al. (2019) tested all three combinations (O_3/ H_2O_2, O_3/UV and O_3/UV/H_2O_2), and also all ozone-based processes associated with ozone micro-bubbles (MB).

The conclusions drawn from this study are not in agreement with the results extrapolated from Lester, et al. (2011) and Garcia-Ac, et al. (2010). In fact, these last two studies concluded that the addition of UV and/or hydrogen peroxide always improved removals when compared to ozone alone. However, Azuma, et al. (2019) obtained the highest removals for O_3/UV, for bicalutamide, and just O_3, for cyclophosphamide. Even so, the removal rates obtained were very low (42.9% for Bica, and 21.5% for Cyc), considering that ozone is fed continuously to the reactor.

Furthermore, this study presents the highest concentration of hydrogen peroxide ([H_2O_2] = 5 $mg \cdot L^{-1}$), which might be enough to drastically increase the removal of the anticancer drugs. As a comparison, in Lester, et al. (2011), only 2 $mg \cdot L^{-1}$ of H_2O_2 are used, and it is sufficient to provide higher removal rates than simple ozonation. Moreover, the addition of microbubbles proved to be useful for the degradation of bicalutamide, achieving almost complete removal (98.9%), when combined with H_2O_2 and UV radiation. For cyclophosphamide, however, microbubbles were not effective, only increasing its degradation from 21.5% (with simple ozonation) to 32% (for ozonation in MB combined with H_2O_2 and UV radiation).

Nevertheless, it is also stated that the consumption of ozone is 2.8 times superior when microbubbles are used, which leads to conclude that, especially for cyclophosphamide, their addition may not prove useful.

As it was mentioned in the first chapter, molecular ozone tends to be more reactive with compounds that possess aromatic systems, double bonds and non-protonated amines. Since cyclophosphamide presents only one double bond, and does not have any aromatic rings, this can justify the low removal rates that are obtained, and confirm that its degradation should, in fact, be more effective through indirect ozonation.

After this extensive analysis, it becomes clear that cyclophosphamide, in most of the studied conditions, cannot, yet, be fully degraded, and given the reduced number of existing studies, most drugs have not yet been analysed regarding their degradation. These compounds will, then, end up in drinking water and surface waters, which proves that this subject is a current issue that needs to be addressed with urgency.

To the best of my knowledge, so far, there aren't any studies regarding the degradation of MPA or MMF, whether by ozonation or another AOP.

The studies that analyse cyclophosphamide often state ozone's inability to fully degrade this compound, and the same can be verified for bicalutamide.

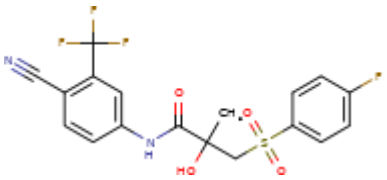
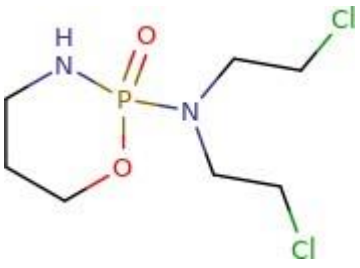
So, in the face of these limitations, and the lack of studies for the other compounds, this dissertation might provide some insight into the degradation of these anticancer drugs, analysing if ozone is, in fact, effective in removing these drugs.

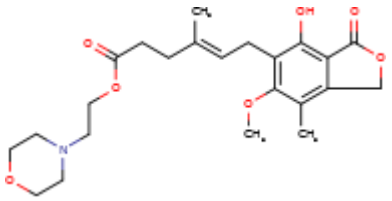
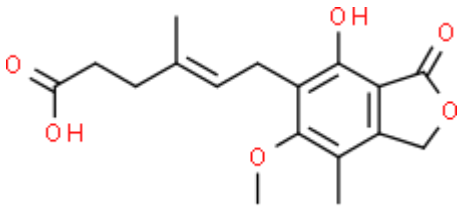
3 Materials and Methods

3.1 Reagents

Table 2 indicates the molecular structures and molecular weights for the studied anticancer drugs, specifically cyclophosphamide, bicalutamide, mycophenolic acid and mycophenolate mofetil.

Table 2: Molecular structure and molecular weight of the anticancer drugs.

Chemical name	Molecular structure	Molecular weight ($g \cdot mol^{-1}$)
Bicalutamide $C_{18}H_{14}F_4N_2O_4S$ (U.S. National Library of Medicine, 2019)		430.3766
Cyclophosphamide $C_7H_{15}Cl_2N_2O_2P$ (U.S. National Library of Medicine, 2019)		261.083

Chemical name	Molecular structure	Molecular weight ($g \cdot mol^{-1}$)
Mycophenolate Mofetil $C_{23}H_{31}NO_7$ (U.S. National Library of Medicine, 2019)		433.498
Mycophenolic Acid $C_{17}H_{20}O_6$ (U.S. National Library of Medicine, 2019)		320.339

Solutions of anticancer drugs were prepared from a methanol-based stock solution of 100 $mg \cdot L^{-1}$ of each compound (bicalutamide, cyclophosphamide, mycophenolic acid and mycophenolate mofetil). Stock solutions were acquired from Sigma Aldrich, with a purity of 98%. To prepare these working solutions, a specific volume of each drug stock solution (100 $mg \cdot L^{-1}$) was transferred to a dark-coloured flask. This flask was then exposed to the environment, to evaporate the methanol. Then, ultrapure water was added to the flask to obtain the desired concentration of each compound. Ultrapure water (18 MΩ) used in all experiments was obtained from a Milli Q system.

Tert-butanol, acquired from VWR Chemiclals, purity of 100%, was used as an $HO^{\bullet}$ radical scavenger

Indigo stock solution was prepared by adding 500 mL of ultrapure water in a 1 L volumetric flask, alongside 1 mL of concentrated phosphoric acid and 770 mg of potassium indigo

trisulfonate ($C_{10}H_7N_2O_{11}S_3K_3$) and completing with ultrapure water to the mark. Indigo reagent II solutions were prepared from the mother-solution, by diluting 100 mL of it in a 1 L volumetric flask, adding 10 g of sodium dihydrogen phosphate (NaH_2PO_4) and 7 mL of concentrated phosphoric acid and diluting to the mark. This solution was discarded when the absorbance at 600 nm was below 80% of the initial absorbance, and was used to analyse the concentration of ozone, through the standard indigo colorimetric method (Bader, 1982).

A solution of ascorbic acid, acquired from Riedel-de Haën, was used to stop the ozonation reaction. For that purpose, 50 mg of ascorbic acid (purity > 99%) was added to a 10 mL volumetric flask, which was, then, completed with ultrapure water, to achieve a stock solution of 5 $g \cdot L^{-1}$. 5 µL of ascorbic acid was added to 500 µL samples, to consume any remaining ozone.

A stock solution of 0.1 M ammonium acetate, using the reagent with a purity of 98% acquired from EMSURE, was prepared by adding 787 mg to a 100 mL volumetric flask and completing with ultrapure water. Ammonium acetate was tested as a buffer for the ozonation solutions.

Sodium hydroxide (4.7 $g \cdot L^{-1}$) and acetic acid (4.96 M) were acquired from PRONALAB and TitriPUR, respectively, and used to adjust the pH of the ultrapure water for the experiments.

LC-MS level methanol and water, acquired from J. T. Baker, were used as eluents for the mobile phase of LC-MS. Formic acid (HCOOH) was also employed, to acidify these eluents.

3.2 Ozonation experiments

To generate O_3, an ozone generator was used with a stream of O_2 connected to it, regulated by a valve.

To obtain ozone dissolved in water, a glass containing ultrapure water and a magnetic bar was placed in a magnetic plate, set to 200 rpm and 30 ºC. Using a perforated cap, two tubes were inserted, one making the connection with the O_3 generator and the diffuser, and another one, closed with a clamp, to allow the collection of samples through a 10 mL syringe. Figure 1 provides a schematic example of the experimental set-up.

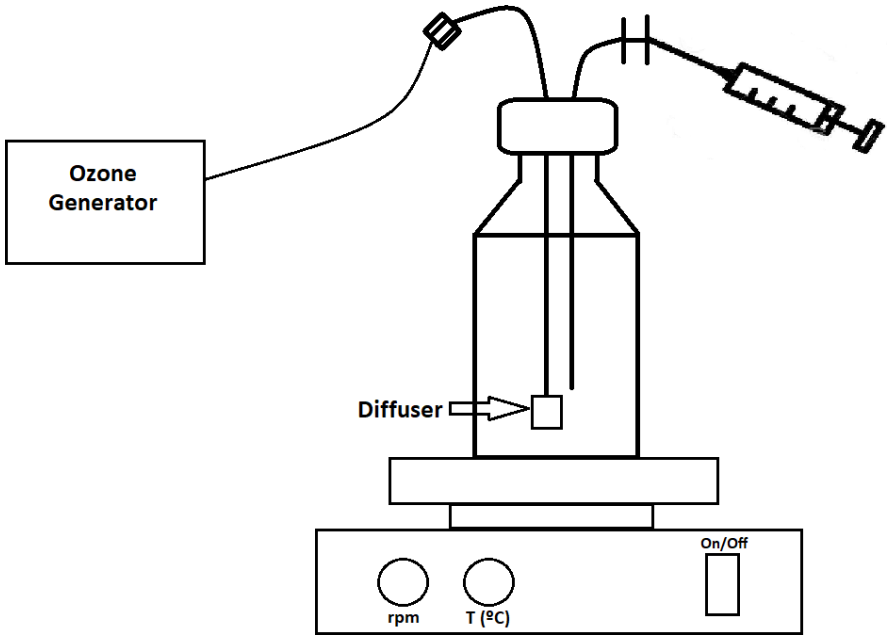


Figure 1: Experimental set-up for ozonation experiments.

3.2.1 Ozonation experiments with anticancer drugs

For the ozonation experiments, ozone was bubbled into the flask containing ultrapure water, until the solution became saturated. Samples were collected and analysed in the spectrophotometer after reaction with indigo reagent (section 3.3.1), to ensure that the concentration of dissolved O_3 was stable.

Simultaneously, aqueous solutions of anticancer drugs were prepared and placed in magnetic plates.

Then, a certain volume of ozonated water was collected and spiked into the anticancer drugs' aqueous solutions, depending on the concentration of O_3 desired.

3.2.2 Study of ozone degradation

The goal of these experiments was to study the stability of dissolved ozone in water over time.

For the analysis of the ozone degradation, ozone was bubbled similarly to the other experiments, until it reached a concentration close to the desired one (5 $mg \cdot L^{-1}$). Then, the generator was disconnected, and the experiment began.

3.3 Analytical methods

3.3.1 Analytical method for ozone measurement

The absorbance of ozonated solutions (after adding indigo) was measured at 600 nm by a Jasco V-530 UV/VIS spectrophotometer, using a 1 cm quartz cell, and recurring to the standard indigo colorimetric method (Bader, 1982). First, a blank solution containing only 1 mL of the indigo solution and ultrapure water was prepared in a 10 mL volumetric flask and measured (blank). Then, a similar solution was prepared, with the addition of a determined volume of the ozone solution, and measured as well. Equation 6 establishes the formula to calculate the concentration of ozone using absorbance values.

$$C(O_3) = \frac{\Delta Abs * V_T}{\varepsilon_b * l * V_S} \quad (6)$$

In the formula, V_T refers to the total volume of the solution (10 mL) and V_S is the volume of the ozone solution taken from the reactor (0.05 mL). ΔAbs defines the difference between the absorbance of the blank solution and the indigo-ozone solutions. Finally, l represents the optical length of the quartz cell (1 cm) and ε_b is the mass absorptivity (0.42 $L \cdot (mg \cdot cm)^{-1}$).

Sample size was hard to determine. At first, 9 mL were collected for each sample, but then it became clear that, with such a large volume, 1 mL of indigo was not sufficient to react with all of the dissolved ozone, and the results were compromised. Then, a new sample volume of 50 µL was defined, and it seemed to achieve the correct results.

Samples of 50 µL were then collected at the allotted intervals of 1 minute until 10 minutes, and then 15, 20, 25 and 30 minutes.

3.3.2 Analysis of the target anticancer drugs by liquid chromatography - tandem mass spectrometry

Analyses of anticancer drugs' concentrations were performed using a LCMS-8040 liquid chromatography-tandem mass spectrometry system (LC-MS/MS). The mobile phase used was a binary mix of J. T. Baker Analysed LC-MS Reagent methanol (B) and water (A), both with 0.1% formic acid (HCOOH).

The equipment included a Luna C-18 column (150 x 2 mmID, particle size 5 µm; acquired from Pnemonenex). The flowrate used was 0.2 $mL \cdot min^{-1}$, for a 50 minutes run.

As for the gradient, initially it corresponded to 5% of B. After 15 minutes, it increased to 20% of B. On minute 30, gradient was 45% of B, and it reached 100% of B at the end of 39 minutes.

This maximum was maintained for 2 minutes, after which it returned to the initial conditions, in 4 minutes.

The sample injection volume was 5 µL, and the linearity range, based on the linear regressions performed, was from 1 $\mu g \cdot L^{-1}$ to 600 $\mu g \cdot L^{-1}$.

This method of analysis was already implemented in the laboratory, and all the experimental data was provided for the analysis of the anticancer drugs.

In order to obtain the calibration curve for each compound, 8 standards containing a mixture of the target anticancer drugs were prepared (1 $\mu g \cdot L^{-1}$ to 600 $\mu g \cdot L^{-1}$).

To prepare these solutions, firstly, an aqueous stock solution (10 $mg \cdot L^{-1}$) was prepared, containing all four compounds.

Then, this standard-solution was used as basis for the 3 samples of higher concentration (200, 400 and 600 $\mu g \cdot L^{-1}$), where 10, 20, 30 µL, respectively, were added to the vials, and then completed with water to reach 0.5 mL. To prepare samples of 25, 50 and 100 $\mu g \cdot L^{-1}$, the standard-solution could not be used, since the required volumes were too low to be properly measured. Therefore, these standards were prepared from a 1000 $\mu g \cdot L^{-1}$ aqueous solution containing all anticancer drugs. Required volumes were 12.5, 25 and 50 µL. Similarly, 50 µL of the 100 $\mu g \cdot L^{-1}$ solution were added to prepare a sample of 10 $\mu g \cdot L^{-1}$, and, finally, 10 µL of the 50 $\mu g \cdot L^{-1}$ solution originated the sample of 1 $\mu g \cdot L^{-1}$.

4 Results and Discussion

4.1 Analytical methodology for the analysis of anticancer drugs in water

The identification and quantification of the target anticancer drugs in water was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The analysis of the four anticancer drugs was performed in ESI (+), using the two highest intensity transitions from $[M+H]^+$ precursor ion (Table 3). Figure 2 represents an example of a chromatogram obtained when a standard solution containing all anticancer drugs at 400 $\mu g \cdot L^{-1}$ is analysed. Eight standards with concentrations of each anticancer drug varying from 1 $\mu g \cdot L^{-1}$ to 600 $\mu g \cdot L^{-1}$ were analysed and the peaks integrated.

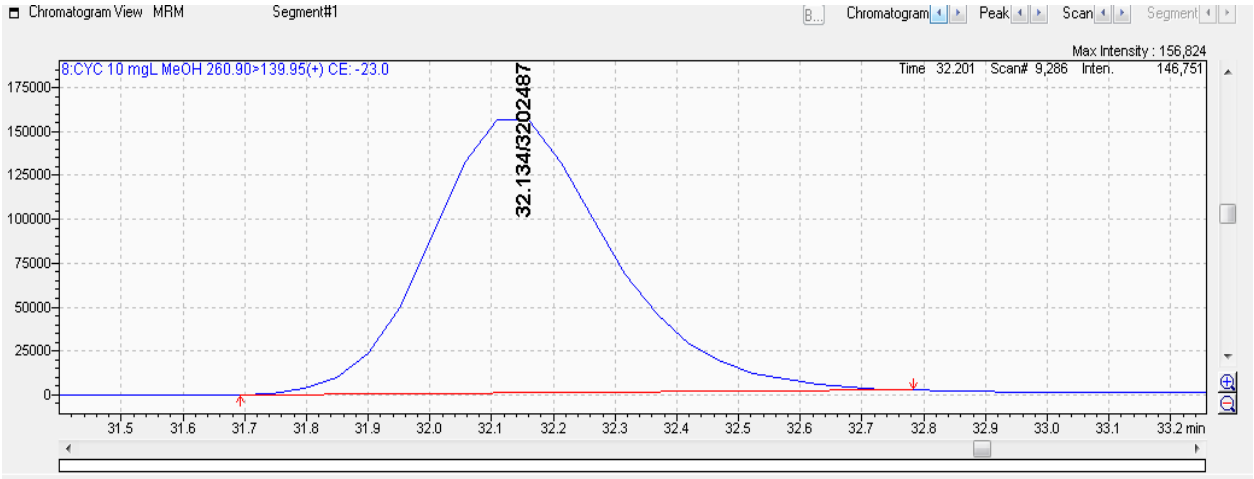


Figure 2: Chromatogram for a cyclophosphamide sample of 400 $\mu g \cdot L^{-1}$ after analysis in LC-MS/MS.

The linear regressions obtained between the instrumental responses and the concentrations of each anticancer drug are compiled in Table 3.

Table 3: Mass spectral information and quality parameters obtained for the analysis of each anticancer drug by LC-MS/MS.

Anticancer drug	Cyclophosphamide	Mycophenolic Acid	Mycophenolate Mofetil	Bicalutamide
Molecular ion (*m/z*)	260.90	321.00	434.10	429.00
Transition 1 (CE, eV)	260.90 → 139.95 (23)	321.00 → 207.00 (23)	434.10 → 114.05 (27)	429.00 → 255.05 (16)
Transition 2 (CE, eV)	260.90 → 106.05 (19)	321.00 → 303.10 (10)	434.10 → 194.95 (36)	429.00 → 184.95 (39)
Retention time (min)	32.134	37.774	37.965	32.723
Linear regression equation	y = 7731x + 34904	y = 8114x + 14233	y = 31312x - 206590	y = 126072x + 929888
R^2	0.998	0.999	0.995	0.989

- CE – collision energy

4.2 Preliminary experiments

These preliminary experiments took place to determine the length of the ozonation reaction and the general effectiveness of ozonation in removing the model drug compounds. They had the duration of 60 minutes, using ozone doses of 0.5, 1 and 5 $mg \cdot L^{-1}$. In these initial experiments, the ozonation was studied using a 20 mL solution that contained the four anticancer drugs, each with a concentration of 1 $mg \cdot L^{-1}$. The experiment took place in transparent flasks, placed in a magnetic plate with a speed of 200 rpm. All flasks were duplicated, i.e., all experiments were made in duplicate. Ozone was spiked into each flask to achieve the desired initial concentration, and samples were collected at every 5 minutes until 30 minutes, and then at 45 and 60 minutes. Ascorbic acid was not added to the sample flasks to stop any possible homogeneous reaction before analysis. Furthermore, there was no pH adjustment performed, so experiments took place at pH ~ 7. Figures 3-5 depict the results obtained for the different initial ozone doses.

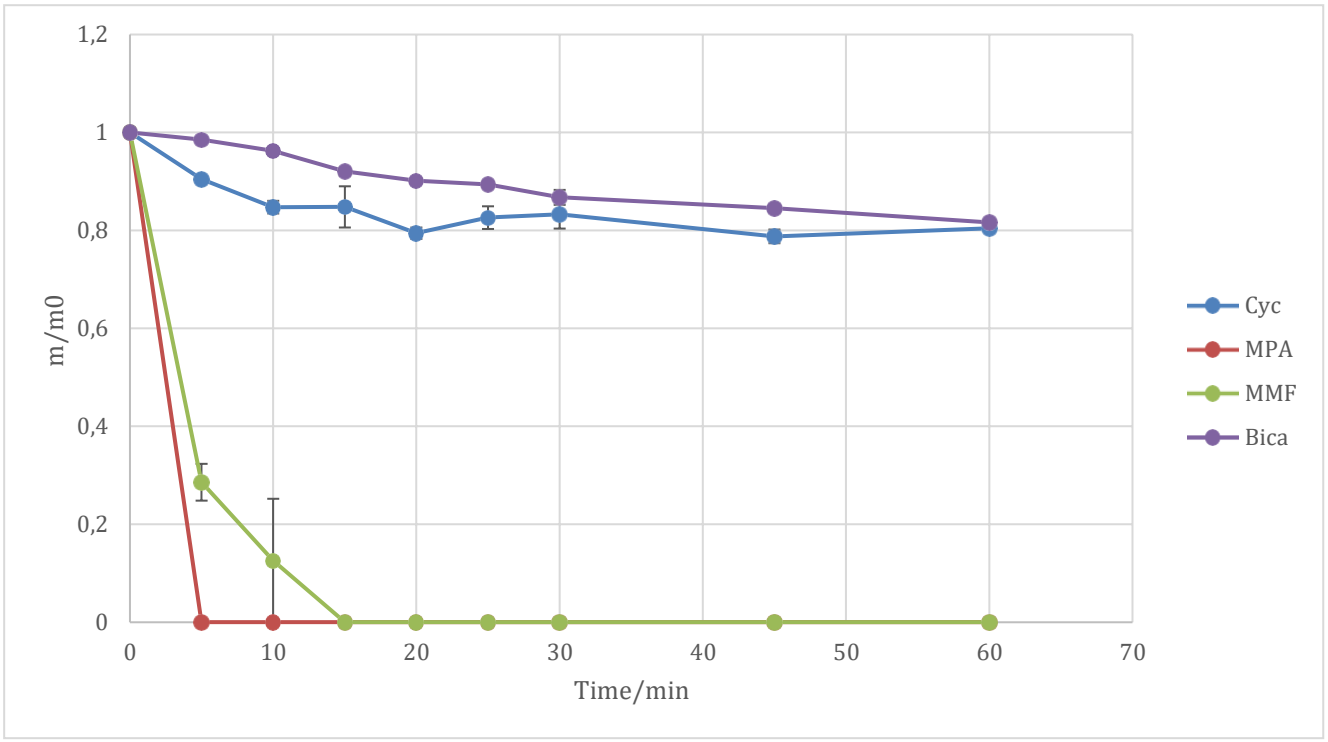


Figure 3 : Preliminary ozonation experiment, using 0.5 mg·L^{-1} of O_3 to degrade 1 mg·L^{-1} of each anticancer drug, T=30 ºC, at 200 rpm.

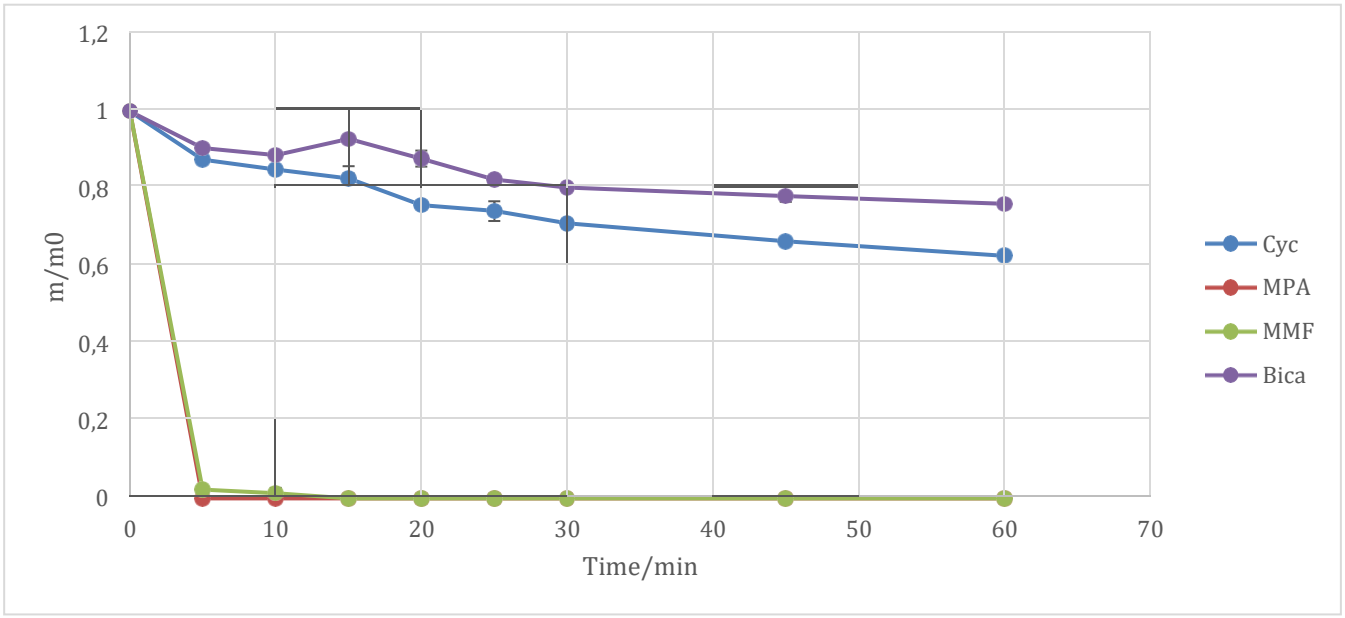


Figure 4: Preliminary ozonation experiment, using 1 mg·L^{-1} of O_3 to degrade 1 mg·L^{-1} of each anticancer drug, T=30 ºC, at 200 rpm.

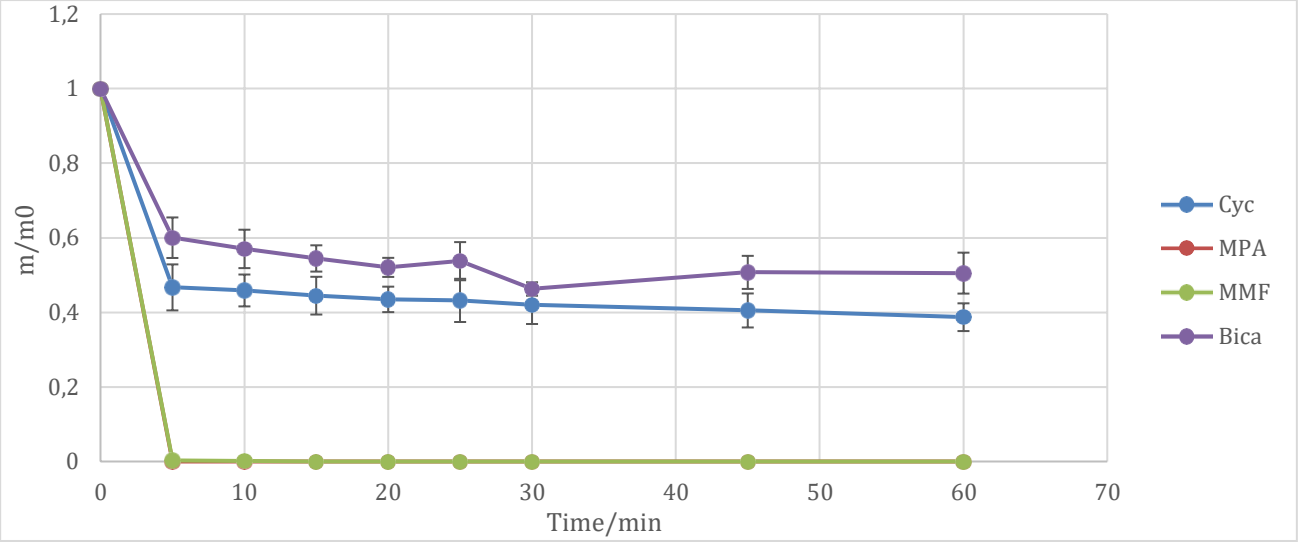


Figure 5: Preliminary ozonation experiment, using 5 mg·L^{-1} O_3 to degrade 1 mg·L^{-1} of each anticancer drug, T=30 ºC, at 200 rpm.

It must be stated that the values presented on the graphs represent the average values between each experiment and the corresponding duplicate, complemented with the respective error bars. It is noticeable that, in most cases, the error bars are reasonably small, which states that both experiments provided similar results.

As observed in Figures 3, 4 and 5, MPA and MMF were completely degraded after a few minutes of reaction (less than 5 min for MPA, while for MMF it was required 5 min for 1 and 5 mg.L^{-1} of O_3 and 15 min for 0.5 mg.L^{-1} O_3). It is clear that MPA and MMF are very easily degradable by ozone under these conditions. However, for Cyc and Bica, it is noticeable that the degradation was incomplete and their concentrations remained nearly constant after 30 minutes of reaction. Cyc and Bica prove to be far more recalcitrant. For the lowest ozone dose, only 20% of each compound is degraded during the 60 minutes reaction time. When the O_3 dose is increased to 1 mg·L^{-1}, Cyc removal increases to 40%, but Bica remains at 25%. Even for the highest ozone dose, their removal does not exceed 65% and 55%, respectively. This suggests that these two anticancer drugs are recalcitrant and that other conditions should be tested.

4.2.1 Ozone degradation throughout time

The first experiments showed that the reaction between the anticancer drugs and ozone happened in very short periods of time, especially for MPA and MMF. Therefore, it was decided that the experiments should be repeated, for a time span of 10 minutes, so that it would be possible to analyse the reaction samples in the first minutes.

The procedure was similar to the first experiment, but the length of the reaction was shortened to 10 minutes. Again, the reaction was not stopped in the sample flasks.

In this case, four ozone doses were tested:

1. 0.5 $mg \cdot L^{-1}$;
2. 1 $mg \cdot L^{-1}$;
3. 5 $mg \cdot L^{-1}$;
4. 5 $mg \cdot L^{-1}$, wherein two spikes were added: the first at t=0, and the second one after the allotted 10 minutes of the experiment, which was extended up to 20 min.

This fourth experiment had the goal of trying to maximise the effectiveness of the degradation, hoping to improve the degradation of the most recalcitrant compounds, i.e., cyclophosphamide and bicalutamide. Figures 6-9 present the results obtained.

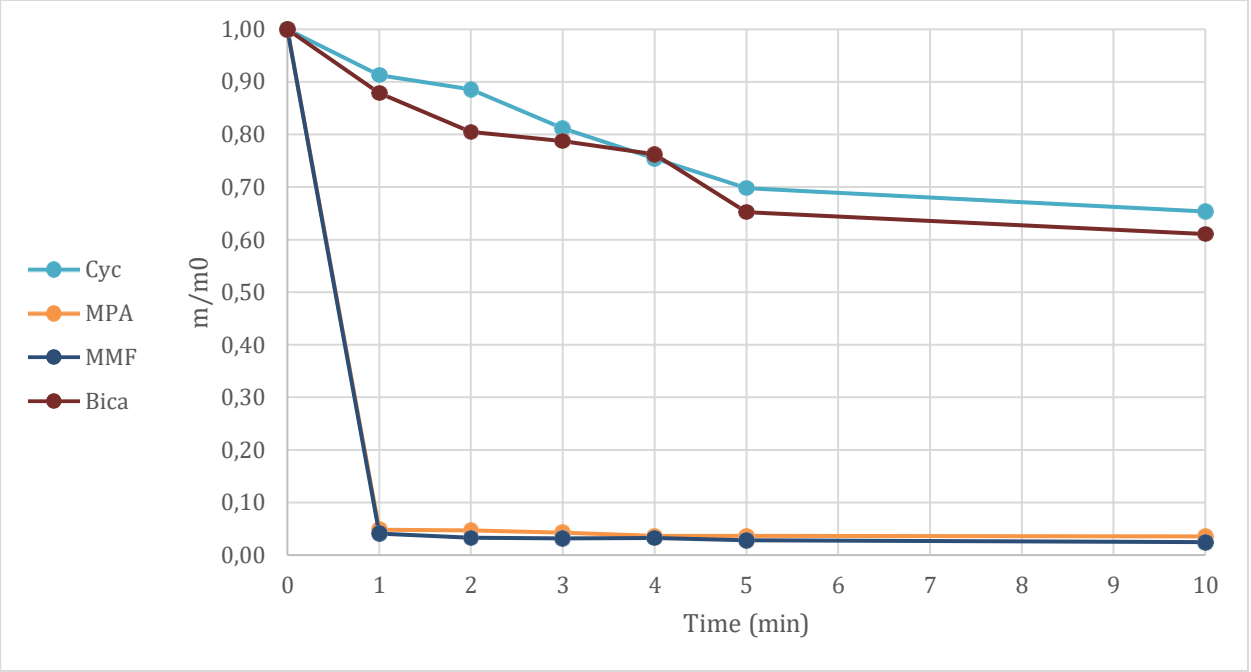


Figure 6: Preliminary ozonation experiment 2, using 0.5 $mg \cdot L^{-1}$ of O_3 to degrade 1 $mg \cdot L^{-1}$ of each anticancer drug, *T=30 ºC, at 200 rpm.*

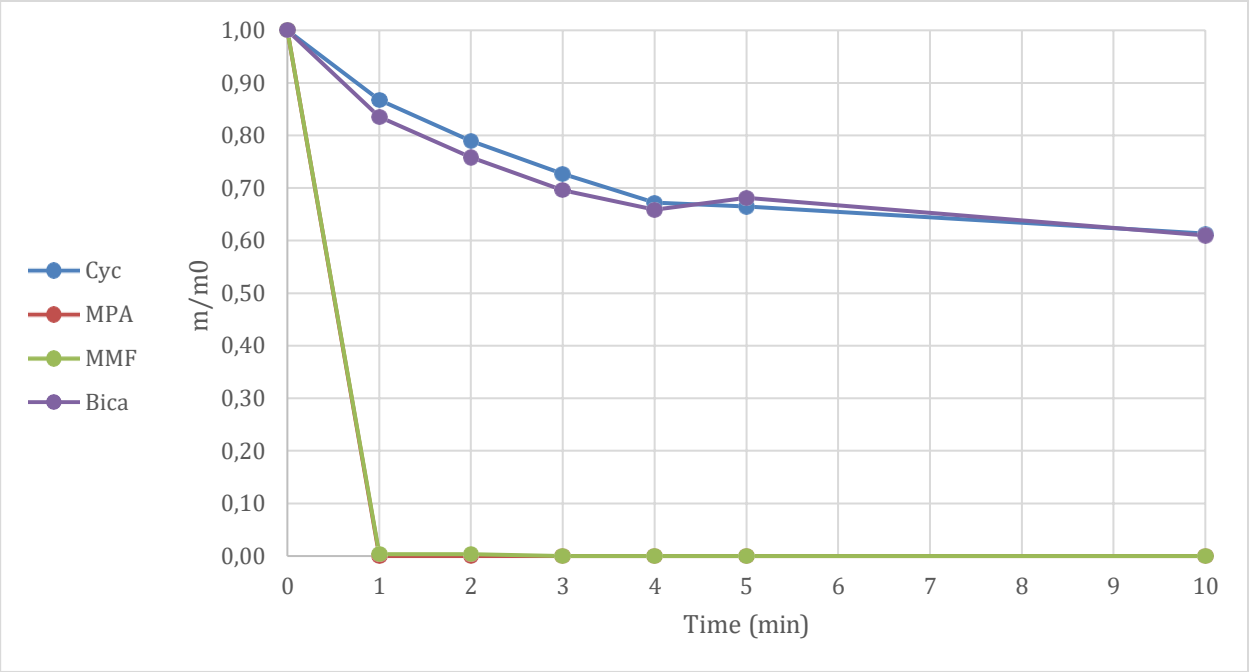


Figure 7: Preliminary ozonation experiment 2, using 1 mg·L^{-1} of O_3 to degrade 1 mg·L^{-1} of each anticancer drug, *T=30 ºC, at 200 rpm.*

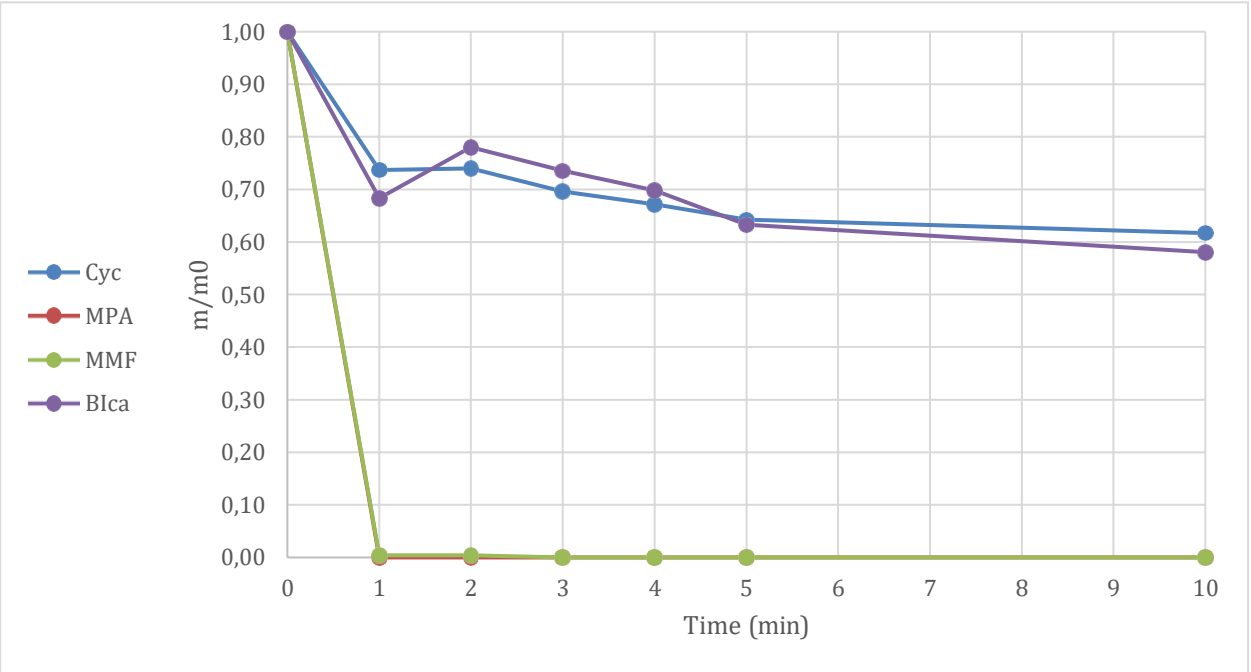


Figure 8: Preliminary ozonation experiment 2, using 5 mg·L^{-1} O_3 to degrade 1 mg·L^{-1} of each anticancer drug, *T=30 ºC, at 200 rpm.*

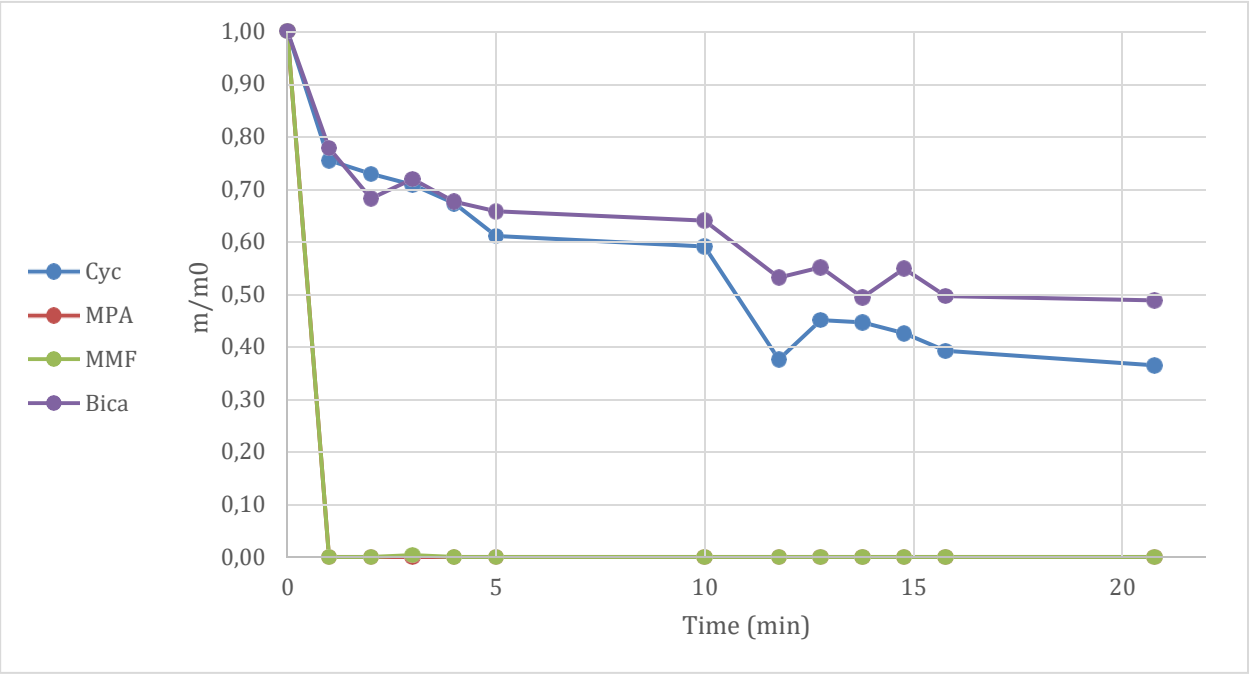


Figure 9: Preliminary ozonation experiment 2, using 2 spikes of 5 mg·L^{-1} of O_3 each (at t = 0 and t = 10 min) to degrade 1 mg·L^{-1} of each anticancer drug, *T=30 ºC, at 200 rpm.*

It is noticeable that the degradation of MMF and MPA always occurs during the first minute(s) of the experiments, either suggesting that the removal is extremely fast or that the reaction continues in the sample flasks.

The results for Cyc and Bica, on the other hand, seem to suggest that degradation continues for 5 minutes, and then stabilises. This information can be correlated to what is shown on figure 10 where two slopes are visible, at minutes 0-5 and 10-15 (after each spike), followed by two stable concentration levels (plateaus) .

The experiment with two spikes of ozone proved effective in increasing both Bica and Cyc's removal. In fact, the removal rate of bicalutamide increased from 36% to 51%, with the addition of the second spike. As for cyclophosphamide, figure 10 shows an increase from 41% to 64%, which, for recalcitrant compounds, can be perceived as an improvement.

The analysis of these results did not provide any insight into whether ozone is stable in ultrapure water, i.e., whether the ozonation reaction would still occur in the samples flasks. Therefore, further tests were performed, which aimed to analyse the degradation of O_3 in ultrapure water.

Furthermore, there was the concern that, by not stopping the ozonation reactions, these could continue in the sample flasks, affecting the veracity of the results. Therefore, tests were performed to determine a suitable agent that could be added to the sample flasks, and that would consume any remaining ozone.

The choice of a reagent to stop the ozonation reaction was made after several attempts. First, indigo was tested, since it is already used for the colorimetric method. Unfortunately, it was discovered that indigo could not be used in LC-MS, for similar reasons to ammonium phosphate. Finally, ascorbic acid was chosen. Testing included adding 100 µL of a 5 $g \cdot L^{-1}$ solution of this acid to a volumetric flask containing 50 µL of a saturated ozonated solution, and then adding 1 mL of indigo reagent II solution, completing with ultrapure water and comparing it to the blank solution containing 1 mL of indigo reagent II solution and completed with ultrapure water. Absorbance values had to be identical, to prove that ascorbic acid had reacted with the ozone.

5 µL of a 5 $g \cdot L^{-1}$ solution were, thereafter, added to all sample flasks, to remove residual O_3 and quench reactive species, such as $^{\bullet}OH$. These volumes were defined in proportion, i.e., if 100 µL of ascorbic acid are capable of reacting with 20 $mg \cdot L^{-1}$ of ozone (which was the average saturated concentration of ozone obtained), then 5 µL added to the samples should be enough to consume residual ozone, that, when measured at the end of the experiment, was always below 1 $mg \cdot L^{-1}$.

4.3 Ozone decomposition in ultrapure water

After performing the preliminary experiments, the stability of ozone when dissolved in ultrapure water was questioned, and experiments were planned for its analysis, at different values of pH (acidic, neutral and alkaline).

For these experiments, ozone was bubbled as usual, into a flask. However, in these experiments the ozonated solution was not saturated. In fact, the concentration of ozone was periodically analysed, and the generator was disconnected when its value reached approximately 5 $mg \cdot L^{-1}$. The experiments were performed in the same flask where ozone saturation occurred.To control the pH of the solution, a buffer of ammonium acetate was tested. Literature suggested a phosphate buffer, but it would generate issues on the LC-MS apparatus, since phosphate is a salt, and it could precipitate and compromise the equipment. Ammonium acetate was set to be used at 0.001 M, in order to maintain pH = 7. The measurement of pH proved its effectiveness in buffering the solution.

Further tests were performed with this buffer, to assure that it would not affect the results of the experiments. However, when the degradation of ozone in ultrapure water with ammonium acetate was tested, it was perceived that, since the buffer is an organic compound, ozone reacted with it (data not shown).

After determining that ammonium acetate could not be used as buffer, it was decided that the pH could simply be controlled by adding a small amount of diluted solutions of NaOH, at 4.7

$g \cdot L^{-1}$ or HCl, at 4.96 M. The pH of the ultrapure water was measured before any experiment, and then adjusted to the desired value. After each experiment, the pH of the water was measured again. It was, then, compared to the pH of the adjusted ultrapure water that was not used in any experiment. This served to analyse whether variations would be due to the experiments, or just caused by variations of the analyser's signal.

This study was performed for a variety of conditions:

1. Ultrapure water, pH=7 (adjusted with NaOH);
2. Ultrapure water, pH=7 (adjusted with NaOH) and *tert*-butanol at 0.025 M;
3. Ultrapure water, pH=3 (adjusted with HCl);
4. Ultrapure water, pH=3 (adjusted with HCl) and *tert*-butanol at 0.025 M;
5. Ultrapure water, pH=11 (adjusted with NaOH);
6. Ultrapure water, pH=11 (adjusted with NaOH) and *tert*-butanol at 0.025 M.

All remaining variables were kept constant, i.e., T=30 ºC and ω=200 rpm. These samples were then analysed on the spectrophotometer.

4.3.1 Ozone decomposition at neutral pH

Figure 10 presents the results for the experiment's that analyse ozone's decomposition at neutral pH.

Figure 10: Ozone decomposition in ultrapure water: pH=7.2 and pH=6.5, with and without tert-butanol (0.025 M), respectively. T=30 ºC and 200 rpm.

Initial concentrations correspond to 9.52 mg·L^{-1} and 9.05 mg·L^{-1} of ozone, respectively, for the experiments with and without *tert*-butanol.

The subsequent decrease can be explained by the system trying to achieve an equilibrium. Therefore, figure 10 shows that there is a decrease in ozone concentration throughout the experiment, from 10.48 mg·L^{-1} to 5.71 mg·L^{-1}, in the experiment with *tert*-butanol, and from 9.52 mg·L^{-1} to 5.24 mg·L^{-1}, in the experiment without *tert*-butanol. After this decrease, dissolved ozone concentration appears to remain stable for the rest of the experiment's duration.

It can be concluded, then, that ozone is not completely stable in ultrapure water, despite the absence of organic matter. In fact, this instability can be related to equations (1) to (5), where the reactions of ozone with OH^-, that is naturally present in water, can be observed. Since pH is neutral, in this case, there is a significant amount of OH^- species that are available to react with ozone.

4.3.2 Ozone decomposition in acidic conditions

Figure 11 represents the variation of ozone's concentration with time, for acidic pH values.

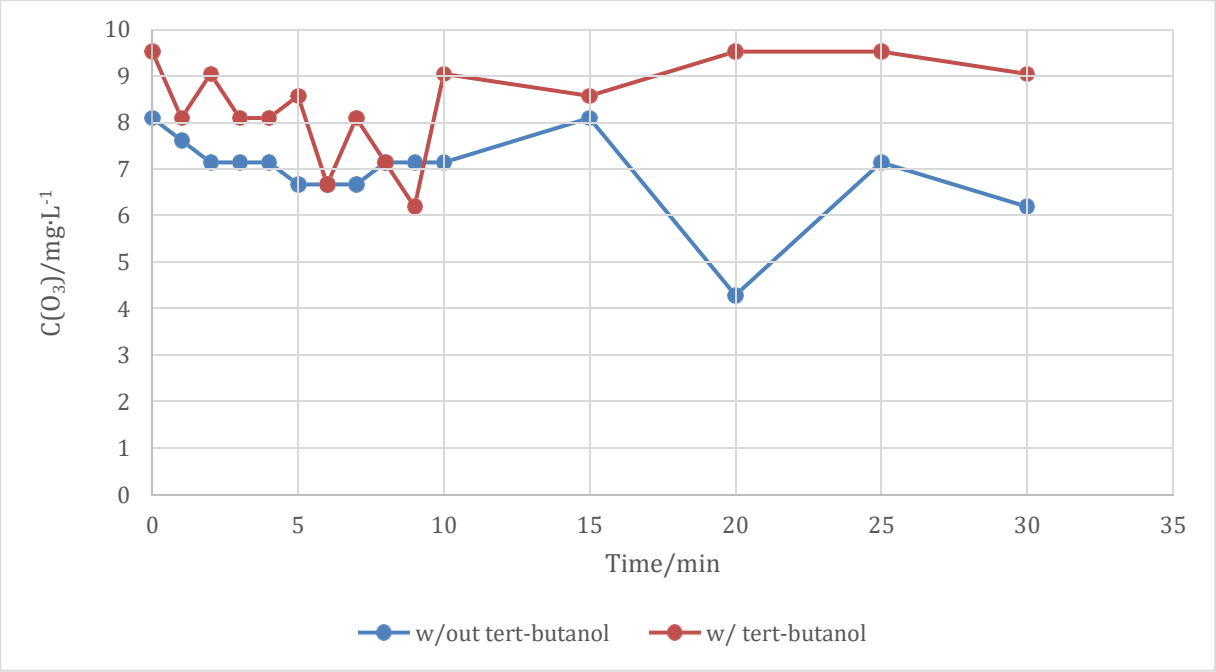


Figure 11: Ozone decomposition in ultrapure water: pH=3.0 and pH=3.1, with and without tert-butanol (0.025 M), respectively. T=30 ºC and 200 rpm.

Initial concentrations of ozone were 9.52 mg·L^{-1} and 8.10 mg·L^{-1} for the experiments with and without *tert*-butanol, respectively.

Unlike what was observed on figure 10, in the experiments represented in figure 11, ozone seems to maintain some stability in ultrapure water. In fact, from the maximum concentration observed for the experiment with *tert*-butanol, that is 9.52 mg·L^{-1}, to the final concentration, that corresponds to 9.05 mg·L^{-1}, variation is minimal. For the experiment without *tert*-butanol, variation occurred between 8.10 mg·L^{-1} and 6.19 mg·L^{-1}. In both cases, these variations are significantly smaller than those observed for the experiments at neutral pH. In fact, since hydroxyl ions are practically inexistent in acidic pH values, this can justify the higher observed stability.

4.3.3 Ozone decomposition in alkaline conditions

The study of ozone degradation in ultrapure water could not be performed for pH = 11, since, at this level of alkalinity, practically all dissolved molecular ozone is converted into ·OH radicals, thus rendering impossible the task of determining the ozone concentration at any point of the experiment.

4.4 Degradation of anticancer drugs by ozonation

4.4.1 Degradation of each anticancer drug by ozone

The degradation experiments were performed for each drug individually. All experiments were performed in 20 mL of ultrapure water, with anticancer drug initial concentration of 100 µM. The flasks were placed on magnetic plates rotating at 200 rpm, with temperature set to 30ºC. The conditions employed were as follows:

- For MPA and MMF, pH was neutral;
- For bicalutamide and cyclophosphamide, degradation was tested for acidic, neutral and basic pH values (pH=3, 7, 11, respectively).

It was decided that it was not necessary to test the removal of MPA and MMF at different pH values since, based on the preliminary experiments, it was expected that these drugs would be completely removed at pH=7. Since bicalutamide and cyclophosphamide are more recalcitrant, testing their removal at acidic and alkaline pH values could provide interesting results, since it is expected that the removal rates suffer great variations with the pH change, considering that the concentration of ·OH radicals is also being altered.

Ozone was spiked into the solution to obtain 5 $mg \cdot L^{-1}$ for Cyc and Bica, and 1 $mg \cdot L^{-1}$ for MPA and MMF. In this case, the decrease is justified by knowing that 1 $mg \cdot L^{-1}$ of O_3 should be enough to completely remove MPA and MMF, and thus there is no need of using a higher concentrate

ion of ozone. Samples of 500 µL were collected at the allotted intervals of 1 min until 10 min, and then 15, 20, 25 and 30 minutes. These samples were placed in vials that already contained 5 µL of ascorbic acid, which was used to stop the ozonation reaction.

At the end of the experiment, a sample was also collected into a 10 mL volumetric flask with 1 mL of indigo reagent II solution, to determine its absorbance and, consequently, the amount of ozone remaining.

Since the solutions of each compound was prepared for 100 µM of each, the following graphs will be expressed in terms of n/n0.

Figures 12 and 13 present the degradation results for MPA and MMF, respectively, at pH = 7.1.

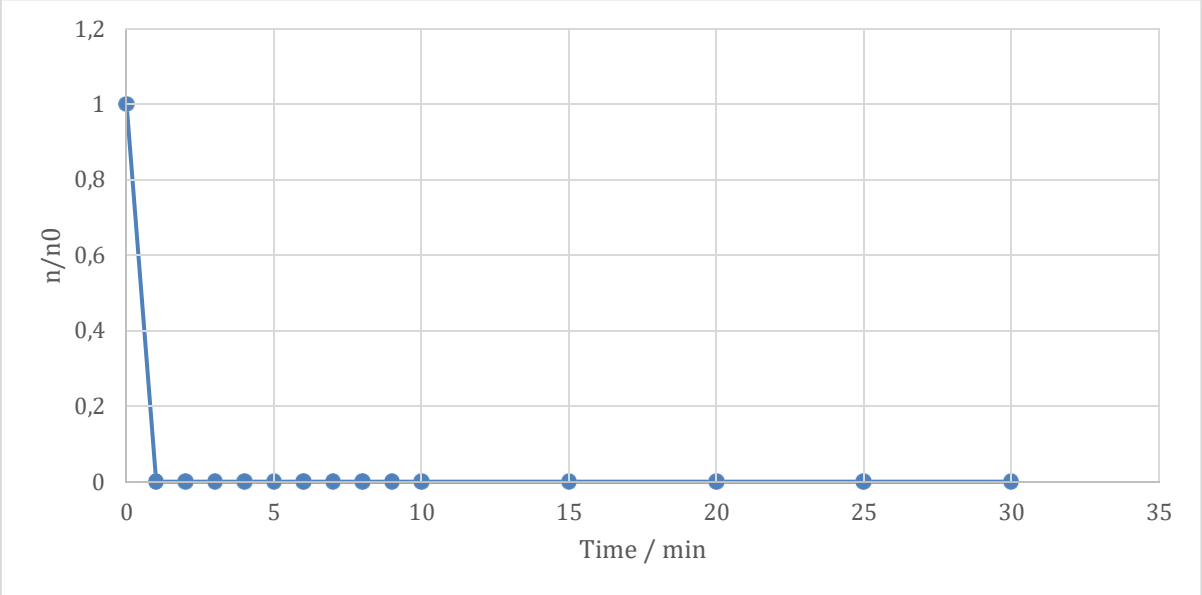


Figure 12: Degradation experiment for 100 µM MPA. $[O_3]_0$ = 1 $mg \cdot L^{-1}$, T=30 ºC, pH=7.1, at 200 rpm.

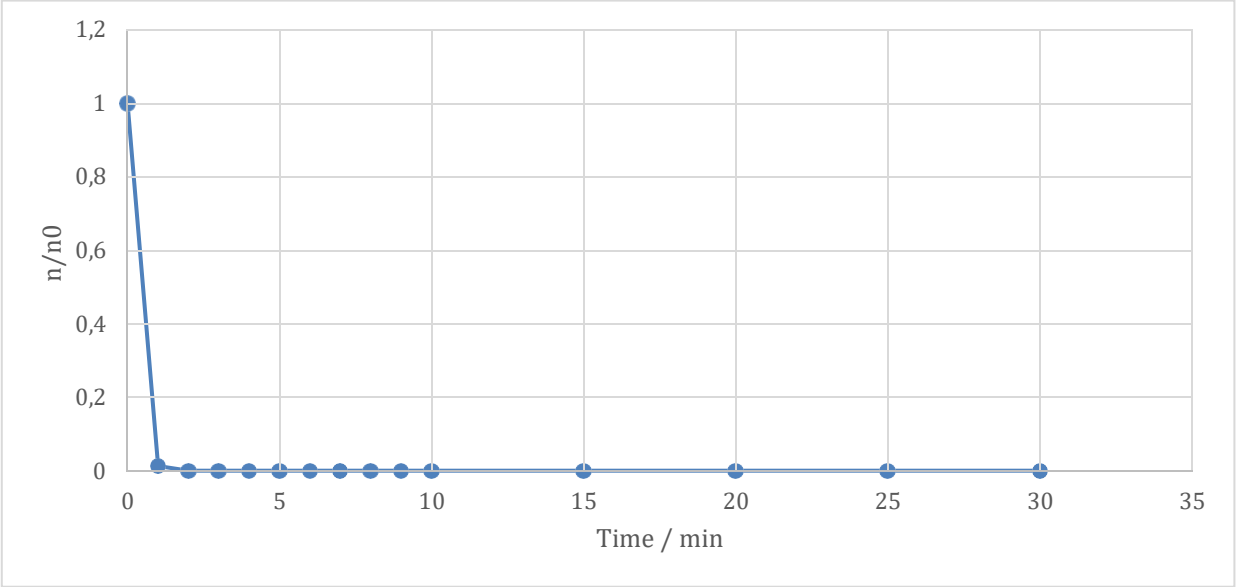


Figure 13: Degradation experiment for 100 µM MMF. $[O_3]_0$ = 1 mg·L^{-1}, T=30 ºC, pH=7.1, at 200 rpm.

As predicted, the removal of MPA and MMF is complete, and the reaction is finished after the first sample is collected (Figs. 12-13). In fact, as Table 2 shows, both of these drugs possess aromatic rings and several double bonds, which makes them highly susceptible to the attack of molecular ozone, thus justifying their very high removal rates.

On figures 14 and 15, the degradation of Bica and Cyc can be analysed, for all 3 pH conditions.

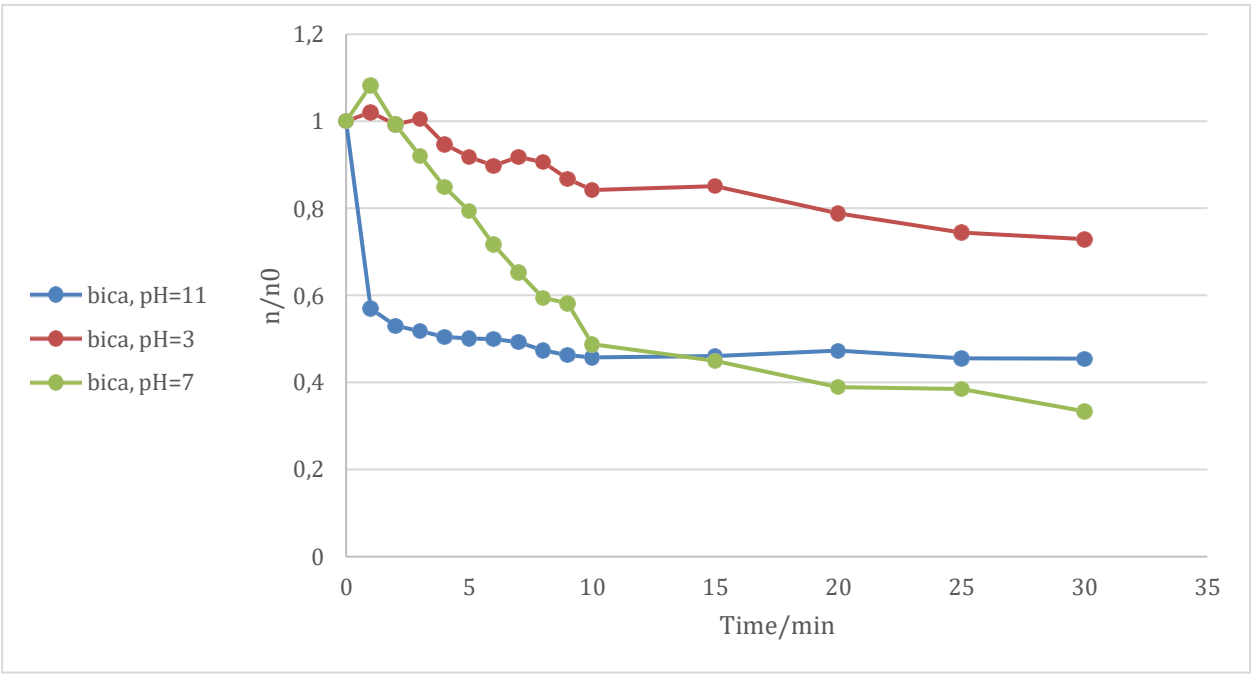


Figure 14: Degradation experiment for 100 µM bicalutamide. $[O_3]_0$ = 5 mg·L^{-1}, T=30 ºC, pH=10.9, pH=7.1 and pH=2.9, at 200 rpm.

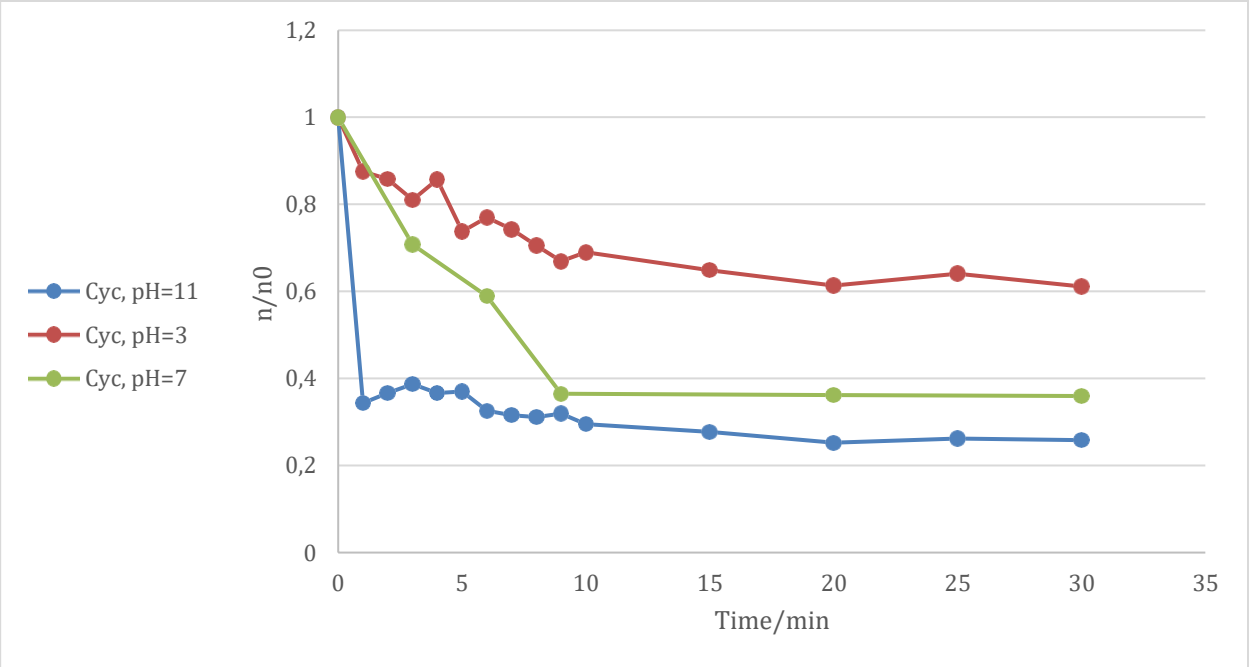


Figure 15: Degradation experiment for 100 µM cyclophosphamide. $[O_3]_0$ = 5 mg·L^{-1}, T=30 ºC, pH=10.9, pH=7.1 and pH=2.9, at 200 rpm.

As predicted previously, the increase of •OH radicals generated by the alkaline pH favours the removal of these compounds. In fact, for pH = 11, around 74.1% of Cyc was removed after 30 min (figure 13), which is higher than in any other experiment that was performed before. As for Bica, its degradation was lower when compared to Cyc, in all experiments at different pHs. However, its removal was still 54.5% at pH = 10.9 (figure 14), which is a satisfactory result since it is a recalcitrant drug. It must be mentioned, however, that there was no control experiment of each compound just in ultrapure water, without the addition of ozone. Therefore, it remains unknown whether, at pH = 11, the anticancer drugs could suffer hydrolysis, thus altering the results of the degradation.

These removal rates can be influenced by the pH of the ozonated water that was added to each solution. In fact, ozone was bubbled into ultrapure water at neutral pH, since it is not possible, as it was previously mentioned, to dissolve ozone in water at pH=11, without being transformed into OH radicals. Therefore, the spike of ozone slightly altered the pH of the solution of each anticancer drug, which may have affected the removal rates.

At lower pH values, the removal rates were lower. In fact, for Cyc, at pH = 7.1, the overall removal rate was 64%, and, at acidic pH, the value decreased to 38.9%. This indicates that, for this anticancer drug, all variations in the concentration of •OH radicals will impact, significantly, the removal of the compound. For Bica, this trend is even more notorious, with removal rates decreasing from 66.6% (pH 7.1) to 27.1% (pH 2.9) during the same 30 min of

reaction time. Since this compound is more recalcitrant than Cyc, it is expectable that the lower concentration of •OH radicals will have a deeper effect on this compound's removal.

It is unclear why the removal rates achieved for pH = 10.9 for Bica are lower than those obtained at pH = 7.1.

4.4.2 Kinetics of degradation of bicalutamide and cyclophosphamide in water by ozone

The following experiments were performed to ascertain the kinetics of the reaction between ozone and each individual anticancer drug.

Three identical experiments were performed for each compound, both in 20 mL of ultrapure water at pH=7.1 and anticancer drug concentration of 100 µM. The flasks were placed on magnetic plates rotating at 200 rpm, with temperature set to 30ºC.

Three experiments were performed for each compound:

1. Anticancer drug solution, 100 µM, 5 $mg \cdot L^{-1}$ of O_3;
2. Anticancer drug solution, 100 µM, with *tert*-butanol at 0.025 M, 5 $mg \cdot L^{-1}$ of O_3;
3. Anticancer drug solution, 100 µM, with *tert*-butanol at 0.025 M, no added ozone.

Tert-butanol was added to act as a scavenger for $^{\cdot}OH$ radicals. This allowed to analyse the difference in removal rates between each solution, thus ascertaining the influence of these radicals (and indirect ozonation) in the process. In the third experiment, no ozone was added. This experiment served to control whether *tert*-butanol would react with the compounds and affect the validity of the results.

Samples of 500 µL were collected at the allotted intervals of 1 minute until 10 minutes, and then 15, 20, 25 and 30 minutes. These samples were placed in vials that already contained 5 µL of ascorbic acid (5 $g \cdot L^{-1}$), that was used to stop the ozonation reaction. For the control experiment, samples were only collected at 0, 10, 20 and 30 minutes.

At the end of the experiment, a sample was also collected into a 10 mL volumetric flask with 1 mL of indigo reagent II solution, to determine its absorbance and, consequently, the amount of ozone remaining.

Figures 16-19 present the results obtained.

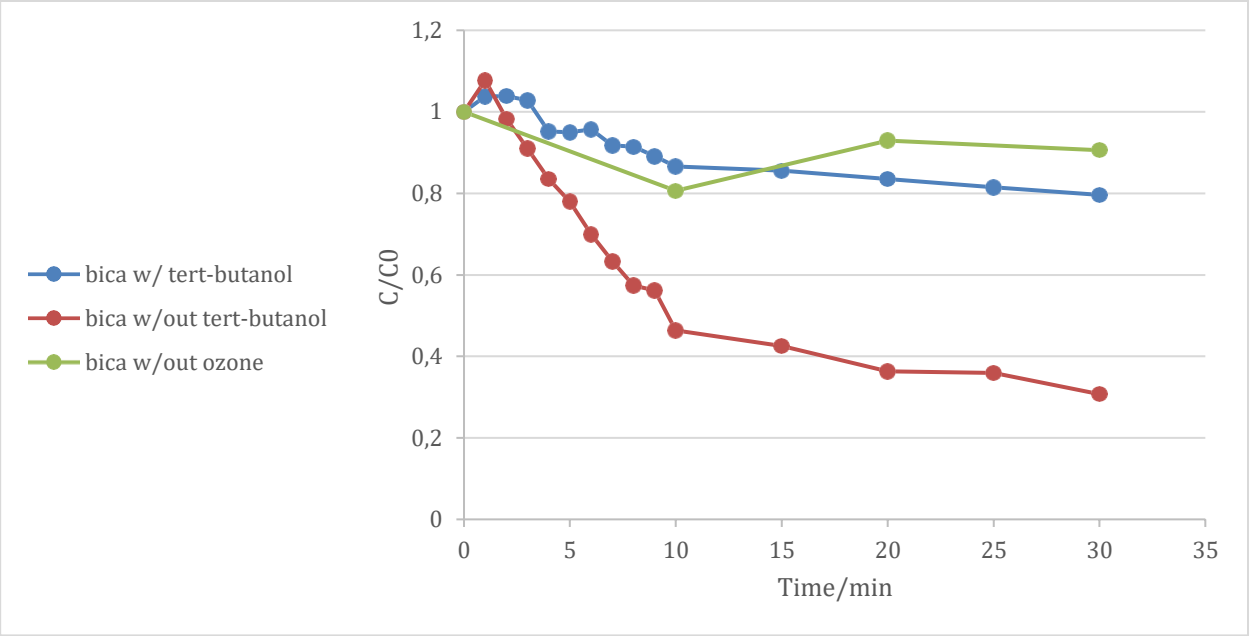


Figure 16: Degradation experiment for 100 µM bicalutamide. $[O_3]_0$= 5 mg·L^{-1}, T=30 ºC, pH=7.14 at 200 rpm.

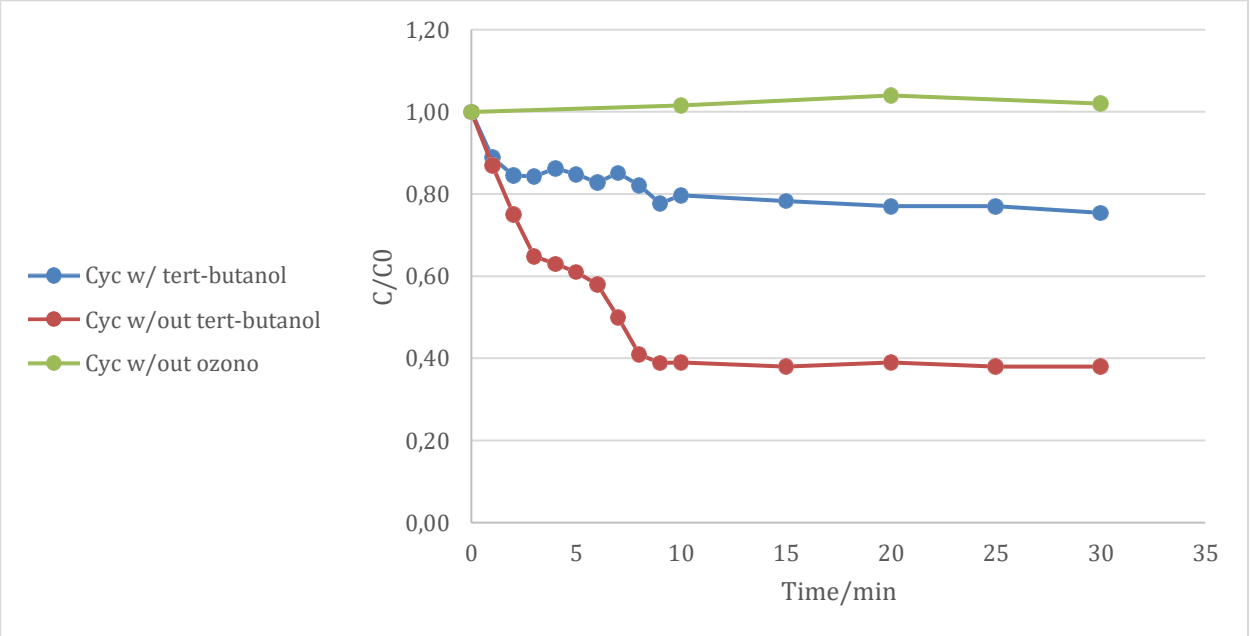


Figure 17: Degradation experiment for 100 µM cyclophosphamide. $[O_3]_0$= 5 mg·L^{-1}, T=30 ºC, pH=7.1 at 200 rpm.

Analysing figures 16 and 17, it is possible to draw several conclusions:

1. *Tert*-butanol does not interact with the anticancer drugs, so their concentration is the blank experiment remains constant;
2. For both Bica and Cyc, it is noticeable that the experiment that doesn't contain tert-butanol leads to a higher removal rate. This suggests that the indirect ozonation pathway is predominant in removing these compounds;

3. For the experiment containing tert-butanol, removal rates are higher for cyclophosphamide, suggesting that this compound is more selective to being attacked by molecular ozone.

For cyclophosphamide and bicalutamide, literature indicates that ozonation follows a second-order rate equation regarding both the anticancer drug and ozone, i.e., first-order for each of the compounds, like equations (7) and (8) exemplify (Y. Lester, et al., 2011).

$$-\frac{d[Cyc]}{dt} = k(O_3, Cyc) * [O_3] * [Cyc]$$
$$\rightarrow ln\frac{[Cyc]_t}{[Cyc]_0} = -k(O_3, Cyc) * \int_0^t [O_3]\, dt \quad (7)$$

$$-\frac{d[Bica]}{dt} = k(O_3, Bica) * [O_3] * [Bica]$$
$$\rightarrow ln\frac{[Bica]_t}{[Bica]_0} = -k(O_3, Bica) * \int_0^t [O_3]\, dt \quad (8)$$

In these equations, $[Cyc/Bica]_0$ and $[Cyc/Bica]_t$ correspond to the initial concentration of each drug and to their concentration after a certain duration of exposure; $k(O_3, Cyc/Bica)$ ($M^{-1}\cdot s^{-1}$) is the second-order rate constant of each compound with molecular ozone, and the integral for O_3 concentration is the measured ozone exposure.

However, it was not possible to use these equations to ascertain the kinetics of the reactions, since it wasn't possible to continuously monitor the concentration of ozone throughout the experiments.

In face of this issue, it was attempted to calculate the kinetic constant as if the reactions were first-order, like equation (9) exemplifies.

$$-\frac{d[drug]}{dt} = k * [drug]$$
$$\rightarrow ln\frac{[drug]_t}{[drug]_0} = -k * \int_0^t dt \quad (9)$$

For this purpose, a graph was made, representing the variation of $ln\frac{[drug]_t}{[drug]_0}$ through time.

However, when the linear regressions were made, it became clear that this approximation could not be applied, since the R^2 presented extremely low values.

Then, a second attempt was performed, assuming a second-order kinetics, but regarding just the anticancer drug's concentration, like equation (10) shows.

$$-\frac{d[drug]}{dt} = k * [drug]^2$$

$$\rightarrow \frac{1}{[drug]_t} - \frac{1}{[drug]_0} = -k * \int_0^t dt \quad (10)$$

The variation of $\frac{1}{[drug]_t} - \frac{1}{[drug]_0}$ through time was also represented graphically, but the results were similar to those obtained in the first attempt. Therefore, it was not possible to determine the kinetic constants for bicalutamide and cyclophosphamide.

4.4.3 Kinetics of mycophenolic acid and mycophenolate mofetil

These experiments are identical to those performed for Bica and Cyc. In this case, ozone was spiked to achieve a desired concentration of 1 mg·L^{-1}. This decrease is justified by the fact that MPA and MMF are far less recalcitrant, so, the decrease of the ozone dose could lead to a slower degradation, that would allow the analysis of the kinetics.

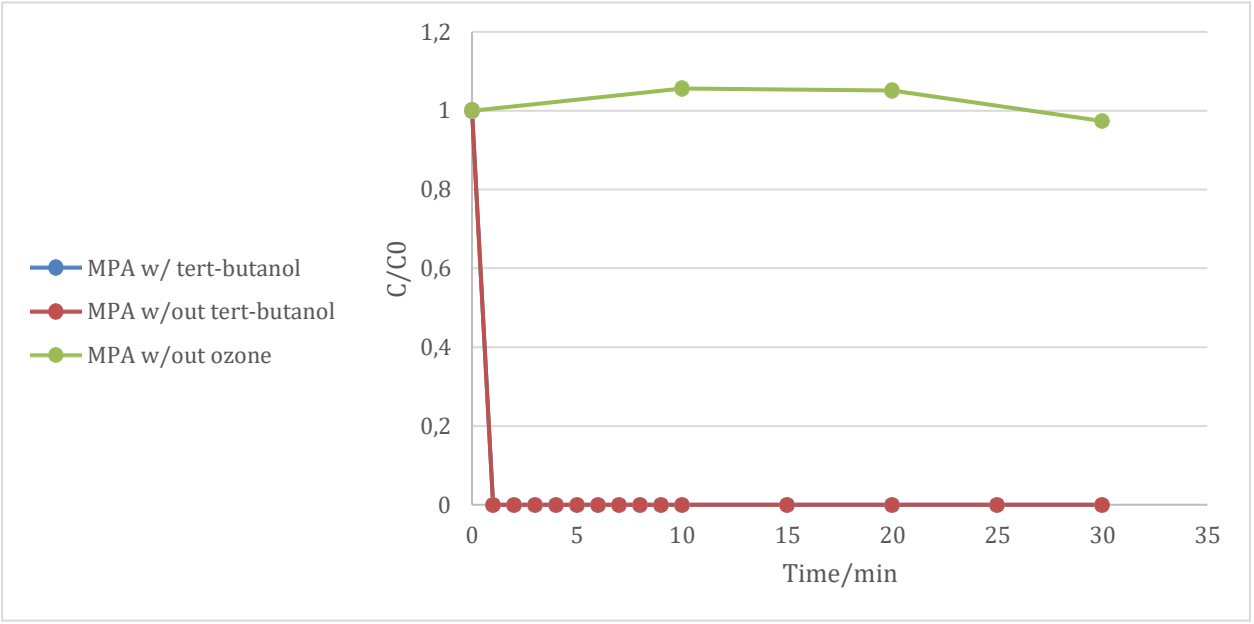


Figure 18: Kinetics experiment for 100 µM MPA. $[O_3]_0$= 1 mg·L^{-1}, T=30 ºC, pH=7.04, at 200 rpm.

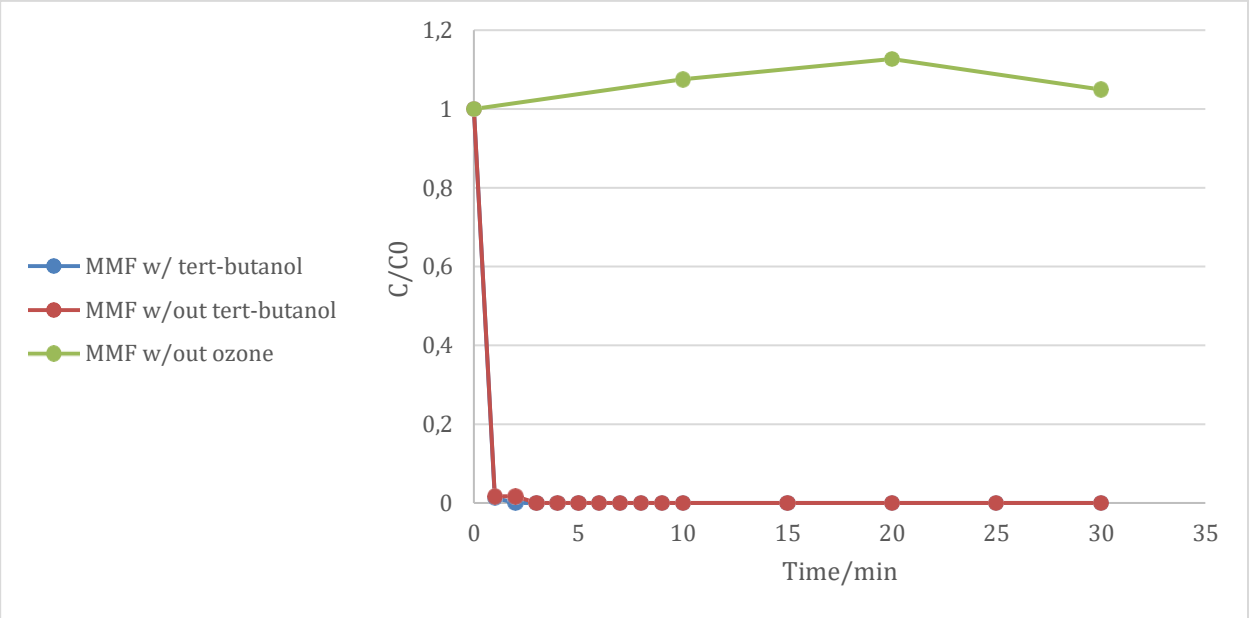


Figure 19: Kinetics experiment for 100 µM MMF. $[O_3]_0$= 1 mg·L^{-1}, T=30 ºC, pH=7.04, at 200 rpm.

Figures 17 and 18 show that the degradation of MPA and MMF occurs in similar ways for the experiments with and without *tert*-butanol. In fact, in both cases, the removal is complete in less than 5 minutes. This suggests that these compounds can be removed either by direct or indirect ozonation, without any differences.

The control experiment also shows, as it did for Bica and Cyc, that *tert*-butanol does not react with the anticancer drugs.

The method employed to analyse the kinetics of Bica and Cyc cannot be used for MPA and MMF, for two reasons: firstly, the removal of these compounds is complete in experiments with and without tert-butanol; secondly, the degradation of MPA and MMF, even using an ozone dose of 1 mg·L^{-1}, is too fast to allow for the analysis of its kinetics. Therefore, there is no way to apply equations (7) and (8), since it is not possible to analyse the variation of these compounds' concentrations through time.

Figures 18 and 19 demonstrate that, after 1 min, the compounds had been fully degraded, and this data is not sufficient to calculate a kinetic constant. To analyse the kinetics of MPA and MMF, a different experiment should be performed, using competition kinetics (CK). However, this method was not used in this dissertation, due to its complexity.

This method is applied to compounds that possess an extremely fast kinetics, since the addition of the competition drug slows the reaction for the main compound, thus allowing its analysis.

5 Conclusions

The goal of this dissertation was to study ozonation as a method to degrade anticancer drugs in water, namely to analyse the behaviour of mycophenolic acid, mycophenolate mofetil, bicalutamide and cyclophosphamide when subjected to this AOP, to ascertain whether their complete removal is possible under the analysed conditions, and to study the effects of certain conditions, such as pH and ozone concentration.

Concerning MPA and MMF, it was demonstrated that the complete removal of these compounds is possible with ozonation, at a neutral pH (as is typical of wastewaters) and ambient temperature, using 1 $mg \cdot L^{-1}$ O_3. It should be remarked that the process is quite fast, and a complete removal was observed during the first five minutes of the experiments.

Regarding Cyc and Bica, it was noticeable that these compounds are far more recalcitrant. In similar conditions to those studied for MPA and MMF, but using 5 $mg \cdot L^{-1}$ O_3, it was only possible to remove approximately 50% of these drugs at pH 2.9 after 30 minutes. This allowed the conclusion that the main form of degradation was indirect ozonation and, to enhance the performance, different pH values were tested. It became clear that, with basic pH, removal was more effective, since there was a higher concentration of $^{\bullet}OH$ radicals in the water. However, it is not sufficient to achieve full removal (and hydrolysis may also play a part in the higher removal rates that were achieved).

In face of these results, it is possible to state that ozonation is a very effective process to remove MPA and MMF that may be present in wastewaters. As for Cyc and Bica, ozonation can be used, but certain conditions must be ensured for the degradation to be effective, i.e. pH > 7 and the addition of another oxidant, to further increase the concentration of $^{\bullet}OH$ radicals.

6 Assessment of the work done

6.1 Objectives Achieved

This dissertation proposed to analyse the process of ozonation for the degradation of four specific anticancer drugs, bicalutamide, cyclophosphamide, mycophenolic acid and mycophenolate mofetil. The aim was to evaluate this process's effectiveness regarding each compound. Furthermore, the ideal conditions to maximise the removal of these compounds should also be analysed, aiming at its full degradation.

In addition, the kinetics of each compound for the ozonation reaction should have been studied, hopefully allowing the identification of the predominant way of oxidation (direct vs. indirect), correlating it with the compound's molecular structure.

This dissertation aimed, firstly, to analyse the degradation of the anticancer drugs cyclophosphamide, bicalutamide, mycophenolic acid and mycophenolate mofetil by ozonation, studying the process's effectiveness for each compound. This goal was achieved, and it was proven that the process is highly effective for both MPA and MMF, allowing for their complete removal, under the studied conditions. As for bicalutamide and cyclophosphamide, it was demonstrated that these compounds are recalcitrant, and that the process may not be ideal to remove these compounds.

Then, another objective was to analyse the effect of the degradation conditions, in order to maximise the removals. In fact, it was proven that MPA and MMF can be fully degraded at pH=7, T=30ºC and with 1 $mg \cdot L^{-1}$ of ozone, and that bicalutamide and cyclophosphamide achieve higher rates of degradation in alkaline solutions (pH=11), with 5 $mg \cdot L^{-1}$ of ozone.

Finally, the kinetics for the ozonation of each compound were also going to be studied. The kinetics of cyclophosphamide and bicalutamide were analysed, but it was not possible to calculate the rate constants, since the reactions followed a second-order kinetics, involving both the anticancer drugs and ozone, and ozone's concentration was not continuously monitored during the experiments. As for MPA and MMF, their degradation is too fast to allow for the calculation of the kinetic constants. In fact, it would be necessary to apply the method of competing kinetics to be able to determine these values, and it was not possible.

6.2 Other Work Carried Out

Throughout the study of ozonation, it became quite clear that the stability of ozone in water was of paramount importance to understand the process and define further experiments,

particularly in what concerns its presence in the sample flasks. Therefore, despite not being an initial objective, ozone stability was studied, to ascertain its presence for a long time or possible degradation when dissolved in ultrapure water. Conclusions lead to believe that it suffers some degradation, due to its reaction with hydroxyl ions present in water, but its concentration tends to stabilise after some time.

Lastly, the testing of buffers and compounds that would stop the ozonation reaction in the sampling vials were minor experiments but, without their existence, the veracity of results would be compromised.

6.3 Limitations and Future Work

Despite the completion of most of the objectives, there were some limitations detected during the experiments.

For once, it became complicated to measure the concentrations of O_3 during the ozonation experiments. Since the standard indigo colorimetric method does not specify the volume of ozonated solution that must be added to measure its absorbance (the volume is defined and then the formula adjusts the values with dilution) (Bader, 1982), this method is not very precise. In fact, during experiments, it was difficult to ascertain the volume of ozone that should be added, since it depends on its dissolved concentration. In sum, it was very complicated to know if the measured absorbance was accurate. Therefore, for future work, maybe the direct measurement of ozone concentration would be a better choice, allowing for the continuous monitoring of O_3 during the reactions.

It was not possible to calculate the rate constants for any of the compounds. For bicalutamide and cyclophosphamide, this was due to the fact that ozone was not monitored continuously during the reactions, and ozonation follows a second-order kinetics that depends on ozone's concentration as well as the concentration of anticancer drugs. For MPA and MMF, the inability to calculate the kinetic constants is related with the method that should be applied (competition kinetics).In the future, the kinetics of MPA and MMF must be studied, using the competition method, to better understand their degradation. Furthermore, regarding bicalutamide and cyclophosphamide, in order for their removal to be complete, the addition of H_2O_2 or UV radiation to the process should be considered.

6.4 Final Assessment

The execution of this dissertation had some set-backs and complications, related to the availability of certain equipment, at first, and then regarding the stability of ozone in ultrapure water (which was in advance not known), since these results were fundamental to steer the course of further experiments.

These drawbacks led to a reduced period of time to perform the experiments, which led to inability to achieved all of the proposed objectives.

Nevertheless, the goals that were achieved provided very interesting results, and allowed the analysis, for the first time, of the degradation of MPA and MMF. Since these results were very promising, this thesis may have provided some new and valuable insight into the degradation of anticancer drugs through ozonation.

7 References

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Annex 1 Reactions of O_3 in water

Annex Table 1: Reactions for the decomposition of ozone in water (Elovitz, et al., 2000).

TABLE III. REACTION MECHANISM FOR THE RADICAL-TYPE CHAIN DECOMPOSITION OF OZONE INCLUDING REACTIONS OF MODEL •OH SCAVENGERS WITH •OH AND O_3.

Rxn. No	Reaction	k_f, k_r or K_a*	ref.
1	$O_3 + OH^- \rightarrow HO_2^- + O_2$	70 $M^{-1}s^{-1}$	(23)
2	$O_3 + HO_2^- \rightarrow O_3^{-}\bullet + HO_2^-$	2.8×10^6 $M^{-1}s^{-1}$	(23)
3	$O_3 + O_2^{-}\bullet \rightarrow O_3^{-}\bullet + O_2$	1.6×10^9 $M^{-1}s^{-1}$	(28)
4	$O_3^{-}\bullet + H^+ = HO_3\bullet$	5×10^{10} $M^{-1}s^{-1}$; 3.2×10^2 s^{-1}	(28)
5	$HO_3\bullet \rightarrow \bullet OH + O_2$	1.4×10^5 s^{-1}	(28)
6	$O_3 + CH_3OH \rightarrow$ products	0.024 $M^{-1}s^{-1}$	(2)
7	$O_3 + CH_3COO^- \rightarrow$ products	3×10^{-5} $M^{-1}s^{-1}$	(3)
8	$O_3 + t$-BuOH $\rightarrow$ products	0.003 $M^{-1}s^{-1}$	(2)
9	$O_3 + HCO_3^- \rightarrow$ products	0.001 $M^{-1}s^{-1}$	(4)
10	$O_3 + CO_3^{2-} \rightarrow$ products	0.01 $M^{-1}s^{-1}$	(4)
11	$\bullet OH + CH_3OH \rightarrow \bullet CH_2OH + H_2O$	9.7×10^8 $M^{-1}s^{-1}$	(27)
12	$\bullet CH_2OH + O_2 \rightarrow O_2^{-}\bullet +$ products	4.9×10^9 $M^{-1}s^{-1}$	(27)
13	•OH + acetate $\rightarrow$ products	7.9×10^7 $M^{-1}s^{-1}$	(27)
14	•OH + t-BuOH $\rightarrow$ products	5.9×10^8 $M^{-1}s^{-1}$	(27)
15	$\bullet OH + HCO_3^- \rightarrow$ products	8.5×10^6 $M^{-1}s^{-1}$	(27)
16	$\bullet OH + CO_3^{2-} \rightarrow$ products	4×10^8 $M^{-1}s^{-1}$	(27)
17	•OH + pCBA $\rightarrow$ products	5×10^9 $M^{-1}s^{-1}$	(22)
18	$O_3 + \bullet OH \rightarrow HO_2\bullet + O_2$	2×10^9 $M^{-1}s^{-1}$	(29)
19	$H_2O_2 + \bullet OH \rightarrow HO_2\bullet + H_2O$	2.7×10^7 $M^{-1}s^{-1}$	(27)
20	$HO_2^- + \bullet OH \rightarrow HO_2\bullet + OH^-$	7.5×10^9 $M^{-1}s^{-1}$	(27)
21	$\bullet OH + \bullet OH \rightarrow H_2O_2$	5×10^9 $M^{-1}s^{-1}$	(27)
22	$O_2^{-}\bullet + \bullet OH \rightarrow O_2 + OH^-$	1×10^{10} $M^{-1}s^{-1}$	(27)
23	$HO_2\bullet + \bullet OH \rightarrow O_2 + H_2O$	6×10^9 $M^{-1}s^{-1}$	(27)
24	$HO_3\bullet + \bullet OH \rightarrow H_2O_2 + O_2$	5×10^9 $M^{-1}s^{-1}$	(27)
25	$H_2O_2 = HO_2^- + H^+$	2.5×10^{-12} M	(30)
26	$HO_2\bullet = O_2^{-}\bullet + H^+$	1.6×10^{-5} M	(23)
27	$HCO_3^- = CO_3^{2-} + H^+$	5.6×10^{-11} M	(30)

* Rate constants for first-order and second-order reactions are give in units of (s^{-1}) and ($M^{-1}s^{-1}$), respectively. For protonation/deprotonation reactions, either 1) a combination of the forward rate constant (k_f, $M^{-1}s^{-1}$) and backward rate constant (k_r, s^{-1}), or 2) the acid-equilibrium constant K_a is given.

Note: Hydroxyl radical scavenging reactions (rxns. 19-24) were included in the kinetic model; however, sensitivity analysis showed that these reactions were of minor importance under the reaction conditions modeled.