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Azithromycin determination using spectrophotometer molecular methods and degradation using an advanced oxidation process.

[1]Rosecler Ribeiro, [2]Fernando José Mallmann Kuffel, [3]Henrique Pretto Etgeton, [4]Cassiano Ricardo Brandt, [5]Daniel Kuhn, [6]Joseane Parizzi Mafioleti, [7]Mariano Rodrigues, [8]Eduardo Miranda Ethur, [9]Simone Stülp, [10]Clarice Steffens, [11]Lucélia Hoehne

[1]Rosecler Ribeiro, undergraduation in industrial chemistry, department of Biotechnology, Univates, Lajeado, Rio Grande do Sul, Brazil.
[2]Fernando José Mallmann Kuffel, undergraduation in chemistry engineer, department of Biotechnology, Univates, Lajeado, Rio Grande do Sul, Brazil.
[3]Henrique Pretto Etgeton, undergraduation in environmental engineer, department of Biotechnology, Univates, Lajeado, Rio Grande do Sul, Brazil.
[4]Cassiano Ricardo Brandt, undergraduation in chemistry engineer, department of Biotechnology, Univates, Lajeado, Rio Grande do Sul, Brazil.
[5]Daniel Kuhn, undergraduation in industrial chemistry, department of Biotechnology, Univates, Lajeado, Rio Grande do Sul, Brazil.
[6]Joseane Parizzi Mafioleti, undergraduation in environmental engineer, department of Biotechnology, Univates, Lajeado, Rio Grande do Sul, Brazil.
[7]Mariano Rodrigues, graduation in Biotechnology, department of Biotechnology, Univates, Lajeado, Rio Grande do Sul, Brazil.
[8]Eduardo Miranda Ethur, doctor in chemistry, department of Biotechnology, Univates, Lajeado, Rio Grande do Sul, Brazil.
[9]Simone Stülp, doctor in Mining, Metallurgical and Materials Engineering, Graduate Program in Environment and Development, Univates, Lajeado, Rio Grande do Sul, Brazil.
[10]Clarice Steffens, doctor in Biotechnology, Technology centre, Universidade Regional Integrada do Alto Uruguai e das Missões, Erechim, Rio Grande do Sul, Brazil,
[11]Lucélia Hoehne, doctor in chemistry, department of Biotechnology, Univates, Lajeado, Rio Grande do Sul, Brazil.

Address For Correspondence:
Lucélia Hoehne, department of Biotechnology, Univates, Univates, Lajeado, Zip code: 95914-014. Brazil.
E-mail: luceliah@univates.br

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ABSTRACT

The concern about the water quality has always been an important factor in human societies, but still, the pollution of this resource has always been present. However, with human evolution and development of new products, the type of pollutant has also evolved, reaching in what is know as micropollutants. Azithromycin is a widely used antibiotic and fits as a micropollutant and it is very difficult to decompose using treatment convencional for water supply. For being present in water matrices, the objective of this study is to analyse two methods for identify Azythromicyn using molecular absorption spectrometry technique and evaluation of the decomposition this micropollutant of aqueous solutions using advanced oxidation process (AOP) with ultraviolet (UV) irradiation. The first quantification method utilized was sulfuric acid with the drug to form compounds which absorb irradiation in the 226 nm range. The second method used alizarin as reagent, which results in a red-orange color when reacting with the antibiotic in a 540 nm. For the decomposition treatment, a reactor for the AOP process was made with two UV lamps for Azythromicyn decomposition tests. A 80 ppm of the antibiotic was used for the irradiation test and the samples were collected each 10 minutes for 1h. The results showed that using the first method with sulfuric acid as reagent, there were some disadvantages, because the spectrum of Azithromycin and acid derivatives absorption bands were close and it was impossible data collection. The second method using alizarin as reagent, it was possible to identify more correctly the Azithromycin and the Alizarin peaks. After the choice of the best method for identify the antibiotic, a reactor for the AOP process was made and tested

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and the preliminary results, it was possible drug degradation about 10%. The best condition was using 10 minutes of irradiation. The results showed that it was higher than those found in the literature, making possible in the future the combination of more AOPs, as Fenton, photo Fenton, photo catalysis or UV with hydrogen peroxide. This proposed paper shows that it was possible determine Azithromycin using spectrophotometry molecular for identification with alizarin as reagent and preliminary test showed that using AOP with UV irradiation is possible to degradation this drug. Further tests will be necessary, but this process could be helpful to other researchers.

INTRODUCTION

Water is an essential substance for all life forms since its beginnings. Despite the Earth's surface being composed of more than 70% water, only about 0.0082% is drinkable and easily accessible to humans. Considering this water deficit, much has been studied about its quality and potential contaminants. Following the human beings' changes of habit over time, the complexity levels of contaminating compounds in water bodies and effluents has increased considerably (Bolong, Ismail and Salim, 2009).

A new class of emerging pollutants has drawn the attention of the scientific community: the micropollutants. Indiscriminate use of drugs, pesticides, insecticides, pharmaceuticals and personal care products has raised the concentration levels of these substances in various environmental matrices (Liu *et al.*, 2014; Pal *et al*., 2010).

Due to the high molecular complexity level of such compounds, these possess high resistance to conventional water and wastewater treatments used by treatment plants (Wols and Hofman-Caris, 2012). Therefore, the micropollutants find ways to the cities water supplies, where they remain unaffected. Even in low concentrations these contaminants can cause several problems to both aquatic fauna as human health in the medium and long term (Gros, Petrovic and Barceló, 2008; Snyder *et al.*, 2003).

Some of the micropollutants that cause the most concern in the scientific community are antibiotics. The presence of those in environmental matrices has increased in proportion to the pharmaceutical industry growth, which is due to the greater demand for medicines by the world population (Uyaguari, 2011). These substances can induce modifications in the bacterial cells, resulting in higher antimicrobial resistance and hindering disease treatments (Sun *et al.*, 2016).

Among the class of pharmaceuticals is Azithromycin, which is an antibiotic with broad range of performance and is used to treat respiratory infections, urethritis, sexually transmitted diseases and other recurrent illnesses (Tong *et al*., 2011). For those reasons, its use is widespread and often advised. However, not being degraded by the human body, this antibiotic joins the waste that is disposed in the sewer system and may end up being improperly discarded in the environment (Iatrou, Stasinakis and Thomaidis, 2014; Bizarro, 2016).

There are some methods to analyzing Azithromycin, such high performance thin layer chromatography (Bouklouze *et al*., 2016), high-performance liquid chromatography method coupled with an evaporative light scattering detector (Zeng *et al*., 2014), or visual colorimetric sensor (Chavada *et al*., 2017). But this methods are expensive and it was necessary sophisticated equipments. A spectrophotometric molecular technique is cheaper and easy operation, and there are methodologies using spectrophotometer for this antibiotic in organic solvent (Ferreira, 2007; Kasten, 2014). Then it would be interesting to evaluate the use of these methodologies for the determination of the drug in aqueous medium.

In addition, a methodology for decomposition this drug should be developed, because it has high resistance to typical drinking water treatments. Thus, the use of more aggressive and specific techniques come into focus (Elmolla and Chaudhuri, 2010; Ganiyu *et al.*, 2015). The employment of advanced oxidation processes (AOP) takes the lead in this segment. Its principle is based on the attainment of hydroxyl radicals (OH) highly reactive (E^o = 2.80 eV) that can oxidize compounds of difficult degradation (Cheng *et al.*, 2016).

Hydroxyl radicals can be formed by various methods classified as AOP, e.g. Fenton and Photo-fenton processes, which use iron in their reactions, ozonation processes (O_3), photocatalysis, using mainly metal oxides, processes with ultraviolet (UV), among others (Ikehata, Naghashkar and El-Din, 2006; Nagel-Hassemer, Coral and Lapolli, 2012). Each one presents greater affinity with a particular contaminant and also varies according to the cost-benefit, requiring an assessment of which method is more efficient in relation to the characteristics of the material to be treated (Comninellis *et al.*, 2008; Kim, Yamashita and Tanaka, 2009).

One of the most commonly used methodologies is the ultraviolet radiation (UV) or photolysis process. This employs a UV light in a reactor (batch or continuous flow) without the addition of chemical reagents. Photolysis can be utilized in effluent treatment (Durán *et al.*, 2013) as well as in the decomposition of pharmaceuticals. According to Tong *et al.* (2011), being very energetic, this radiation is able to generate the cleavage and disruption of complex molecules such as Azithromycin, thereby engendering simpler and less aggressive compounds due to absorption of light, thus inactivating the properties of the drugs (Tokumura *et al.*, 2016).

Even though Azithromycin is a complex molecule, the application of photolysis can degrade this kind of antibiotic, transforming it in an environmental friendly molecule. Sometimes UV alone is not efficient to remove the antibiotic from aquatic matrices, so UV/H_2O_2 AOP can be used. This process produces more hydroxyl radicals and usually presents bigger removal of micropolluant concentration. (Kim, Yamashita and

Tanaka, 2009). In the future, this process might be employed in wastewater treatment plant (Michael *et al.*, 2013; Luo *et al*., 2014 ;Yuan *et al*., 2009).

Owing to the environmental concerns cited, the aim of this paper was evaluate two spectrophotometry methods for azithromycin determination and analysis of degradation of the drug using a reactor and AOP with UV radiation.

2 Methodology:

This study was adapted on two different Azithromycin determination methodologies: using sulfuric acid (Ferreira, 2007) and then utilizing Alizarin (Kasten, 2014).

Initially, the solubility of dihydrate Azithromycin was tested in water, methanol and acetonitrile. Solubility tests of the drug with no impurities presented complete solubility in methanol and acetonitrile.

A Shimatzu spectrophotometer and quartz cells were used for the tests. All other reagents were obtained from Sigma-Aldrich and were analytical grade.

2.1 Spectrophotometric method of Azithromycin with sulfuric acid:

The method was adapted from Ferreira (2007) and it is based on the reaction of sulfuric acid (H_2SO_4) with Azithromycin sugars groups, forming compounds which absorb light at 226 nm. A standard of dihydrated Azithromycin 98% purity without excipients was applied.

A solution of concentration 1000 $mg.L^{-1}$ was prepared. From this, the calibration curve was built at concentrations of 0, 20, 40, 60, 80 and 100 $mg.L^{-1}$. The samples analyzed were drawn up in 25 mL volumetric flasks with 15 mL of deionized water, 400 uL of sulfuric acid 98% purity and swelled with acetonitrile. After, the ultrasound bath was employed for 5 minutes. A UV spectrophotometer scanning at 190-500 nm was later executed, obtaining the maximum peak at 233 nm.

2.2 Spectrophotometric method of Azithromycin with Alizarin:

This method was adapted from Kasten (2014) and is based on the reaction of Azithromycin with Alizarin, thereby obtaining a red-orange color sample.

At first, a dihydrated Azithromycin 98% purity standard of concentration 1000 $mg.L^{-1}$ was prepared and diluted in 0, 20, 40, 60, 80 e 100 $mg.L^{-1}$ standards to obtain the calibration curve. These standards were prepared in 10 mL volumetric flasks using 4 mL of deionized water, 1 mL of Alizarin and were swelled with methanol. After executing a UV spectrophotometer scanning, the maximum peak was obtained at 540 nm.

The Alizarin solution was prepared by adding 19.2 mg of Alizarin in a 50 mL volumetric flask. Dimethyl sulfoxide (DMSO) was used for dilution and bulking.

2.3 Reactor for AOPs tests:

A reactor developed by Mafioleti (2014) was used (Figure 1). This consists of two low pressure germicidal lamps (254 nm) of 15 W (Philips). The lamps were connected at 220 V for 30 minutes for stabilization, and then the samples (80 $mg.L^{-1}$ of Azithromycin) were added to the reactor's 100 mL quartz tube. The temperature was constantly monitored using a digital thermometer sensor..

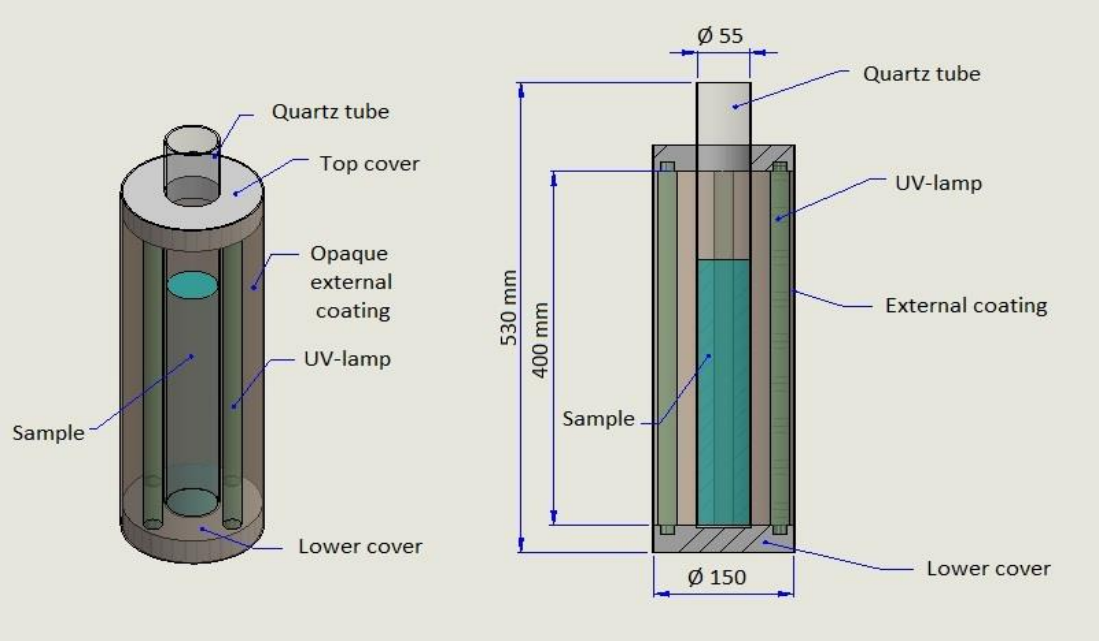

Fig. 1: Reactor project used in the Azithromycin degradation.

For irradiation tests using methodology with sulfuric acid, using the reactor, tests with different pH levels (3, 4, 5, 6, 7, 8 and 9) were proposed. After the rescan of spectrum peaks, the treatment with pH=9 was selected for presenting the best peak and being close to the pharmaceutical's pH.

For irradiated samples, a 100 mL solution of concentration 80 $mg.L^{-1}$ was prepared from the dihydrate Azithromycin 98% purity standard. The solution was introduced into the reactor at pre-determined times of 0, 10, 20, 30, 40, 50 and 60 minutes. The pH was adjusted to 9 by adding sulfuric acid 0,002 M or sodium hydroxide (NaOH) 0.01 M. After undergoing irradiation, the samples were swelled with sulfuric acid and acetonitrile to remain according to the calibration curve. To verify the drug's degradation, readings by UV-VIS were performed.

For irradiation tests using alizarin method, the sample was irradiated in the reactor. For analysis, 5 mL aliquots were withdrawn before and after the irradiation process and diluted with 4 mL of methanol and 1 mL of Alizarin in a 10 mL volumetric flask. The drug's degradation or not was verified from the initial and final concentrations found.

2.4 Temperature test:

The temperature was measured at different times of irradiation, since according to Timoumi *et al.* (2014) this is a variable that can significantly interfere in the drug degradation.

2.5 Statistical analysis:

The trust level of the results obtained in this study were evaluated using Analysis of Variance (ANOVA) according to Hair *et al.* (2005) and Dancey and Reidy (2006). For validation purposes, the level of statistical significance was set at $P<0.05$ and the confidence level was established at 95%.

RESULTS AND DISCUSSION

3.1 Spectrophotometric method of Azithromycin with sulfuric acid:

By adjusting the pH to 9, shifts in peak length and peak deformations were noticed, as shown in Figure 2. According to Cienfuegos and Vaitsman (2000), this can be attributed to the action of the compounds from the acid, such as hydrogen ions (H^+). These compounds absorb light in the same wavelength as the sample, causing an incorrect reading by the equipment. Zhang et al. (2009) found in their study that the degradation rate constants of Azithromycin are very dependent on pH and concentration.

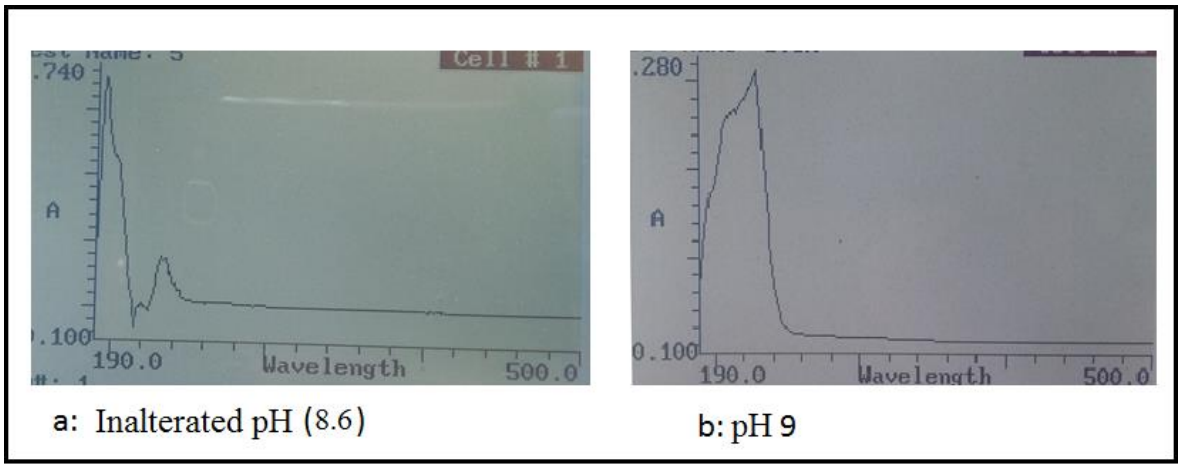

Fig 2. Peak alterations with pH=8.6 and 9

In the reading of dihydrated Azithromycin 98% purity, the peak was obtained at the wavelength of 233 nm, close to what was found by Ferreira (2007) and by Nyola and Jeyabalan (2012), in wavelengths of 226 nm and 235 nm, respectively. The calibration curve made with 20 to 100 $mg.L^{-1}$ standards resulted in a 0.9885 correlation coefficient, as shown in Figure 3. The difference can be because of water presence in the solution.

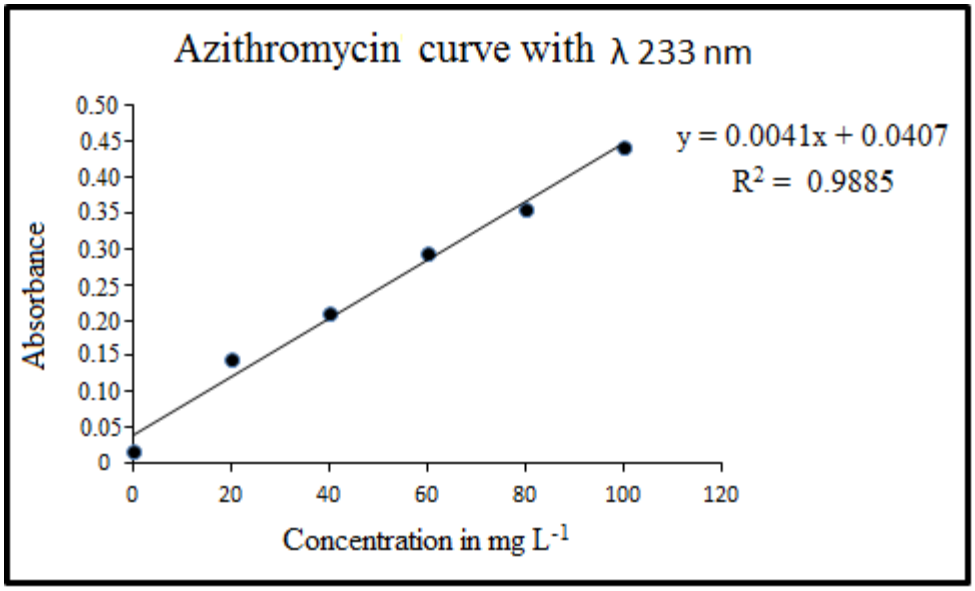

Fig. 3: Calibration curve of dihydrated Azithromycin 98% purity evaluated with the wavelength of 233 nm.

From the analytical calibration curve, the absorbances of the samples before and after UV irradiation were calculated. Concentrations of samples irradiated at 10, 20 and 30 minutes were estimated as shown in Table 1.

Table 1: Concentration (ppm) evaluation of dihydrated Azithromycin 98% purity in 10, 20 and 30 minutes of UV irradiation.

UV Irradiation	10 min	20 min	30 min
Initial time	80.90 ± 4.11	80.54 ± 5.47	79.57 ± 0.81
Final time	94.04 ± 1.91	100.12 ± 9.98	200.35 ± 0.80

Source: the author

The results found for absorption and concentration can be justified by the presence of compounds that absorb light in the range of 250 to 380 nm, according to Silverstein, Bassiler and Morril (1994). The organic compounds and sulfides from the sulfuric acid (Silverstein, Webster e Kiemle, 2013) and the sugars groups and macrolactones of the antibiotic (Tong *et al.*, 2011) absorb light in nearby reading tracks of Azithromycin and may have interfered in the readings, generating the results shown in Table 1.

Only the absorbances were calculated for the samples irradiated for 40, 50 and 60 minutes, since their concentrations were outside the calibration curve and their peaks were deformed, as shown in Figure 4.

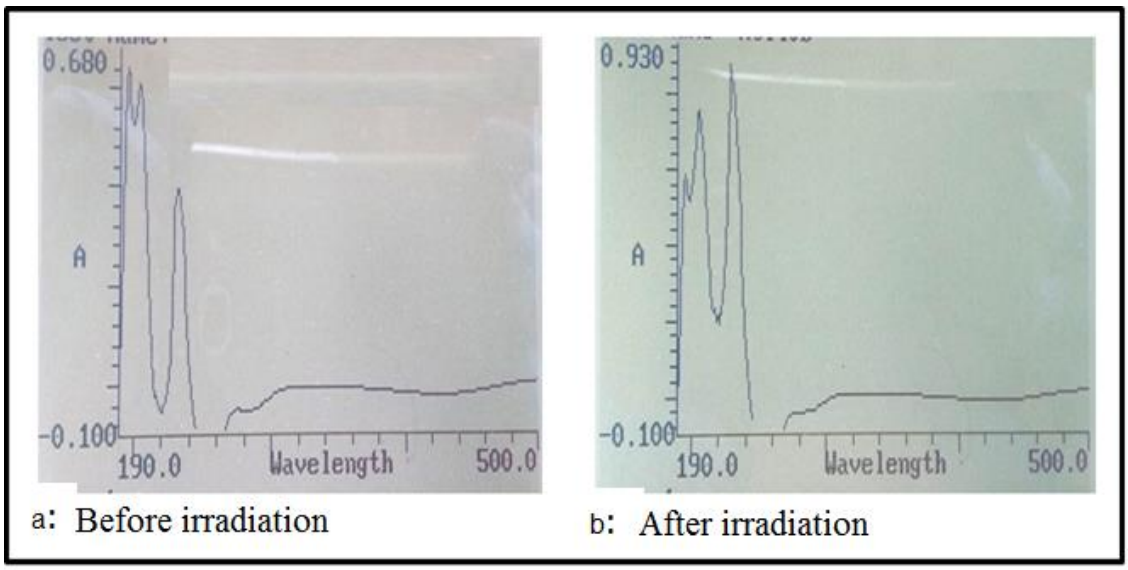

Fig. 4: Superposition of peaks after 60 minutes of UV irradiation

As suggested by Tong *et al.* (2011), the formation of a by-product of Azithromycin was observed (Figure 5). The higher the irradiation time, the higher the concentration of the by-product. Therefore, the increase in

absorbance and concentration can be attributed to the absorption of light by the by-product and not directly by the drug. Theoretically, as stated by Silverstein, Webster and Kiemle (2013), the cleavages of Azithromycin tend to occur where there are hetero atoms in the same period and where there is less electronegativity. In this case, the most susceptible regions for cleavage would be carbon and oxygen bonds, where interactions are weakened. The methyl on the Azithromycin molecule extremities makes it difficult to break the bonds. Conforming to Silverstein, Bassiler and Morril (1994), CH_3^+ ions are more stable than the branched carbons such as $R'CH_2^+$, $R_2'CH^+$ and $R_3'C^+$.

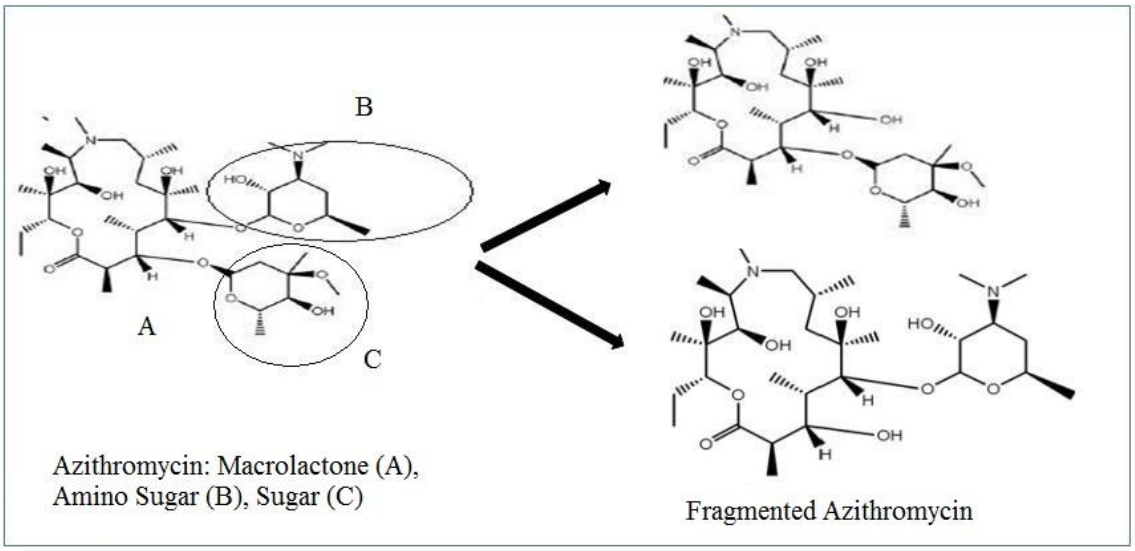

Fig. 5: Cleavage of Azithromycin and its by-products.

The method using sulfuric acid was not suitable to analyze Azithromycin in water samples. The mineralization of the samples was desired. When that occurs, the absorbance decreases, but this was not observed in the experiment.

In order to compare the results of this method, the methodology using Alizarin was conducted.

3.2 Spectrophotometric method of Azithromycin with Alizarin:

Two peaks in the samples readings were detected utilizing Alizarin, the first being of Alizarin and the second of Azithromycin. The samples exhibited color change, from yellow to purple, in accordance to the concentration (Figure 6). This, according to Kasten (2014), is observed because Azithromycin acts as a donor of electrons that yields an Alizarin mono anion, which produces the color noticed.

Fig. 6: Calibration curve color from Alizarin samples with different concentrations.

As Martins, Sucupira e Suarez (2015), the color is obtained as a function of the chromophoric groups concentration and the visible color is different from the absorbed. The purple color is absorbed in green wavelength of 500 to 565 nm

Scanning from 190 to 700 nm peak of the calibration curve solutions was performed, defining the peaks of Azithromycin and Alizarin as shown in Figure 7. The maximum peak was obtained at 540 nm. Thus it can be

used to determine Azithromycin, since conforming to Brasil (2010), to verify the concentration of a substance, reagents can not absorb light at the same wavelength of the sample and the peaks should remain constant.

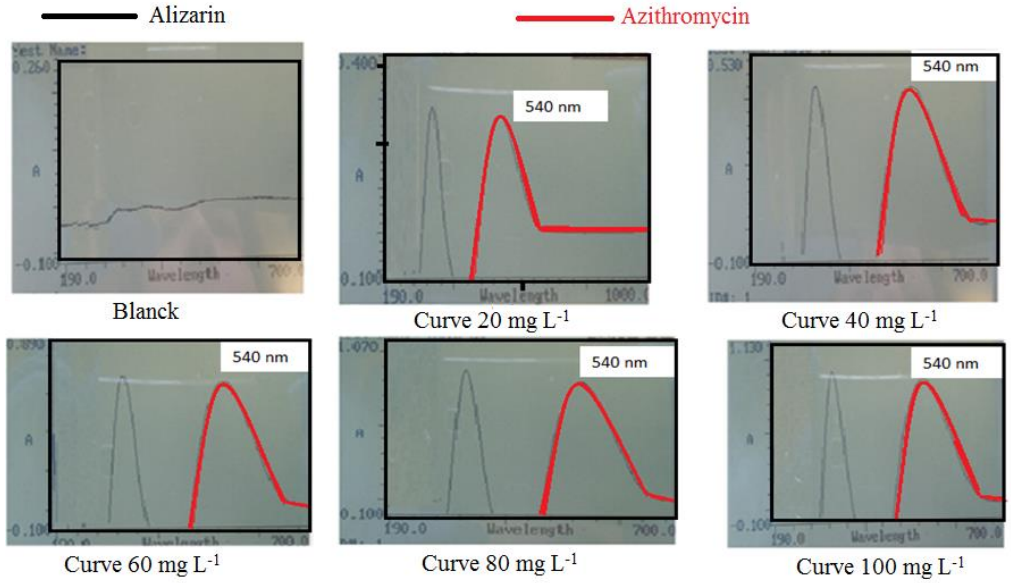

Fig. 7: Evaluation of calibration curve peaks of dihydrate Azithromycin 98% purity.

After determining the wavelength more suitable for Azithromycin analysis, it was fixed at 540 nm for UV irradiation. As shown in Figure 8, a calibration curve of coefficient R^2=0.9954 was achieved.

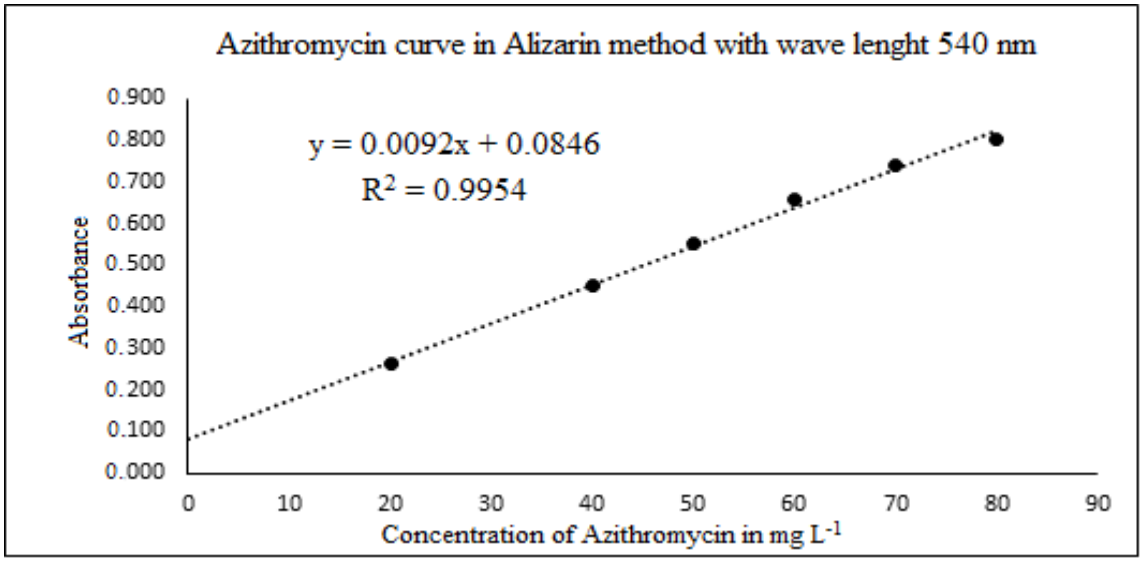

Fig. 8: Concentration curve of Azithromycin in wavelength of 540 nm

From the calibration curve, the Azithromycin degradation after undergoing UV irradiation was calculated (Figure 9).

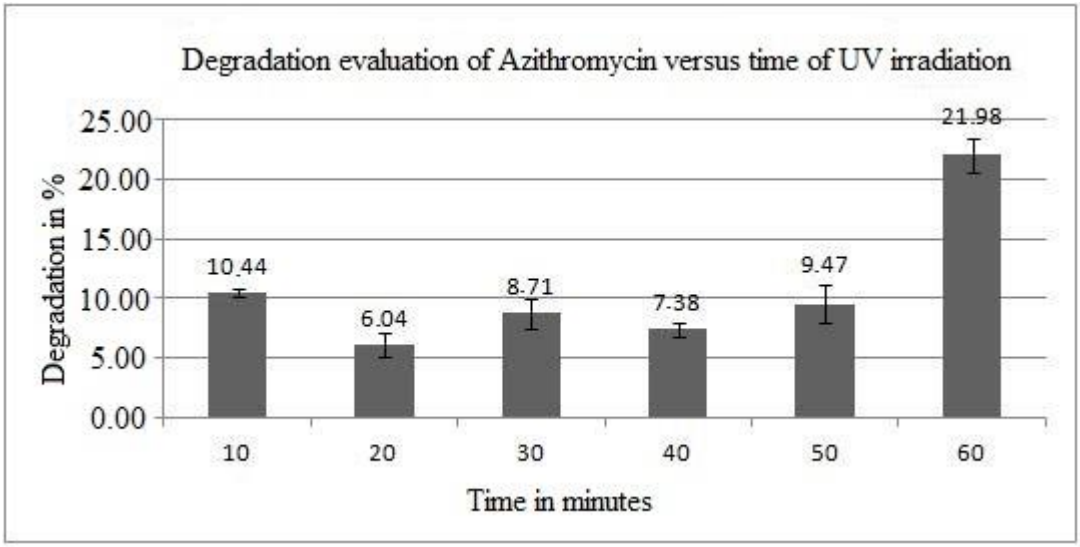

Fig. 9: Degradation evaluation of the Azithromycin with Alizarin method

The results ranged from 6 to 21.9% of deterioration. Analysis of Variance (ANOVA) was used and the averages presented statistical differences P <0.004 and F (8.17) greater than critical F (3.68). Although being low, degradation rates were higher than those found by other authors such as Kim, Yamashita and Tanaka (2009), who obtained removal from 4 to 7%, and De la Cruz *et al.* (2012), who did not achieve removal of the drug within 10 minutes of UV irradiation. However, both used more powerful lamps than this study, these being 60 W and 25 W, respectively. In view of this, the removal difficulty of the drug is emphasized. The removal rate of this research was higher than those found in the literature.

A decrease in the antibiotic removal rate after 10 minutes was noticed, except in the 60 minutes irradiation. As stated by Tong *et al.* (2011), this fact can be associated to competing reactions of Azithromycin and its derivatives.

After 60 minutes of UV irradiation, a greater degradation (21.98%) than the one found in 10 minutes (10.44%) was achieved. Notwithstanding, the exposure time was 6 times higher and the degradation rate was not proportional. Therefore, as the irradiation and degradation times are not proportional, it is not advantageous to keep the samples exposed for 60 minutes, as the cost-benefit is not maintained.

Better efficiency can be accomplished through a combination of more sorts of AOPs. Souza (2013) achieved a near 100% degradation combining hydrogen peroxide (H_2O_2) with UV irradiation. In this manner, a modification in the method by adding hydrogen peroxide without its interference in the analysis is suggested.

3.3 Temperature test:

Conforming the temperatures measured during the irradiation process, the higher the UV irradiation time, the higher the temperature reached in the sample. The temperature conditions are close to the room temperature of Rio Grande do Sul state, Brazil, during the summer season. The values found during the process ranged from 24.5 ° C to 38 ° C, as shown in Table 2.

Table 2: Temperature evaluation in UV irradiation times

UV irradiation time (min.)	Temperature (ºC)
0	24.5
10	25.0
20	30.5
30	34.0
40	35.0
50	37.1
60	38.0

Source: the author

The results obtained in this study were similar to those found by Tong *et al.* (2011). This was based on temperatures between 25 to 35 °C, not observing micropollutants degradations due to the temperature increase. However, Timoumi *et al.* (2014) found that Azithromycin is stable at temperatures up to 70 °C.

Conclusion:

Azitromicyn is a micropollutant because the molecular structure is complex and it was very difficult to decompose using treatment convencional for water supply. The antibiotic presents risks of producing more resistant bacteria to the drug and thus hinders the treatment of simple bacterial infections if consumed frequently. Therefore, effective treatments must be carried out so that it does not have residues of this sample in supply water. There are many methods of drug determination, but it was necessary expensive equipments and trained professionals. In spite of this, the present paper evaluated two adapted spectrophotometric methods for Azithromycin determination in aqueous media: one using sulfuric acid and other using alizarin as reagents and one molecular spectrophotometer UV/VIS. And, it was possible to conclude the best method was the second. The method presented linearity in the analytical calibration curve (obeying Beer's law) and well-defined wavelengths, since the higher wavelength suppressed the problem with the interferents. The wavelength used in the method was set at 540 nm and the methodology proved to be simple, fast and easy to work with. In contrast, the spectrophotometric method using sulfuric acid displayed several variations such as the increase in absorbance, possibly by the presence of chromophore groups coming from UV degradation. It was important use quartz cell for determination for intereference elimination in the analysis.

Then, a reactor could be made and used for irradiation test using UV lamps with 80 ppm of the sample. The drug degradation technique using irradiation treatment attained a maximum degradation of 21.97% after 60 minutes of irradiation. Nonetheless, regarding the irradiation time and degradation obtained, irradiation for 10 minutes was defined as the best treatment condition, as this reached 10.44% degradation. The results obtained in the study were higher than those found in literature. About the solution temperature after the best condition irradiation ranged from 24.5 ° C to 38 ° C and in this temperature the Azithromycin is stable. This confirm the importance of this paper to show the difficult to develop a efficient method for drug decomposition. Seeking a higher degradation in the process, the implementation, in the future, of other AOPs can be used, for example: Fenton, hydrogen peroxide or ozone, can increase the process efficiency and have already been studied by many authors. Also, it is important to analyze the toxicity of the solution after irradiation, because the subproducts formed can be more toxics than the initial product. Tests using microcracks or some vegetables can be used for this purpose. Besides that, there are many other AOPs to be used and each one has different reactions with the samples under tests, so a study over the best option of AOP to be used and their lowest cost is important to optimized the method. The best method preferably should be easy to operate, use cheap equipment, non-destructive technique, selective, efficient and do not leave by-products more toxic than the originals. Future studies of Azithromycin detection in complex environmental matrices, such as rivers and lakes, will be performed later. This paper can help others researchs involving studies on the determination and degradation of azithromycin in aqueous media.

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