



**DETERMING THE FATE OF SE-
LECTED ANTIBIOTICS DURING NI-
TROGEN RECOVERY VIA UREA-
FORMALDEHDYE SYNTHESIS**

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Environmental Engineering

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ABSTRACT

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Determining fate of the selected antibiotic during nitrogen recovery via Urea Formaldehyde synthesis

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The work presented here focused on the determination of fate of four selected antibiotics: enrofloxacin, oxytetracycline, sulfamethoxazole and tylosin during nitrogen recovery from source separated urine via urea formaldehyde synthesis. The experimental pH was at 2 and temperature at 25°C throughout the chemical reaction, preventing urea hydrolysis. Five main chemical reactions: aqueous + antibiotics, Urine + formaldehyde + antibiotics (UF synthesis experiment), urea-formaldehyde polymer + antibiotics (adsorption experiment), formaldehyde + antibiotics and dried Urea formaldehyde polymer dissolved in the nitric acid + antibiotics (absorption experiment) were set up. The spiked antibiotics concentrations were analyzed for each reaction. Solid phase extraction (SPE), liquid chromatography and mass spectrometer (LC/MS) were used to extract and measure the antibiotics. According to the data obtained, enrofloxacin was not hydrolyzed, thus its degradation behavior was not analyzed in details. Oxytetracycline's concentration fluctuated and indicated that at some point chemical reaction occurred with presences of alpha and beta epimers. The results for sulfamethoxazole showed that the antibiotic was very stable and did not undergo any chemical reaction except during UF synthesis and adsorption experiment. Tylosin in all experimental chemical reactions showed vulnerability to hydrolysis due to its instability at pH 2 and it was completely degraded in each experiment. In addition two more chemical reactions: urine + antibiotics and selected urine (no Cl and Ca) + antibiotics were set up in order to determine the role of inorganic and organic compounds during the reaction and their effect on the fate of the antibiotics. The results showed that inorganic ion, absence or presence of Cl and Ca and organic compounds had no effect on the concentration decrease and the fate of the antibiotics. The results helped to validate and support the five main chemical reactions analysis.

Key words: Urea; Formaldehyde; UF; Antibiotics; SPE; Temperature; pH; ; Hydrolysis; SPE; LC/MS; Degradation; Experiment; Epimers.

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GLOSSARY or ABBREVIATIONS AND TERMS

| | |
|--------------------------------|--|
| TAMK | Tampere University of Applied Sciences |
| ERX | Enrofloxacin |
| OTC | Oxytetracycline |
| SMX | Sulfamethoxazole |
| CLWE | Conditioning, Loading, Washing and Elution |
| STP | Sewer Treatment Plant |
| WWTPs | Wastewater Treatment Plants |
| WHO | World Health Organization |
| P | Phosphorous |
| Mg | Magnesium |
| Ca | Calcium |
| NH ₃ | Ammonia |
| SMX | Sulfamethoxazole |
| MgO | Magnesium oxide |
| MgOH | Magnesium hydroxide |
| H ₂ SO ₄ | Sulphuric Acid |
| IBDU | Isobutylaldehyde-Diurea |
| ERX | Enrofloxacin |
| OXT | Oxytretracycline |
| OH | Hydroxide |
| UFR | Urea- Formaldehyde Reaction |
| UF | Urea Formaldehyde |
| U | Urea |
| F | Formaldehyde |
| OM | Organic Matter |
| ATB | Antibiotic |

1 INTRODUCTION

In the recent years the consumption of pharmaceutical products has increased worldwide and it is approximated that the use of these products will be between half tons to tons per year per each compound. However, the increase on the use of the products depends on location and country's population ([Kujawa, 2008](#)).

According to the recent pharmaceutical data released, it is expected that the use of the pharmaceutical product will keep on increasing for as long as good health and longer life expectancy is promoted. Figure 1, shows the data of pharmaceuticals sales worldwide.

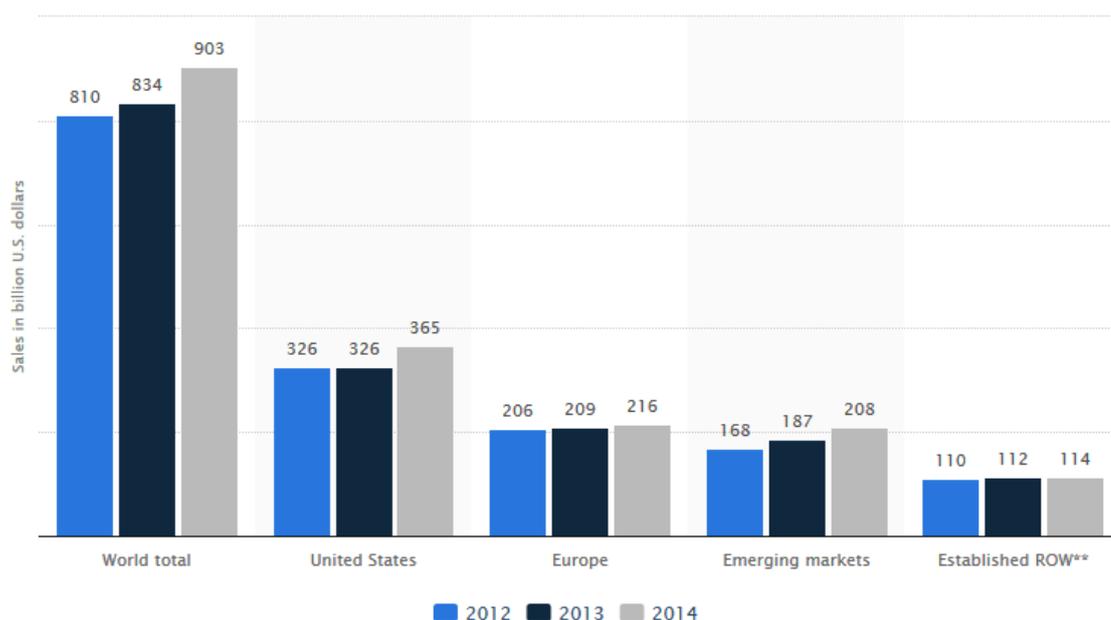


Figure 1: Latest data of pharmaceutical sales around the globe.

Source: Statista.com

Most of the pharmaceutical products used today are in the category of veterinary and are used to treat and enhance animal growth, and others are used to treat human's diseases, infections, and prevent reoccurrence of infections.

Once the drug is administered it undergoes four main stages of pharmacokinetics namely: absorption, distribution, metabolism and excretion. The excretion of the pharmaceuticals occurs into two parts, first, is the elimination of pharmaceuticals such as antibiotics via urinary tract and more than 50% of the ingested drugs are eliminated as part of urine. The second elimination occur via faecal matter and the excretion is usually less than 50%.

Pharmaceuticals and other pollutants manage to get into the environment via sewer system. This is due to the current sewer system design which is not adequate in dealing with micro-pollutants, and other problem is the mixing of urine and faeces thus making it more complicated to treat this type of wastewater. Once these compounds get into the environment they may harm the aquatic ecosystem and destroys beneficial microorganisms in the environment ([Kujawa, 2008](#)).

The toilet design play a vital role in separating urine from faeces. For an example, no mix toilet design separate faecal matter from urine. The main concept is to make sure that the separation and treatment of urine and faeces is possible and effective. Since urine is used as a fertilizers by some farmers due to its rich in nutrients, precaution on its use should be taken seriously due to the presence of pharmaceutical and other micro pollutant compounds. Thus, appropriate measures should be considered before exposing or using it as a fertilizer. The faecal matter may contain pharmaceutical residues and other micro pollutants as well, as pathogens. Therefore aggressive treatment may be the best approach to treat sources with faecal matter contamination ([Kujawa, 2008](#)).

1.1. Pharmaceuticals and their effects

The antibiotics are synthesized to target the specific metabolic and molecular pathways of microbial cells by inhibiting its functions. The main benefit of the antibiotics is to eradicate pathogen, but beside that they contain negative effects which can be lethal to humans, animals and environment. When these antibiotics are ingested and later eliminated from the host system, they contain active compounds that are introduced into the environment through faecal matter and urine. However, the fate of the antibiotics in the environment is not well known. Antibiotic compounds (ATBC) with low volatility may be associated with aqueous channel distribution whilst those with high volatility may be associated with air channel distribution. When present in the environment they may alter the microbial ecology and biodiversity, and also may negatively affect humans, animals and aquatic species ([Fent et al. \(2006\)](#)).

1.2. Pharmaceuticals in human and animals

According to [Fent et al. \(2006\)](#), pharmaceutical products are emerging environmental pollutants with increased consumption for the past decade. This is due to the promotion of good health and management of farm animals. The chemical composition of these antibiotic compounds (ATBC) varies, few of them have chemical compositions that are easily degradable whilst the majority of them are not easily degradable and they are recalcitrant to hydrolysis. Physicochemical and degradation properties such as hydrophobicity and hydrophilicity make the ATBC react differently when released into the environment. Thus, there is a need to study these compounds and evaluate their effects on the environment and impacts on human health.

For an example, in European countries, 3000 types of pharmaceuticals are used for human treatment ranging from analgesics, anti-inflammatory, contraceptives, antibiotics, beta-blockers, lipid regulators and neuroactive substances. The residues of these compounds end up in the environment, and the utilization of these classes of pharmaceuticals keeps on increasing. The presence of these compounds and their interactions with the host system such as human or animal are categorized into two, 1) pharmacodynamics characterized by what the chemical compound does to the body and consist of biochemical and physiological properties, and 2) pharmacokinetics characterized by what the host system does to the chemical compound and consist of absorption, distribution, metabolism and excretion process ([Fent et al. 2006](#)).

Beside human excretion of ATBC, hospital wastewater, industrial wastewater and landfill leachates may contain high concentrations of these compounds of which some may be recalcitrant to hydrolysis thus do not undergo biodegradation. The removal of ATBC from the sewer system poses a lot of challenges since it requires a lot of effort, energy and money. Therefore, it is recommended to treat the waste on site efficiently before discharging it into the environment. If the waste containing ATBC is not properly treated it may contaminate the rivers, lakes, estuaries, and to some extent groundwater and drinking water, and the environment at large. The application of animal manure into agricultural land from animals that have been treated or on treatment with ATBC may lead to contamination of soil, surface water due to runoff and sometimes drainage ([Fent et al. 2006](#)).

The main usage of veterinary antibiotics is to treat animal infections and enhance growth. Upon administering of ATBC, processes such as absorption and metabolism occur one after another before the residue of ATBC are eliminated from the body via urine and faecal matter. The faeces as the by-product of animal's digestion contain high level of nutrients which the farmers uses as a slurry or manure in their agricultural land or gardens. But depending on the farmer's choice, some farmers may store the manure for couple of days or weeks before applying it to the field. The percentage of active residue ATBC present in the faeces and urine are quite high, and for those farmers who apply faeces as a manure direct into the field, the active compound dose may be higher compared to farmers who store it for days or weeks or months. Therefore, the presence of this active residue compound in the faeces and urine may threaten the human health and environment at large ([Boxall et al. 2000](#)).

But luckily there are laws and regulations that have been formulated to deal with environmental pollution although, limited legislation have been developed to control the pollution originating from micro-pollutants such as pharmaceutical compound and product care. However, the directive of EU 81/852EEC, was passed to all member state to carry out an environmental risk assessment of veterinary antibiotics and other related pharmaceutical compounds, ([Boxall et al. 2000](#)).

The effects of veterinary antibiotics has not been studied extensively compared to pesticides, nutrients and other pollutants. It is a well-known fact that the methodology that have been developed to study chemical classes have never been useful for determining antibiotic compounds and other related pharmaceutical products. The risk assessment study carried out by [Boxall et al. \(2000\)](#), on selected veterinary antibiotics showed that Oxy-tetracycline containing the active ingredient of tetracycline was excreted via urine and faeces as the parent compound with approximated percentage of 40-70% of the administered dosage, and 30-95% of sulphonamides were suggested to have been excreted as unchanged. They discovered that oxy-tetracycline had low potential to leach into the soil, although the rate of degradation in aqueous media such as water indicated that the drug can be recalcitrant to hydrolysis and can persist in the aquatic environment.

In their summary, [Boxall's et al. \(2000\)](#) showed, that the predicted concentration of the selected antibiotics in the soil and water had a very minimum concentration level to cause any toxicity to aquatic life and terrestrial organisms, however, further studies need to be carried out to vindicate their analysis.

1.3. Source separation based sanitation, urine treatment and nitrogen recovery

Households produce different types of wastewater due to various activities carried out by humans such as laundry, bathing, cooking and flashing. Some wastewater treatment plant (WWTPs) collect all sources of wastewater in one pipe system, making it difficult to treat all sources of wastewater accordingly because the content of each source differ.

The concept of source separation and sanitation is centrally focused on the idea that wastewater coming from different sources can be separated based on their concentration and composition. For an example, both wastewater that comes from the toilet known as black water and urine referred to as yellow water contains different types of nutrients, organic matter, micro-pollutant and presence of microorganisms in black water, whilst wastewater originating from domestic use like bathing, washing and dishwashing known as grey water has less nutrients concentration ([Kujawa, 2008](#)).

Much of the organic matter, inorganic substances, and major fraction of nutrients such as nitrogen and phosphorus are contained in domestic wastewater and urine, thus separation is needed to recover the essential nutrients. The separation based concept is vital in concentrating the high risk sources of wastewater in a very small volume in case of urine by using reverse osmosis or other techniques where appropriate measures can be instituted to make sure that eradication of pathogens, elimination of organic micro-pollutant is guaranteed. The use of dry toilet and no mix toilet as figure 2, shows, are cardinal in promoting black water separation from yellow water and provide easy management of wastewater. Thus, it is imperative that such toilet are utilized and promoted. The resource and energy recovery can easily be instituted from this wastewater when they are clean from pathogens and micro-pollutant, thereby limiting the negative environmental effects ([Kujawa, 2008](#)).



Figure 2: No-mix toilet design

1.3.1 Urine



Figure 3: Urine sample

Figure 3, shows urine with its composition of water, organic solutes that includes; urea, creatinine, uric acid, traces of enzymes, carbohydrates, hormones, fatty acids, pigments, Pharmaceutical residues, Inorganic ions; Na, K, Cl, Mg, Ca, NH₄, P and sulfate.

According to the study carried out by [Maurer et al. \(2006\)](#), demonstrated that separating urine from other waste sources has numerous advantages such as promotion of the sustainability of wastewater management, provide best mechanism of eliminating organic micro-pollutants, and urine can be used as a fertilizer since it contains nutrients such as nitrogen and phosphate required by plant growth. However, the use of urine as a fertilizer has to follow WHO guidelines in order to manage any risk associated with its use in farm land and gardens. Nutrients such as nitrogen and phosphorous can be recovered in form of urea-formaldehyde and struvite respectively among other processes, and can be used

as a sustained release fertilizer. But the use of urine as a fertilizer remain a challenge and unresolved matter ([Maurer et al. 2006](#)).

Since urine contain nutrients needed for plant growth, its use as a fertilizer in farm land is well accepted. The urine collection system differs due its composition within the specified time, chemical alteration which occurs in a non-sterile environment, and flushing water dilute and add substances such as magnesium, potassium and calcium to the urine and further alter the composition of its content. The composition of stored urine is different from the fresh urine and under non sterile condition, urea present in urine undergo hydrolysis producing ammonia/ammonium and carbonate due to microbial activity and the action of urease enzyme. The production of ammonia/ammonium and carbonate is associated with pH increase from 6 to 9. The pH increase causes the precipitation of calcium and magnesium in the form of carbonates and phosphates. But under sterile condition the activities of urease enzyme and microbes are prevented from taking place due to the maintenance of pH below 6, under this pH, the urine content is stable and there is no production of ammonia/ammonium and carbonate ([Maurer et al. 2006](#)).

Urine usually contain low heavy metal and the concentration of nitrogen and phosphate in relation to industrial fertilizer is quiet low. To achieve the use of urine as a fertilizer, the concentration of nitrogen and phosphate has to be high enough to meet the required amount needed by the plant to grow. On the other hand, the use of urine as a fertilizer as raised a lot of questions for the past few years because of detected presence of synthetic organic micro pollutant such as pharmaceutical compounds. However, many researchers still doubt whether this micro-pollutant concentration in urine can cause negative effect on the soil, aquatic species in case of leaching and on human health through crop consumption and symbiotic relationship ([McAdell, 2013](#)).

The excretion pathway of many pharmaceutical compounds like antibiotics is through renal and faecal route, and the water solubility of these compounds are essential for the liver detoxification. The absorbed compounds are actively prepared and removed by the kidney through elimination process as part of urine. Urine contain a majority of dissolved micro-pollutants. For example, 80% of the natural oestrogen and 67% of the synthetic

hormone 17 α -ethinyl estradiol are excreted via urine and more than 50% of antibiotics are eliminated from the body through urine, ([McAdell, 2013](#)) and ([Maurer et al. 2006](#)).

1.3.2 Urine treatment units

The possible treatments units for urine are presented in Figure 4.

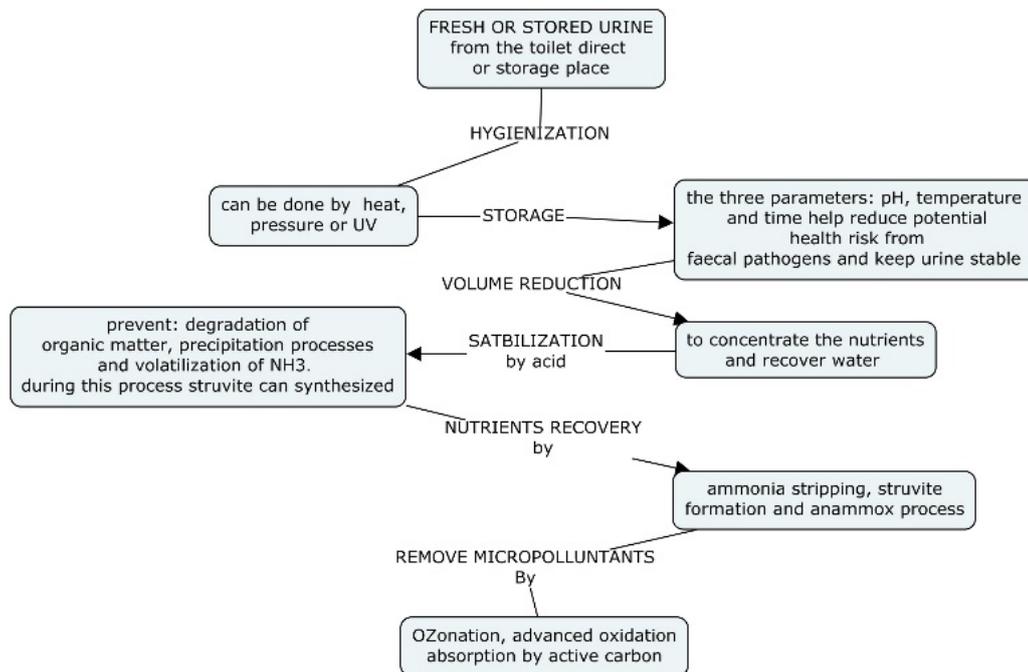


Figure 4: Urine treatment process (after [Maurer, et al., 2006](#)).

Urine is collected and transported either as fresh or stored and the transportation depends on the condition and situation such as location of the plant. The urine hygienization phase is usually done by heat or pressure or UV and it is important in making sure that microorganisms are inactivated. The storage phase of urine is done for the purpose of reducing health risk and eradication of microorganisms that may be present in case of urine contamination by faecal matter. The parameters such as pH, time and temperature are essential in determining the urine condition such as acidic or alkalinity ([Maurer et al. 2006](#)).

The volume reduction phase during urine treatment process is required in order to concentrate the nutrients, water recovery and easy storage. The process is done by evaporation, freeze – thaw (freezing the urine at 14°C), and reverse osmosis. Thereafter, urine is stabilized to prevent the hydrolysis of the urea and the degradation of organic matter (OM). The stabilization process is essential in preventing the release of ammonia

(NH₃) and increase of urine pH from 6 to about 9, thereby helping in controlling odour which may result from OM degradation. The action of urease enzyme and microbial activity causes the hydrolysis of urea molecule resulting into the formation of NH₃ and pH increase. The NH₃ which is formed escapes due to its high volatility and high pH environment whilst compounds such as Mg, Ca and P precipitate due to their low volatility. Thus, stabilization process is vital in blocking the urea hydrolysis and pH increase thereby preventing the formation and escape of NH₃, and precipitation of Mg, Ca and P ([Maurer et al. 2006](#)).

The acidification of the urine is essential and mainly done by keeping the pH below 4, thereby preventing the action of urease enzyme which causes urea hydrolysis. According to the research conducted by [Maurer et al. \(2006\)](#) showed, that the concentration of 60 mmol of hydrogen ions in one litre of urine supplied in form of sulphuric acid and kept for 250 days can keep the acidic pH environment constant and can prevent the hydrolysis of urea from taking place. At acidic pH of 4 or below, certain synthetic and micro-pollutant compounds may be degraded with respect to time of exposure. [Butzen et al. \(2005\)](#), studied selected pharmaceutical compounds such as sulfamethoxazole, tetracycline and diclofenac at pH 2. The results were that the three drugs were inactivated between the level of 50% and 95%. Nitrification is another process that can be used to lower the pH since the process itself occurs in an acidic environment, and it can also help in inactivating certain organic micro-pollutant compounds.

The recovery of nutrients such as P and N from urine are essential for enhancing plant growth. But the recovery of these nutrients are challenged with the presence of ATBC and other micro-pollutants. Therefore, before the commencement of recovery process, treating the urine is important to make sure that micro-pollutants available are eliminated. Thus preserving the biodiversity of the environment and preventing any human health risks that might be associated with drug resistance as the result of food consumption fertilized by urine. The recovery of P is mainly done by struvite techniques, where the magnesium ammonium phosphate known as struvite is recovered. The mechanism of action is that Mg is added to the urine in the form of magnesium oxide (MgO) or hydroxide (MgOH), thereby enhancing the precipitation process of the phosphate. The process usually occurs at alkaline pH. It is cardinal to note that the change of pH does not affect the recovery of P, if it is the only nutrient being recovered. However, the struvite

process is not effective in the recovery of N due to hydrolysis of urea at alkaline pH environment thereby, resulting into the escaping of NH₃ being formed ([Maurer et al., 2006](#)).

The recovery of Nitrogen as a nutrient can be done by using different methods and techniques. One of this method is the use of ion exchanger whereby, zeolite such as clinoptilolite as a natural ion exchanger is used to recover N since they have high affinity for ammonium. In addition this method can be coupled with P recovery by adding MgO to the media, this method of recovery is not widely used. Other methods includes; anammox, Nitrification, ammonia stripping (use of vacuum) and Isobutylaldehyde-diurea (IBDU) were IBDU is added to the urine to allow the reaction between urea and IBDU to occur and form a complex structure (polymer) which can be used as a slow release fertilizer ([Maurer et al., 2006](#)).

The removal of micro-pollutants from the urine is usually done by Nano-filtration, Electro-dialysis, advanced oxidation and Ozonation. Overall ozonation treatment has been proven to be more effective among other method in removing synthetic organic pollutants such as pharmaceutical compounds from urine ([Maurer et al., 2006](#)).

1.3.3 Other work on nutrients recovery

Resource recovery has become the centre of many research work and industrial focus work today. The world natural resources keep on diminishing due to population growth, human activities such as eutrophication and increase in the use of synthetic organic material. Many scientists and researchers are now focusing on the recovery of resources from already used materials such as waste biomaterial and non-biomaterial, thereby, helping to sustain the world's natural resource and allow the environment to recover.

The researchers such as [Pronk](#) and [Kone, \(2010\)](#), carried out the study which focused on collection of nutrients from urine and the use of urine after treatment. The recovery of nutrients from urine has attracted a lot of attention from both industry and research field. The main objective is to recover nutrients such as N and P from urine that are essential for agricultural purposes. Urine constitute a large percentage of N and P. However, the recovery of these nutrients are not easy because of the presence of micro pollutants and to some extent urine may be contaminated by faecal matter containing pathogens. The

elimination of this micro pollutant may be a challenge due to inefficient technology. Although the technology has advanced so much and the detection of certain micro pollutant has been carried out effectively, more is needed to be done.

Furthermore, [Pronk and Kone, \(2010\)](#), stated that the use of urine as a fertilizer, its collection and application for agricultural purposes has been in use since decades traditionally. [Maurer et al. \(2006\)](#), also indicated that different techniques on how to treat and recover nutrients from urine have been there since decades, some of each have been described earlier in this thesis. Other researchers such as [Udert et al. \(2006\)](#), [McArdell, \(2013\)](#) and [Hussein and Mona, \(2013\)](#), had similar study and experiment in their research work. All the research work outlined above emphasised on the use of N and P as a recovered nutrients from urine for agricultural purposes, and they all concluded that urine should be considered as important resource for providing nutrients needed for plant growth and treatment of urine should be prioritized.

1.3.4 Nitrogen recovery via urea formaldehyde synthesis

The recovery of nitrogen from urine can be obtained by using different techniques as described earlier by [Maurer et al. \(2006\)](#), and other researchers. However, in this research experiment the focus was on the recovery of N by adding formaldehyde to the urine content. The addition of formaldehyde to the urine is coupled with two stage reactions, the first stage is hydroxymethylated of urea (U) molecule present in urine. The mechanism of action is that, addition of formaldehyde to the media under acidic environment causes the formaldehyde molecule to be ionized thereby increasing the electrophilicity of the carbonyl group thus creating the condition for nucleophilic urea nitrogen to attack. This result into addition of OH group from protonated F to the amide group of U molecule resulting into the formation of monomethylurea molecule. Furthermore, this molecule attract or combine with each other to form dimethylurea and tetramethylurea molecule as a polymer, figure 5, shows the process of this reaction ([Conner, 1996](#)).

The second stage proceeds with condensation process by which methylureas formed are reduced to a low molecular weight polymers but in our case since the pH was at 2, the molecular weight was increasing due to series of reaction that resulted into the formation of methylene bridges with release of water molecule, the reaction between methylol and amino groups on reacting species, see figure 5, ([Conner, 1996](#)).

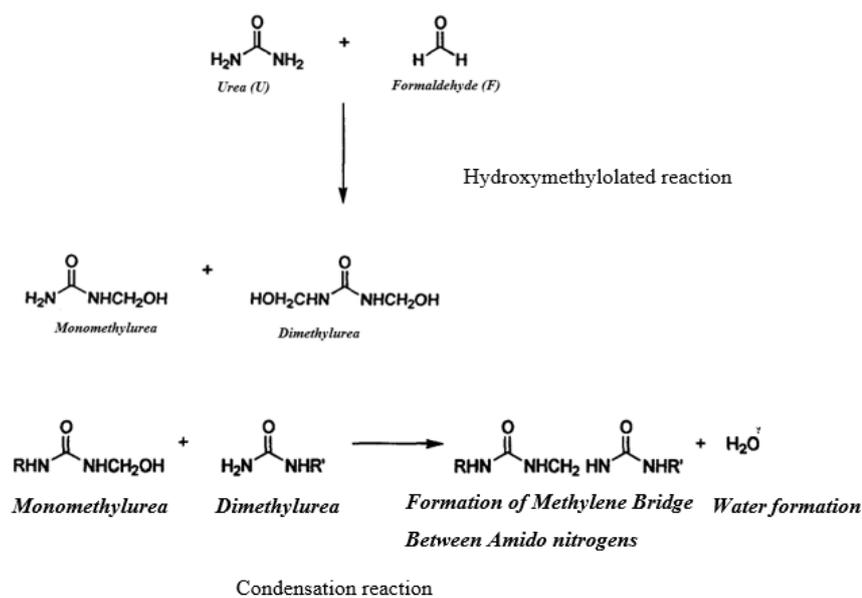


Figure 5, Hydroxymethylated and condensation process of U-F reaction

The two stages of reaction of UF synthesis depicted in figure 5, were used in the analysis of experimental results and played a significant role in determining the degradation behaviour of ERX, OTC, SMX and Tylosin during recovery process when present in the reaction. The role of acid dissociation constant (pKa) was based on determination of the ionization state of each antibiotic at pH 2, an indication that determined whether the reaction took place or not at set pH. In addition extra experiments were carried out to validate the results. The molar ratio of F to U was (1:1) in the urine content, and reaction time was restricted to 24hrs period.

Figure 6, is the schematic diagram that shows how the process of UF formation were done in the recovery of nitrogen from urine at Hokkaido University laboratory scale, starting from the initial step of making synthetic urine up to the final step of obtaining UF polymer as sustained release fertilizer. In this paper the discussion was based on the chemical process in relation to the presence of four antibiotics in urine. Thus, the reaction proceeded with additional of ATBC to the media at the chemical reaction process level where series of experiment were carried out. During chemical process samples were taken out for the analysis and monitoring of the ATBC behaviour.

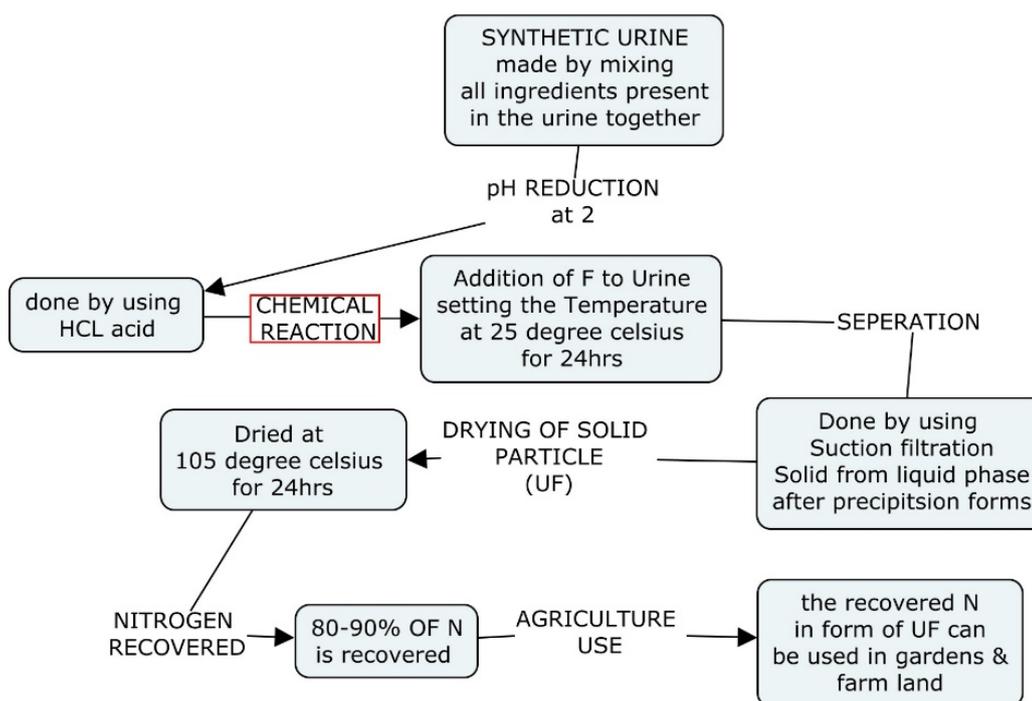


Figure 6: Nitrogen recovery process using synthetic urine

1.4. Selection of antibiotics

The fate of ATB in the urine are determined by their characteristics and how easily they can biodegrade. In this thesis our focus is on the four selected antibiotics namely; ERX, OTC, SMX and Tylosin. The selection of these four antibiotics were based on their physiochemical properties, as table 1, indicates below.

Table 1: Antibiotic selection criteria

| | |
|---|---|
| 1 | Physical – chemical characteristic (e.g. hydrophobicity and hydrophilicity) |
| 2 | Availability and usage around the globe |
| 3 | Reported eco-toxicity such as acute and chronic |
| 4 | Susceptibility to degradation |
| 5 | Belong to the therapeutic class |
| 6 | Compatible with LC/MS device |

The characteristics of each antibiotic was evaluated and noted down for experimental purposes and their variation in physiochemical characteristics had an effect on degradation behaviour. Table 2 and 3, give the main physiochemical characteristics of each antibiotic and their structure. However, due to the nature of the research experiment

conducted on this antibiotics, pKa value was the most significant characteristic considered among other characteristics highlighted. Beside the physiochemical characteristics of each antibiotic, their structure variation had an effect on the degradation rate. For example, tylosin had more hydroxide (OH) group compared to the rest of the antibiotics and it was vulnerable to hydrolysis despite having complicated structure.

Table 2: Characteristics of selected antibiotics

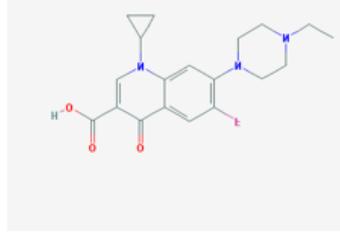
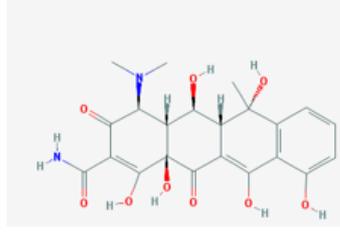
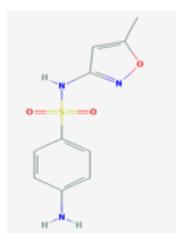
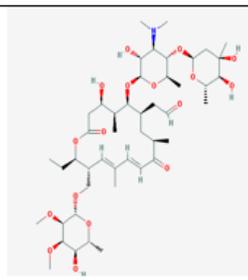
| COMPOUND | ENROFLOXACIN | OXYTETRACYCLINE |
|----------------------|---|--|
| STRUCTURE |  |  |
| Pharmaceutical Class | Antibacterial/antibiotic | Antibacterial/antibiotic |
| Log Kow | 0.70 | -0.90 |
| Pka | 6.2/7.9 | 3.2/9.5 |
| MW | 359.4 | 460.4 |
| W. Solubility | Slightly soluble at pH 7 | 313mg/l at 25°C |
| Renal Excretion | 30-40 % | 70 % |

Table 3: Characteristics of selected antibiotics

| COMPOUND | SULFAMETHOXAZOLE | TYLOSIN |
|----------------------|---|--|
| STRUCTURE |  |  |
| Pharmaceutical Class | Antibacterial/ Antibiotic | Antibacterial/antibiotic |
| Log Kow | 0.89 | 1.73 |
| Pka value | 5.7 | 7.73 |
| MW | 254.3 | 916.1 |
| W. Solubility | 610mg/l at 37°C | 5mg/l at 25°C |
| Renal excretion | 84.5 % | 70 % |

1.4.1 Mode of action of the selected antibiotics

The four selected antibiotics have different mode of action due to their chemical composition as described earlier from table 3-4. Below is the mechanism of action of each antibiotic on the bacteria cell. These selected antibiotics act on bacteria's protein synthesis, metabolic pathway and DNA or RNA synthesis to inhibit the function of the bacterial cells as table 4, shows.

Table 4: mechanism of each antibacterial agent on microbial cell

| Antibiotic Name | Mode of Action at Cell level |
|--------------------------------|--------------------------------------|
| Enrofloxacin (Quinolones) | DNA or RNA synthesis inhibitor |
| Oxytetracycline (Tetracycline) | Protein synthesis inhibitor |
| Sulfamethoxazole (Sulfonamide) | General metabolic pathways inhibitor |
| Tylosin (Macrolide) | Protein synthesis inhibitor |

2 SCOPE OF THE SUDY

The main focus of this study was to determine the fate of four selected antibiotics namely; enrofloxacin (ERX), oxytratracycline (OTC), sulfamethoxazole (SMX) and Tylosin during nitrogen recovery through urea-formaldehyde synthesis from urine. The analysis and determination of each antibiotic's fate was done by following the decrease in concentration in different media and how each content of the media affected the concentration decrease of antibiotics. During the experiment, temperature was kept constant at 25°C and pH at 2. Therefore, basing on the outcome of the results, analysis were done to determine the main causes of the concentration decrease and possible fate of these antibiotics during the formation of urea-formaldehyde polymer.

3 METHODS AND MATERIALS

3.1. Method

According to the basic rule of reaction from the chemistry point of view, the addition of F and selected antibiotics in the urine solution was assumed to trigger series of reaction during the process of UF synthesis. Thus, the following reaction were expected to occur.

1. Inorganic and organic compound in the Urine \rightarrow Urine + antibiotics
2. Formaldehyde (F) + antibiotics (present in urine) \rightarrow F + Antibiotics
3. U (CH_4N_2O) + F (H_2CO) \rightarrow UF ($C_2H_6N_2O_2$)
4. Adsorption on UF \rightarrow UF + antibiotic reaction
5. Absorption (During UF polymer synthesis) \rightarrow UF +antibiotic reaction

The above expected reactions were set according to the need and focus of the experiment and the order did not necessarily matter. The reaction 2 and 3, reactions were only valid when F is present. According to the requirement of the experiment, the expected chemical reaction were set as experimental reaction to determine the behaviour of each antibiotic when introduced to different media content. Reaction 5, was difficult to analyse as the polymer did not dissolve completely in the available acids, and the main chemical process was the reaction 3, between U and F to produce UF polymer. Figure 8, shows the list of chemical reactions and their objectives with reference to the above expected reactions that was experimented on.

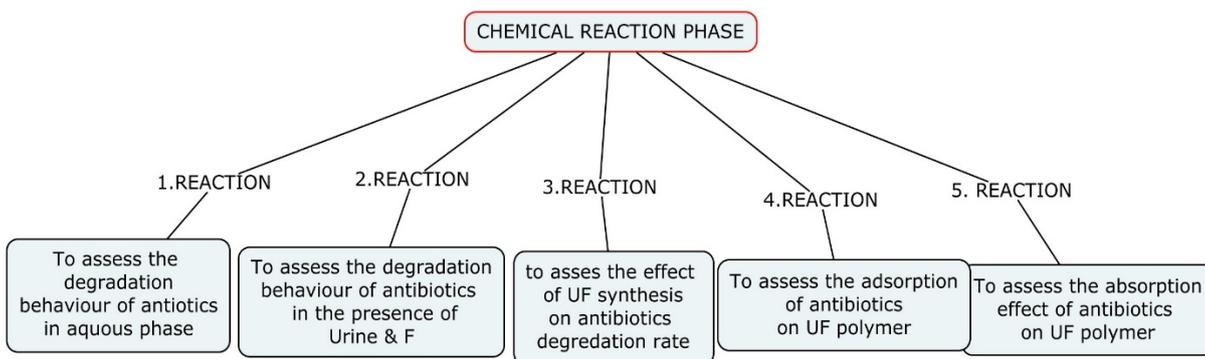


Figure 8. Reaction performed in order to assess the degradation behaviour of antibiotics

In additional to the already established experimental data, following reaction were conducted as extra experimental reaction, to further understand and monitor each media

content influence on ATBC degradation behaviour, thus ensuring the validity of the results.

- 1) Urine + Antibiotics → Inorganic and organic compounds present (from expected reaction above)
- 2) Urine (absence of Calcium and Chlorine) + antibiotics → selected urine inorganic and organic compounds

All the series of experiment were prepared by spiking 10mg/l of OTC, SMX and Tylosin, and 5mg/l of ERX into one liter beaker irrespective of their media content with constant temperature and pH throughout the experiment. The sampling was done periodically with time variation. During the sampling period, the sample was taken and directly processed using SPE process via the CLWE techniques up to the drying stage and then stored below 0 °C. After 24hrs of sampling all samples were processed further and eluted before the LC/MS detection.

3.2. Materials

In order to proceed with experiments, materials such as chemical reagents for making synthetic urine and mobile phase for LC/MS, antibiotics, SPE material and LC/MS device were required. Table 5, shows the material used such as antibiotic's concentrations, SPE cartridge, and filter and solvents types. Type of SPE equipment used can be viewed latter in figure 12 and 13.

Table 5: Essential material required for the experiment

| Antibiotics | Stock. Conc (mg/l) | SPE- Cartridge | Filters | Solvent types |
|------------------|--------------------|----------------|------------------|-----------------|
| Sulfamethoxazole | 200 | 3cc (60mg) | 0.45µm (Suction0 | Deionized water |
| Oxytetracycline | 200 | And | | And |
| Enrofloxacin | 100 | 6cc (500mg0 | 0.20µm (LC/MS | Methanol |
| Tylosin | 200 | | | |

Oxytetracycline hydrochloride was obtained from ICN Biochemical Company, Eschwege, Germany. Enrofloxacin was obtained from Tokyo Jinsei Kogyo (Japan), Company, Japan. And Sulfamethoxazole was obtained from Fluka of Sigma-Aldrich Company, St. Louis, USA. The solvents of mobile phase of LC/MS namely; acetonitrile and formic acid (99%) were purchased from Wako Company and these solvents are known as HPLC high grade, and methanol solvent was also obtained from Wako Company. The stock concentration of the selected antibiotics were prepared by weighing 20mg of OTC, SMX, Tylosin, and 10mg of ERX and put into 100ml volumetric flask and filled with methanol solvent up to the mark to obtain 200mg/l and 100mg/l respectively. Methanol was used because of solubility of the selected antibiotics. The stock solution were stored at -30°C.

3.2.1 Material preparations

Table 6, below describes the method used in order to clean the tools or equipment available in the laboratory in order to avoid contamination of instruments. This is because of many laboratory users and equipment exposure to other element such as chemicals.

Table 6: steps of cleaning the equipment or instrument

| steps | Do the following before using any equipment to avoid sample contamination |
|-------|--|
| 1 | Wash the equipment with soap and rinse |
| 2 | Fill the beaker, volumetric flask and others with tap water and add a detergent known as contaminant |
| 3 | Place the filled beaker, volumetric flask and others in the ultrasonic(to separate or loosen the particles from the glass) bath for about 10 -15 minutes |
| 4 | Rinse the beaker, volumetric and others from the ultrasonic bath with tap water and later deionized water |
| 5 | And rinse again with methanol, then proceeds to the weighing of the sample |

3.2.2 Protocol of the experiment and sample analysis

Figure 9, shows the detailed protocol used to carry out the experimental reactions.

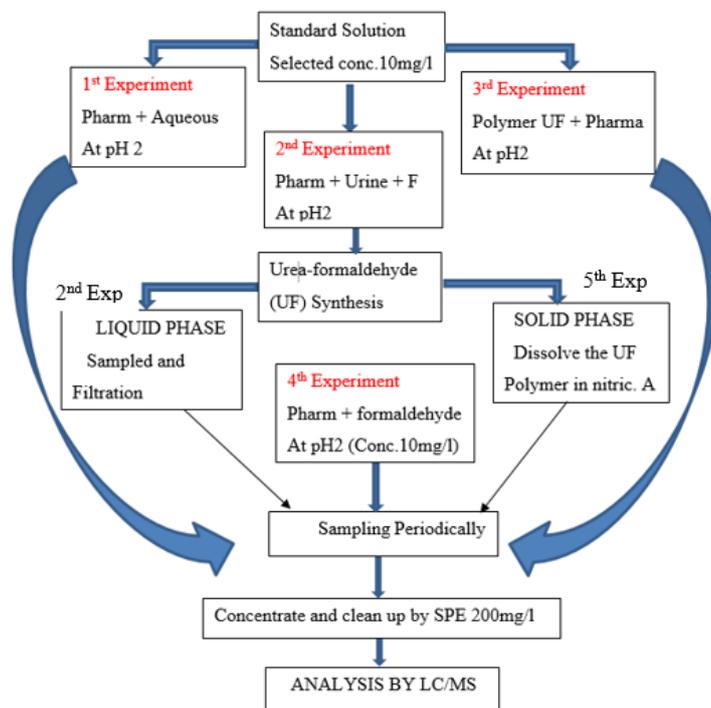


Figure 9. Experimental methods for the set reactions

The Sample preparation was done in four different stages as follows; 1) stock solution, 2) 1st dilution of the stock solution, 3) standard solution and 4) Sample extraction by SPE. Figure 10, summarizes all the steps involved before LC/MS analysis.

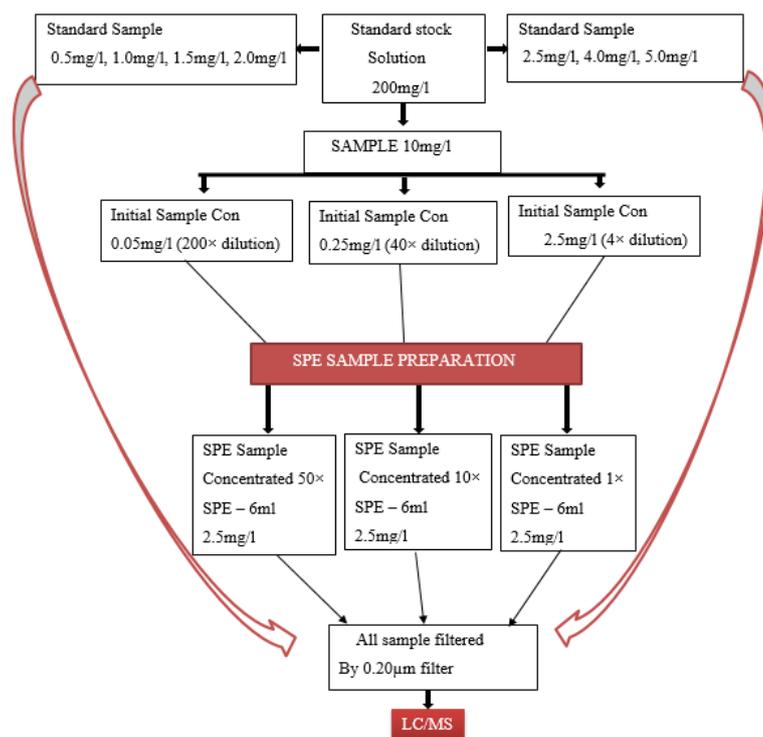


Figure 10. Detailed method of sample preparation before LC/MS analysis detection

3.2.3 Solid phase extraction (SPE)

Solid phase extraction is very important in sample preparation for LC/MS device detection. The use of SPE techniques can help solve problems such as incomplete phase separation, less than quantitative recoveries, and use of expensive breakable specialty and disposals of large quantities of organic solvents. The techniques is used to prepare samples for LC/MS detection as figure 11 and 12, shows. The SPE techniques are best for sample extraction, concentrating and clean up, and for easy detection in the LC/MS without damaging the column. Table 6, outlined series of steps necessary for carrying out SPE process effectively.

Table 6: Solid Phase Extraction (SPE) protocol using 3cc cartridge of 60ml

| STEPS | SOLVENT | VOLUME (ml) | LOADING VOLUME (ml) |
|----------------|------------------|---|---------------------|
| Conditioning | Methanol | 3 | 3*1 |
| | Pure water (pH3) | 6 | 3*2 |
| Sample loading | Sample pH3 | 6 | 3*2 |
| Washing | Pure water | 3 | 3*1 |
| | Drying | Use manifold or welch vacuum to dry the samples | |
| Elution | Methanol | 4 | 4*1 |

The SPE cartridge was first conditioned with the addition of 3 ml methanol and allowed the wetting process to take place just for few minutes or seconds depending on the volume loaded, during this stage the cartridge was protected from drying up. This allows the wetting of the reversed verse packing to be successful. After wetting, the 6 ml sample was added to the cartridge and retention of the desired compound took place, allowing the undesired compound to drip off into the waste collection flask, see figure 12. The process was then followed up by adding 3 mls of pure water (miliQ) to the cartridge in order to remove all unwanted compound and only retain the desired compound. The use of miliQ water as a washing solvent was effective in removing the weak compounds and leaving the strong compounds intact. Thereafter, drying of the sample was conducted by using Welch vacuum, the drying took 10-15 minutes. The final step was done by adding 4 ml

of methanol to the dried cartridge and for the extraction of the desired compound into 6ml collection tube thereafter, 2 ml of miliQ water was added to the 6 ml tube up to the mark in order to achieve 2/3:1/3 ratio concentration ([Waters, 2014](#)).



Figure 11. Shows water bath at 25°C and SPE-sample loading using Uni concentrator



Figure 12. Shows SPE-sample washing and SPE- extraction

3.2.4 Liquid Chromatography and Mass Spectrometer Conditions (LC/MS)

The selected antibiotics were analysed by using a dC18 reversed phase column (sunfire, US μm 2.1 \times 50mm) as stationary phase at 40°C with Waters Alliance system column oven and a cooled auto sampler (Waters 717 plus, at 4°C). The mobile phase gradient was 100% acetonitrile for phase B, and 10 mM ammonium formate, 0.3% formic acid for phase A, aqueous solution. The flow rate was 0.30 ml/min, and the injection volume was set at 10 μl .

The selected antibiotics were quantified by external standards and all the corrections, settings and view of chromatographic data were done by Waters Empower software application. The optimization of these antibiotics and cone voltage settings were done successfully. Other setting configurations were positive ion electrospray mode at 120°C, desolvation temperature at 350°C, gas flow rate at 550L/hr and flow rate of cone desolvation gas set at 50L/hr.

Figure 13, below shows the gradient program flow rate configured only to detect the selected antibiotics. The change in Mobile phase A and B had significant impact on the detection of antibiotics by LC/MS. Also table 7, contains optimization and cone voltage data of selected antibiotics.

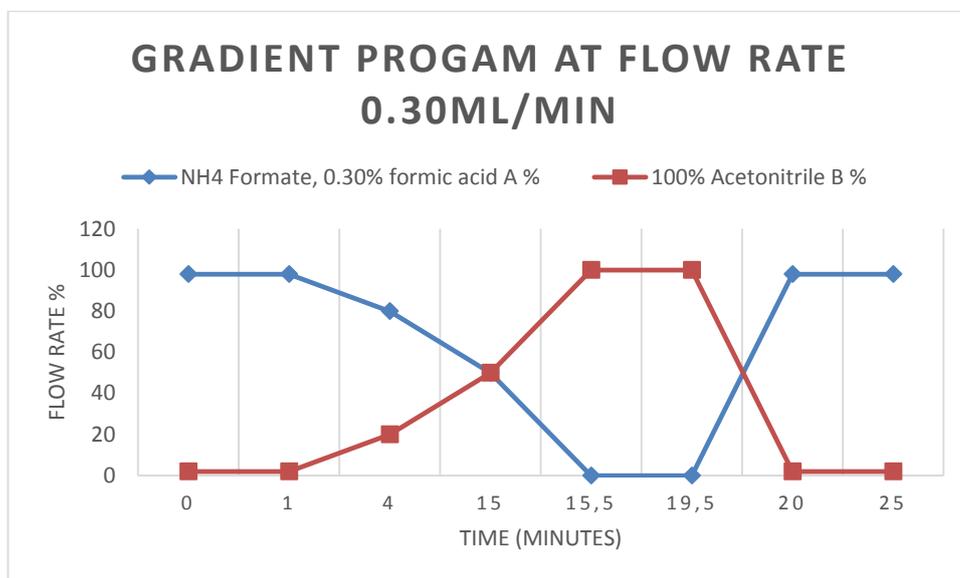


Figure 13: Gradient program for liquid chromatography

Table 7. Optimization of m/z and Cone voltage for spectrometer determination

| Selected | m/z | Cone |
|----------|-------|------|
| OXY | 254.2 | 28 |
| ERX | 360.3 | 40 |
| SMX | 461.4 | 30 |
| Tylosin | 916.9 | 63 |

3.2.5 SPE – LC/MS Recovery and Correlation data for the Experiments

Initially before the commencement of the set up experiment, recovery rate of the selected antibiotics were determined. Table 8, shows the recovery rate after six trials, see all data on appendix 1.

Table 8. Recovery rate percentage of N trials

| RECOVERY PERCENTAGE RATE (%) N=6 | | | |
|-----------------------------------|-----|-----|---------|
| OTC | ERX | SMX | Tylosin |
| 93 | 86 | 108 | 113 |

The settings and calibration of the LC/MS was done before carrying out the set experiments. According to correlation factor represented in table 9, it was more evident that the data was correct, reliable and reflected the well-functioning of LC/MS device.

Table 9. Shows the correlation factor of four main experiment conducted using LC/MS

| CORRELATION FACTOR (R ²) at pH 2 and Temperature 25°C | | | | |
|---|---------|------------------------|----------|--------------|
| Types of Exp. | ATB+ AQ | ATB + Urine + Forma | ATB + UF | ATB + Formal |
| Enrofloxacin | 0.998 | 1 | 0.989 | 1 |
| Oxytetracycline | 0.997 | 0.977 | 0.990 | 0.994 |
| Sulfamethoxazole | 0.999 | 0.998 | 0.997 | 0.997 |
| Tylosin | 0.998 | 0.997 | 0.998 | 0.986 |

4 RESULTS AND DISCUSSION

4.1. First experiment (Antibiotics + Aqueous solution)

The media contents of this experiment were antibiotics spiked in one liter beaker containing aqueous water. Table 10, shows the results obtained after LC/MS analysis.

Table 10: LC/MS results for 1st Experiment

| 1st EXPE. REPEAT AQUEOUS + PHARM CONC(mg/l) | | | | |
|---|-----------|------------|------------|-------------|
| Time(min) | 0.05ERX | 0.05OTC | 0.05SMX | 0.05Tylosin |
| 0 | 5 | 10 | 10 | 10 |
| 10 | 5.78±0.08 | 9.25±0.51 | 9.74±0.34 | 9.42±0.18 |
| 40 | 6.05±0.09 | 9.60±0.25 | 11.05±0.19 | 9.75±0.59 |
| 120 | 5.69±0.09 | 9.66±0.28 | 10.30±0.26 | 8.38±0.21 |
| 1320 | 6.27±0.03 | 10.50±0.11 | 10.89±0.53 | 0.59±0.06 |
| 1440 | 5.94±0.12 | 11.35±0.49 | 11.64±0.47 | 0.46±0.01 |
| 1 st EXPE. REPEAT AQUEOUS + PHARM CONC(mg/l) | | | | |
| Time(min) | 0.05ERX | 0.05OTC | 0.05SMX | 0.05Tylosin |
| 0 | 5 | 10 | 10 | 10 |
| 10 | 5.78±0.08 | 9.25±0.51 | 9.74±0.34 | 9.42±0.18 |
| 40 | 6.05±0.09 | 9.60±0.25 | 11.05±0.19 | 9.75±0.59 |
| 120 | 5.69±0.09 | 9.66±0.28 | 10.30±0.26 | 8.38±0.21 |
| 1320 | 6.27±0.03 | 10.50±0.11 | 10.89±0.53 | 0.59±0.06 |
| 1440 | 5.94±0.12 | 11.35±0.49 | 11.64±0.47 | 0.46±0.01 |

Table 10, indicates that, ERX, OTC and SMX in aqueous solution did not undergo:

1). chemical reaction, 2). The concentration was increasing, though the causes was undefined. In this case the decline of tylosin concentration was due to hydrolysis of the molecule under strong acidic pH.

In aqueous media, the behaviour of Oxytetracycline was relative stable and did not show any signs of degradation. The pKa values for OTC is -1.22, 4.5 – 9.2, this simply means that at pH 2, OTC molecule might have been in unionization state thus, no reaction took

place, [Snow et al](#) and, [Zhimin and Adams, 2004](#)). SMX's concentration remained stable despite small fluctuations in concentration throughout the experimental period. It is possible that there was no reaction that took place and SMX was not ionized. The pKa value for SMX is in the range of 1.7 – 5.6, and at pH 2 the molecule was more likely to be neutral, and the acidic environment had no effect on SMX concentration reduction ([Chen, et al., 2011](#)).

Tylosin antibiotic compound throughout the experiment was unstable and gave a lot of trouble during LC/MS analysis. The concentration varied a lot and it was observed that at pH 2, Tylosin was extremely unstable and degraded so rapidly within 24hrs as seen in table 10. The results and observations obtained were supported by [Remington's, \(2006\)](#), [Loftin's, et al \(2008\)](#) and [Paesen's, et al, \(1995\)](#), experiments and observations that tylosin at acidic pH 2 is unstable and can be completely degraded within 12 hrs of reaction although, in our case it took 24 hrs for tylosin to degrade may be this was due to late elution in the LC/MS column and other undefined reasons.

4.2. Second experiment (Urine + Formal + antibiotics)

The media contents of this experiment were antibiotics spiked in one liter beaker containing formaldehyde and urine in solution form. Table 11, shows the results obtained after LC/MS analysis

Table 11: LC/MS results for 2nd experiment

| 2nd EXP. REPEAT PHARM+FORMAL+URINE (mg/l) | | | | |
|---|------------|------------|-----------|-------------|
| Time(min) | 0.05ERX | 0.05OTC | 0.05SMX | 0.05Tylosin |
| 0 | 4,83 | 9,66 | 9,66 | 9,66 |
| 10 | 5.92±0.05 | 8.76±0.37 | 9.55±0.49 | 6.55±0.14 |
| 40 | 5.63±0.04 | 8.67±0.17 | 1.75±0.01 | 3.59±0.09 |
| 180 | 5.51±0.12 | 8.57±0.23 | 1,36 | 2.11±0.08 |
| 300 | 6.158±0.12 | 10.11±0.50 | 1,35 | 1.40±0.04 |
| 1320 | 6.52±0.03 | 9.21±0.27 | 1,34 | 0,00 |
| 1440 | 7.32±0.06 | 9.86±0.07 | 1,34 | 0,00 |

| 2 nd EXP. REPEAT PHARM+FORMAL+URINE (mg/l) | | | | |
|---|------------|------------|-----------|-------------|
| Time(min) | 0.05ERX | 0.05OTC | 0.05SMX | 0.05Tylosin |
| 0 | 4.83 | 9.66 | 9.66 | 9.66 |
| 10 | 5.92±0.05 | 8.76±0.37 | 9.55±0.49 | 6.55±0.14 |
| 40 | 5.63±0.04 | 8.67±0.17 | 1.75±0.01 | 3.59±0.09 |
| 180 | 5.51±0.12 | 8.57±0.23 | 1.36 | 2.11±0.08 |
| 300 | 6.158±0.12 | 10.11±0.50 | 1.35 | 1.40±0.04 |
| 1320 | 6.52±0.03 | 9.21±0.27 | 1.34 | 0.00 |
| 1440 | 7.32±0.06 | 9.86±0.07 | 1.34 | 0.00 |

The concentration of ERX and OTC was much stable throughout the experiment although there was some fluctuation in concentrations. ERX and OTC behaviour was hard to determine due to complex physiochemical interactions in the solution. The concentrations were observed to be increasing. Gajurel, Et al. (2004) also observed an increase in the final concentration compared to the initial concentration during their experiment. SMX concentration reduced remarkably after 10min, possibly due to interaction or reaction between intermediate products of UF and SMX to form a conjugate or different chemical compound with different chemical structure.

According table 11, tylosin concentration decreased rapidly as seen in table 10 previously. This results were expected since tylosin under pH of 2 was observed to be unstable in aqueous media. The notable observation was that tylosin concentration reached 0mg/l rapidly, an indication that the process of UF formation might have accelerated the concentration decrease and completely degraded the tylosin molecule. The results and observations made during the experimental process was similar to Adams', et al, (2008), observations, where the degradation pattern was not predictable.

The OTC concentration was observed to be fluctuating and with a decrease at 180 minutes which can be ignored or analysed, however the concentration remained constant for the rest of the experiment. Although, the decrease in concentration was considered as a negligible it is possible that partial reaction could have occurred due to OH group present on the molecule or conformational change which may have resulted from epimerization. [Morrison and Boyd, \(1983\)](#), [Mayr and Ofial, \(2005\)](#), [Loftin et al., \(2008\)](#) and [Xuan et al., \(2010\)](#), in their OTC degradation experiment, observed three types of

OTC degradation pattern referred to as epimers namely; alpha-OTC, beta-OTC and 4-epi-OTC, see figure 18 later. Alpha epimer was observed at the initial phase of the reaction and beta epimer later and finally the 4-epimer a bit later. Thus, looking at our results it is possible that the variation in concentration was triggered by partial reaction which might have resulted into formation of alpha epimer, but with time this epimer reverted back to parent OTC compound thus the concentration remained constant.

The mechanism behind the SMX concentration reduction and partial reaction of OTC as well as tylosin degradation behaviour is further discussed in the later subject (the behaviour of selected antibiotics).

4.3. Third experiment (Urea- Formaldehyde + antibiotics)

The media contents of this experiment were antibiotics spiked in one liter beaker containing Urea-formaldehyde in solution form after 24hrs of UF synthesis. The experiment was set up to determine the adsorption of antibiotics on the UF polymer. Table 12, shows the results obtained after LC/MS analysis.

Table 12: LC/MS results for 3rd experiment

| | 3rd experiment UF + PHARM | | | |
|-----------|---------------------------|-----------|-----------|-------------|
| Time(min) | 0.05OTC | 0.05ERX | 0.05SMX | 0.05Tylosin |
| 0 | 9.5 | 4.8 | 9.5 | 9.5 |
| 10 | 10.94±0.66 | 5.44±0.11 | 7.29±0.25 | 9.37±0.39 |
| 180 | 12.01±0.32 | 6.35±0.19 | 4.39±0.17 | 6.12±0.15 |
| 1320 | 6.48±0.17 | 3.78±0.06 | 1.85±0.01 | 0.76±0.04 |
| 1440 | 10.69±0.59 | 6.34±0.08 | 2.31±0.07 | 0.75±0.01 |

From table 12, it was evident that adsorption occurred for SMX during the 24hrs reaction time. The adsorption might have occurred due to interactions between neutral charge of sulfamethoxazole and Urea-formaldehyde intermediate species and Van der Waals forces might have played a major role between stabilized UF and SMX. It is possible that both ERX and OTC underwent physiochemical reaction at 1320 minutes which resulted into

concentration fluctuation. Tylosin degradation behaved as observed in other two experiments.

During formation of urea-formaldehyde, OTC concentration decreased at 1320 minutes an indication that some reaction might have taken place and both alpha and beta OTC could have been present in the media as earlier discussed. However, the concentration after 1320 minutes started to increase, an indication that at sampling time the sampled solution might have contained unstable molecule which could have undergone partial reaction thus resulting into unstable conformation either by partial ionization or reacting partially with other molecules available in the media ([Loftin et al., 2008](#)).

The adsorption of the SMX on the UF polymer might have taken place between the stabilized UF and neutral charge of the SMX. According to the experimental data given in table 12 and observations, the rate of degradation of SMX was slower compared to UF synthesis process given in table 11, this was attributed to the UF molecule of not having enough energy to carry out further reaction and having limited number of intermediate molecules compared to UF reaction. However, both reactions proved to have degradation effect on SMX, and both Van der Waals forces and amide nucleophilic charge properties played significant role. In this case epimerization was not the stopping point of degradation for SMX. The mechanism of adsorption of SMX on UF polymer is further discussed later in this paper.

4.4. Fourth experiment (Antibiotics + Formaldehyde)

The media contents of this experiment were antibiotics spiked in one liter beaker containing formaldehyde and aqueous water. The main purpose of this experiment was to rule out the effect of formaldehyde on antibiotic's concentration decrease. Table 13, shows the results obtained after LC/MS analysis.

Table 13: LC/MS results for 4th experiment

| 3rd EXP. REPEAT PHARM+ FORMAL CONC(mg/l) | | | | |
|--|-----------|-----------|------------|-------------|
| Time(min) | 0.05ERX | 0.05OTC | 0.05SMX | 0.05Tylosin |
| 0 | 5 | 10 | 10 | 10 |
| 10 | 5.42±0.05 | 8.99±0.05 | 9.54±0.16 | 11.31±1.26 |
| 40 | 5.64±0.08 | 9.09±0.26 | 9.65±0.65 | 9.39±0.19 |
| 180 | 5.68±0.11 | 9.53±0.14 | 10.57±0.30 | 6.62±1.02 |
| 1320 | 5.58±0.16 | 6.75±0.27 | 11.04±0.37 | 1.37±0.04 |
| 1440 | 5.85±0.10 | 7.16±1.38 | 11.58±0.15 | 1.26±0.01 |

| 3rd EXP. REPEAT PHARM+ FORMAL CONC(mg/l) | | | | |
|--|-----------|-----------|------------|-------------|
| Time(min) | 0.05ERX | 0.05OTC | 0.05SMX | 0.05Tylosin |
| 0 | 5 | 10 | 10 | 10 |
| 10 | 5.42±0.05 | 8.99±0.05 | 9.54±0.16 | 11.31±1.26 |
| 40 | 5.64±0.08 | 9.09±0.26 | 9.65±0.65 | 9.39±0.19 |
| 180 | 5.68±0.11 | 9.53±0.14 | 10.57±0.30 | 6.62±1.02 |
| 1320 | 5.58±0.16 | 6.75±0.27 | 11.04±0.37 | 1.37±0.04 |
| 1440 | 5.85±0.10 | 7.16±1.38 | 11.58±0.15 | 1.26±0.01 |

From table 13, it was observed that the behaviour of tylosin was still the same as observed in aqueous media and other two experiments. The continued concentration decrease of tylosin was an indication that if time was prolonged for more than 24hrs, it may have reached 0mg/l. ERX and SMX was stable and the concentration remained constant despite variation. While OTC showed some decrease in concentration at 1320 minutes the sign that some reaction might have taken place. Overall, the presence of formaldehyde in the media did not affect the ERX and SMX concentration decrease.

4.5. Fifth experiment Absorption (UF + Antibiotics)

The media contents of this experiment were the UF polymer dissolved in nitric acid and later lowered the pH to 2 and thereafter spiked the antibiotics. The main purpose of this experiment was determine if there was any presence of antibiotics in the polymer after drying it at 105°C for 24hrs, and the experiment was referred to as absorption. Figure 14, shows the results obtained after LC/MS analysis

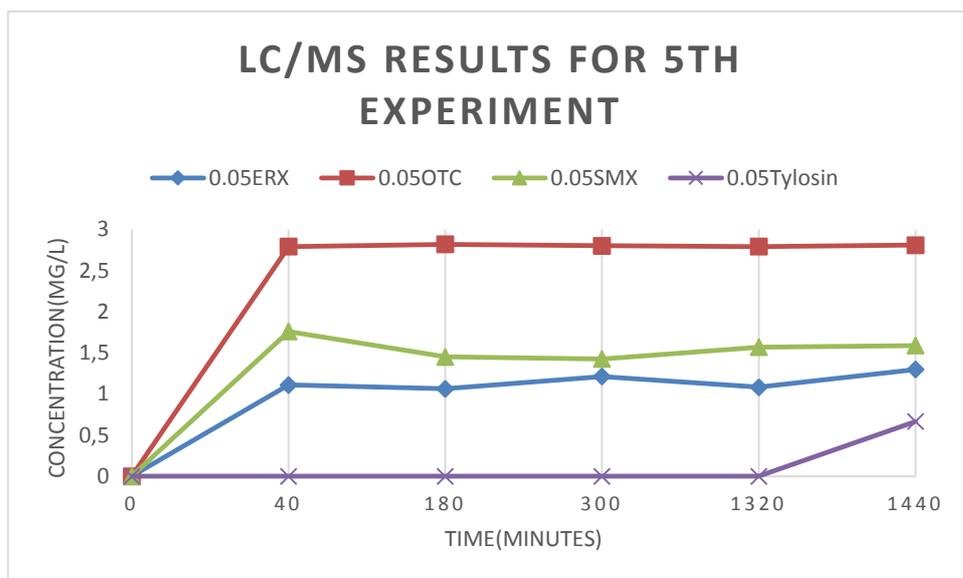


Figure 14. The traces of antibiotics molecule in the UF polymer

From figure 14, it was observed that the antibiotics were present in the UF polymer, an indication that absorption took place. The concentration of OTC, ERX and SMX were relative stable and the highest concentration was 2.82mg/l, 1.21mg/l and 1.76mg/l respectively. Tylosin concentration was not detected until after 24hrs at which 0.67mg/l was detected. This could have been late elution or other reasons unknown, however the detected concentration value of tylosin was neglected.

4.6. Additional experiment (Urine + antibiotics)

The media contents of this experiment were antibiotics spiked in one liter beaker containing urine in solution form. The main purpose of carryout this experiment was to supplement the already obtained knowledge during the analysis and evaluation of above experiments in order to assess if the inorganic and organic substances present in the urine had effect on the antibiotics concentration. Figure 15, shows the results obtained after LC/MS analysis.

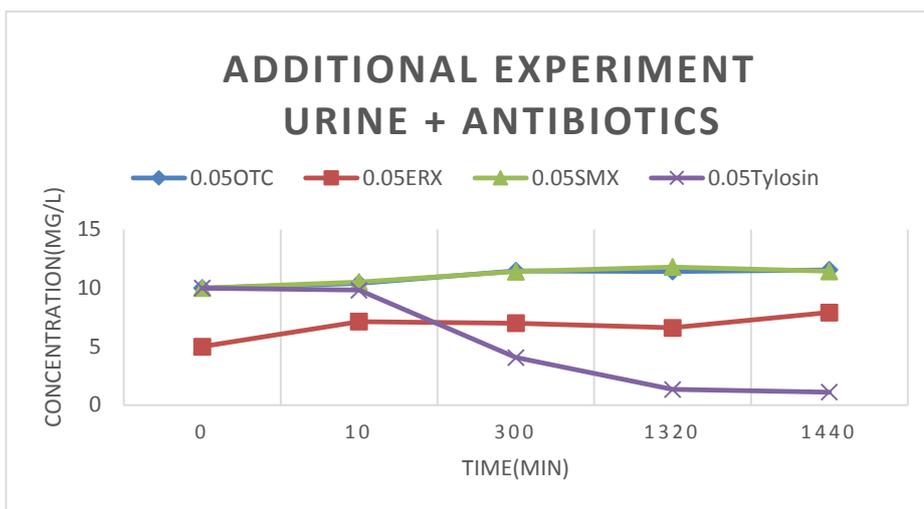


Figure 15: Antibiotics behavior during in the urine media after 24 hrs.

According to figure 15, ERX, OTC and SMX did not undergo chemical reaction the concentration remained relatively stable throughout the experimental period. But for tylosin as usual observation, its concentration decreased rapidly.

4.7. Additional experiment (absence of Cl and Ca (in the urine) + antibiotics)

The media contents of this experiment were antibiotics spiked in one liter beaker containing urine with no Cl and Ca in solution form. The main purpose of carryout this experiment was to supplement the already obtained knowledge during the analysis and evaluation of above experiments in order to observe if absence of Cl and Ca had an effect on antibiotics concentration decrease. Figure 16, shows the results obtained after LC/MS analysis

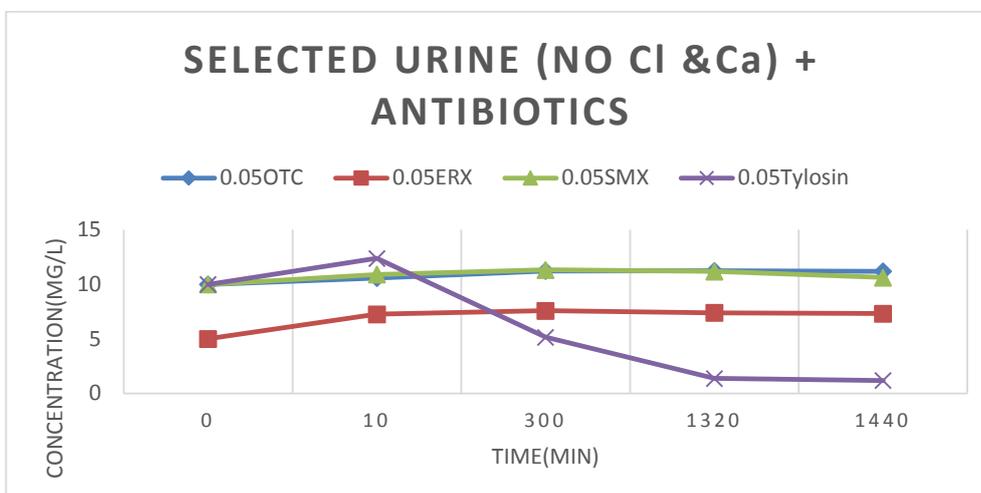


Figure 16: Antibiotics behaviour in the absence of chorine and calcium after 24 hrs.

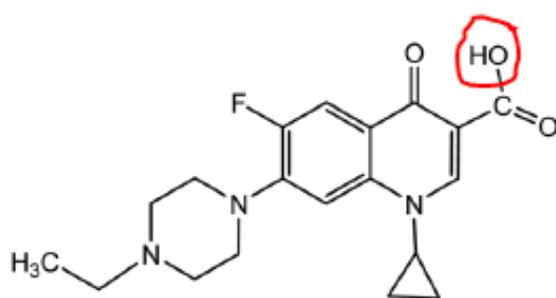
As figure 16, shows, the concentration of ERX, OTC and SMX compounds remained constant and stable after 24 hrs of reaction whilst tylosin's concentration decreased as usual. An indication that the absence of Cl and Ca did not affect the antibiotics concentration.

4.8. Behavior of antibiotics under different condition-discussion

The behaviour of the enrofloxacin, oxytetracycline, sulfamethoxazole and tylosin during series of experiment was analysed and evaluated basing on the outcome of the results. The analysis of these antibiotics were centered on the chemical reaction of the given media content. For more information on the peaks and standard curve of these antibiotics, see sample on appendix 2.

4.8.1 Enrofloxacin

Enrofloxacin was not much evaluated due to recalcitrant to hydrolysis. The antibiotic belongs to quinolones family that consist of carboxyl group at one end and methyl group on the other end as figure 17, shows below. The pka values of this antibiotic molecule falls in the ranges of 2.74, 2.74 – 7.11, an indication that at pH 2, the antibiotic did not ionize thus rendering it difficult to be hydrolysed and its functional group such as hydroxyl and methyl group were not easily substituted ([Zhimin and Adams, 2004](#)).



Enrofloxacin

Figure 17, enrofloxacin chemical structure

Figure 18, summarizes the trend of enrofloxacin in different media content during the experimental reactions. As depicted in figure 18, the concentration of the enrofloxacin in all the media was relative stable except in urea-formaldehyde media where the decrease in concentration was observed though it was negligible.

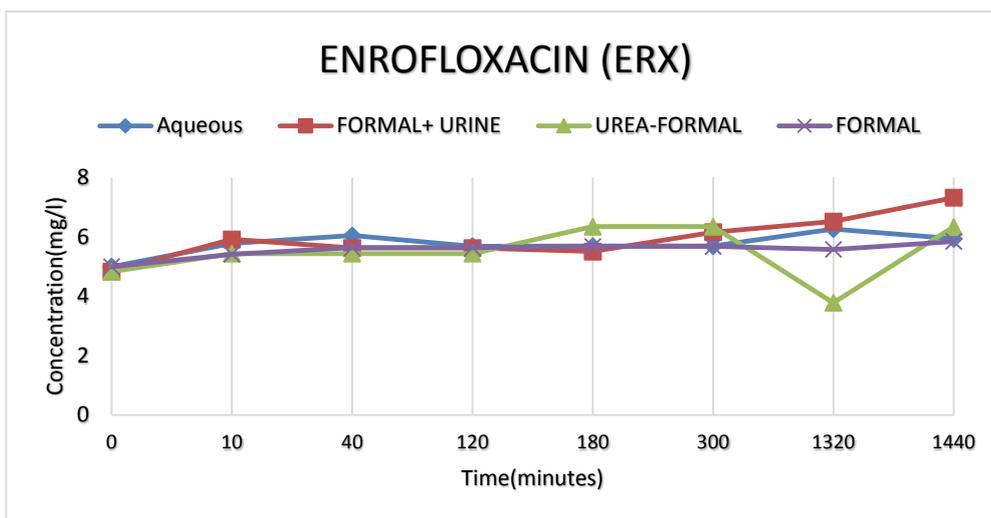


Figure 18. Shows the behaviour of Enrofloxacin during four main experiment

Enrofloxacin at this state cannot undergo chemical reaction thus remained neutral and recalcitrant to hydrolysis. During the experimental analysis, it was observed that at pH 2, enrofloxacin remained relative stable throughout all series of experiment as observed earlier in the results section. The experiment was conducted for twenty four hours (24hrs). During ionization it is possible for the hydroxyl group to be replace with another group such as halogens for example, chlorine because of weak acid function of carboxyl group. However, it was not the case for ERX.

4.8.2 Oxytetracycline

The behaviour of the oxytetracycline varied depending on content of the media. Figure 19, represents the trend of OTC during experimental reaction.

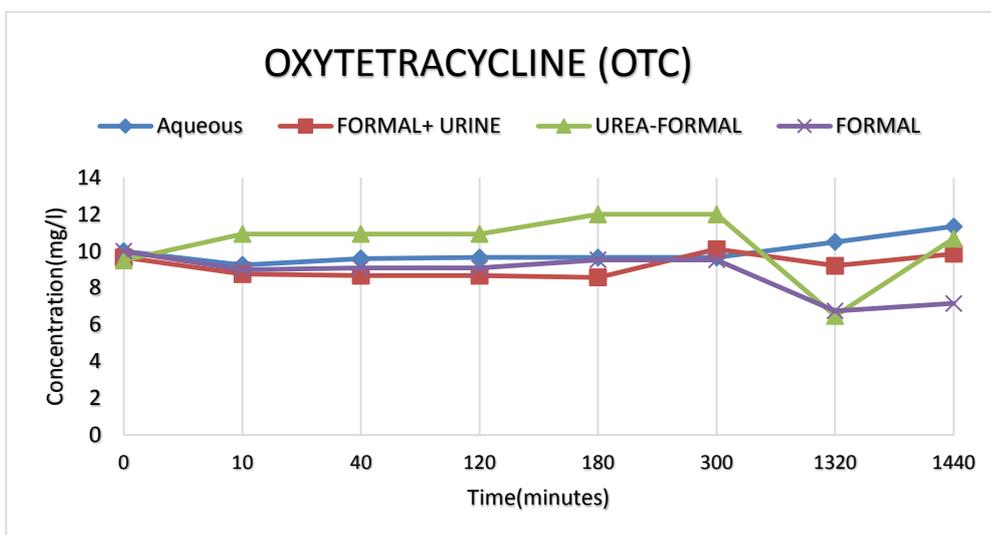


Figure 19. Shows the behaviour of Oxytetracycline under different media content

The analysis of the OTC characteristics during reaction of different media content was based on the formation of epimers. Epimers are characterized by change of chemical structure due to conformation or end product of degradation compared to the original or parent molecule. During the experimental reaction it was possible that following epimers of the OTC parent drug were formed with respect to time either due to molecule conformation or partial ionization. Figure 18, shows three types of epimers with likelihood of beta and alpha epimer being formed during the experimental reactions.

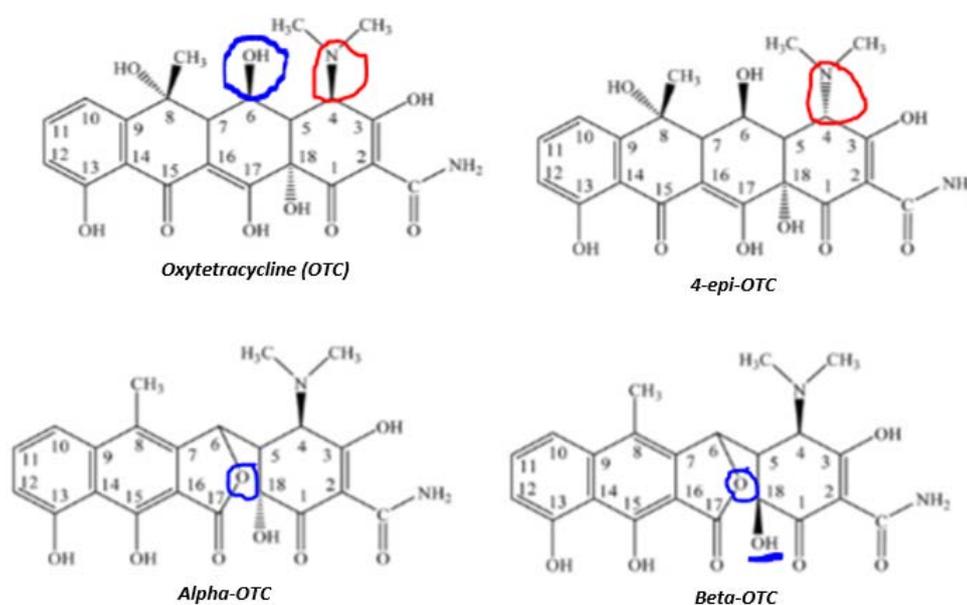


Figure 20. Three types of epimers during OTC degradation

Source: [Loftin, A. K, et al., \(2008\).](#)

According to [Loftin, A. K, et al., \(2008.\)](#), study on OTC degradation behaviour, it was assumed that during the fluctuation of the concentration especially in UF (4th Exp) and F (3rd Exp) media content, the alpha epimers and beta epimers were present during the concentration decrease at 1320 minutes. But this epimers disappeared with time with the possibility that they reverted back to original OTC molecule. Thus they did not form a stable conformation compound. The functional group OH at carbon six (C-6) determines the basicity and acidity of the molecule. According to experimental analysis of [Zhimin and Adams, \(2004\)](#), [Xuan, et al., 2010](#)) and, [Loftin, A. K, et al., \(2008\)](#), explained that the structure rearrangement or replacement of OH group with other group may induce steric hindrance which may prevent the molecule from forming conjugate with other molecule present in the media and block other group from being attached to the molecule.

4.8.3 Sulfamethoxazole

Sulfamethoxazole was one of the antibiotic used in the experiment that was stable and acted as indicator of the reliability of the results and method efficiency. The behaviour of SMX in different media depended on the type of elements present in the media. Generally, according to [Morrison and Boyd, \(1983\)](#), sulphonamide functional group are more difficulty to hydrolyse due to steric factors such as 3D-size and shape. This fact can be seen from the four experiment conducted on SMX, only two experiment was capable of influencing the concentration reduction. Figure 21, shows how the media content influenced SMX concentration reduction.

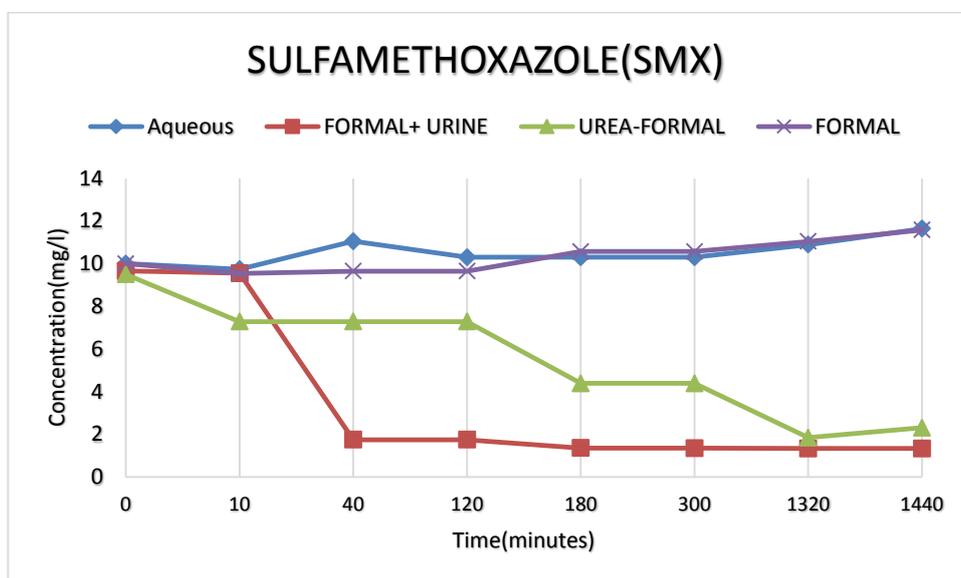


Figure 21. Shows the Sulfamethoxazole behaviour under different condition

Therefore, during the UF synthesis (2nd Exp) and U-F polymer (4th Exp) the concentration of the SMX reduced remarkably from the initial phase of the experiment. The reason for this decrease was attributed to the nucleophilic properties of the SMX molecule and the presence of Urea formaldehyde (UF) intermediates which was positively charged. The mechanism of action was that during the formation of UF, SMX molecule was neutral but carried a negative charged nucleus. Thus, the presence of positive UF intermediates created the attraction between the two molecules, thereby forming a conjugate molecule which resulted into the degradation of original SMX molecule, and forces such as Van der Waals were equally at play between the stabilized UF and SMX as both carried neutral charge. Figure 22, Shows the reaction that might have taken place between two molecules ([Mayr and Ofial, 2005](#)).

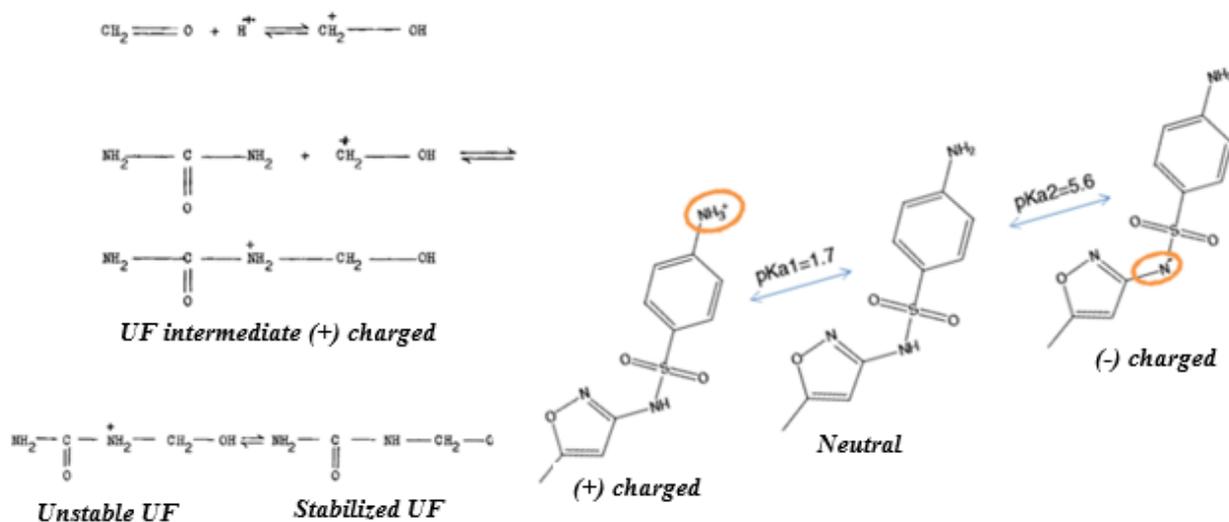


Figure 22, Synthesis of Urea- formaldehyde with its intermediate and ionization state of the SMX molecule at different pKa values

Urea formaldehyde reaction was very effective in the degradation rate of sulfamethoxazole, an indication that during nitrogen production via the synthesis of urea formaldehyde polymer, SMX as an antibiotic can be degraded rapidly within 24hrs although epimerization seemed to be the stopping point of degradation because the concentration remained constant 1.34mg/l at 22hrs and 1.34mg/l at 24hrs, however longer time experiment needed to be conducted to confirm this fact. According to the results obtained indicates that UF polymer can have little or no antimicrobial effect on both humans and environment when used as a sustained release fertilizer in farms and gardens. Thereby reducing the possibilities of SMX resistant in treating various kind of infections in humans and preserve the alteration of the biodiversity of the microorganisms and other symbiotic organisms.

4.8.4 Tylosin

Tylosin as an antibiotic is used to treat animals, and throughout the experiment it was unstable and gave a lot of trouble during LC/MS analysis. The concentration varied a lot and it was observed that at pH 2, Tylosin was extremely unstable and degraded so rapidly within 24hrs. From figure 23, below, it was observed that at all condition Tylosin's concentration was reduced remarkably.

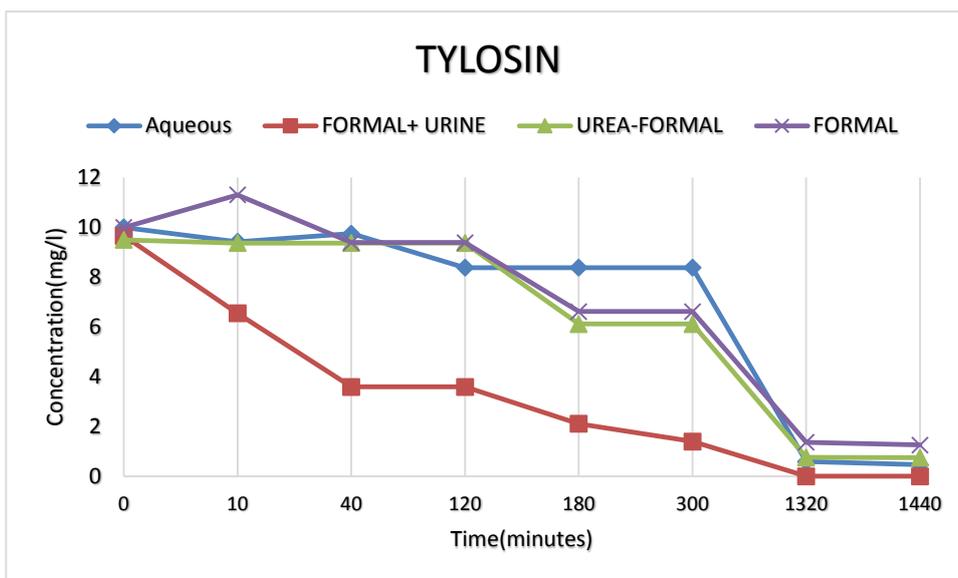
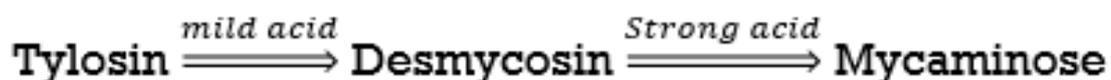


Figure 23. Depicts the behaviour of tylosin at different Condition

[Korzybski's, et al, \(1967\)](#) and [Hamill's, \(1965\)](#), during analysis of tylosin, they discovered that at mild acidic level tylosin parent compound was hydrolysed to desmycosin which contains antimicrobial activities and under strong acidic level such as (pH 2) it was completely hydrolysed to release a non-antimicrobial agent known as mycaminose sugar as depicted below.



The aqueous (1st Exp), U-F (3rd Exp) and F (4th Exp) experimental reactions had little or no effect on the tylosin degradation because at pH 2 tylosin in the aqueous media was hydrolysed and degraded. The minor observation was noted during the experimental reaction of UF synthesis (2nd Exp) were the concentration of tylosin reached 0mg/l after 22hrs of reaction. This may have been an indication that UF synthesis acted like a catalyst to speed up the hydrolysis and degradation rate of tylosin to reach 0mg/l faster than in U-F polymer (3rd Exp).

Therefore, if we analyse the data of [Korzybski, et al, \(1967\)](#), [Loke, et al, \(2000\)](#) and [Hamill, \(1965\)](#), we may assume that during the experimental reaction at pH 2, mycaminose sugar could have been the only molecule present in the media after 24 hrs of reaction. Since this molecule have no antimicrobial effects, and it cannot alter the

biodiversity of microorganisms, the use of UF as a fertilizer cannot be harmful to environment and humans in the case of tylosin.

4.8.5 Absorption and adsorption of antibiotic on UF polymer

The absorption experiment was carried out to vindicate if the UF polymer may contain antibiotic residue and what concentration may be present. According to the test performed it was observed experimentally that OTC, ERX and SMX residue were detected by LC/MS device in a low concentration while for tylosin no concentration was detected. The detected concentration was too low to cause any harm to the environment and humans, however, further absorption experiment should be carried out to substantiate this fact. Despite the polymer not dissolved completely in the acid, the detection of antibiotic residue compound in the UF polymer was an indication that absorption occurred thus precaution on the use of UF as a fertilizer should be taken into consideration.

During the absorption experiment the UF polymer was dried up at 105°C for 24hrs. Thus the heating of the polymer might have played a role as well in concentration reduction of the antibiotics. It was noted that the structure of each antibiotic had effect on the degradation behaviour. Tylosin was vulnerable to hydrolysis and its concentration dropped sharply due to its structure as observed in figure 23, and figure 24, shows tylosin's structure with hydroxyl group as a functional groups which make the molecule unstable under strong pH. ERX, SMX and OTC have relative stable structure as depicted in figure 24, and they were very stubborn to hydrolysis. Their structure consist of amide and carboxyl group which make them very stable under acidic condition.

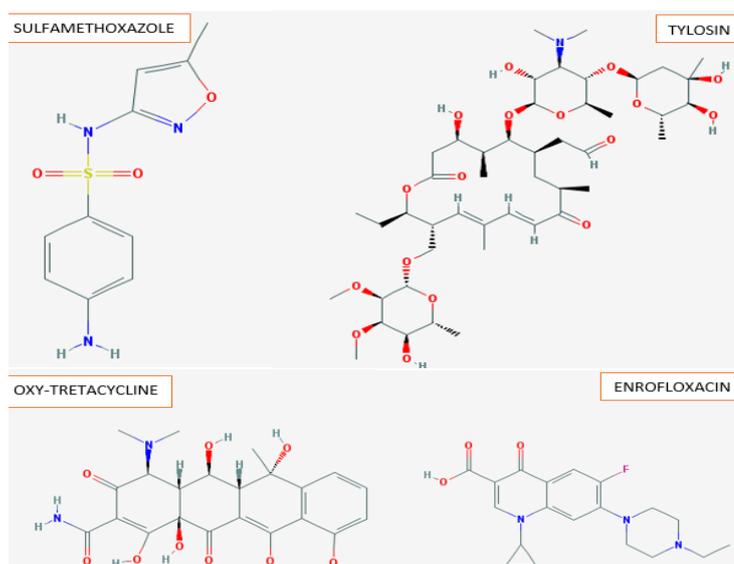


Figure 24. Shows the structure of ERX, OTC, SMX and Tylosin

5 CONCLUSION

In summary, after conducting series of experiment it was evident that UF synthesis had effect on the degradation rate of SMX and the adsorption experiment confirmed that SMX molecule was adsorbed on UF polymer. UF synthesis had little or no effect on the degradation pattern of ERX and OTC due to their recalcitrant behaviour. But Tylosin proved to be vulnerable to hydrolysis in all media under strong acidic pH of 2, and the concentration reduction was independent of any media content. Tylosin's final product was assumed to be mycaminose sugar according to series of studies conducted before.

The absorption experiment showed that residue of antibiotic compounds might be present in UF polymer but at a lower concentration. It was noted and observed that the structure of each antibiotic molecule had effect on the degradation behaviour. Overall, the results obtained are more interesting and need further analysis. To some extent series of experiment should be carried out to determine exactly the mechanism of degradation of SMX and evaluate why ERX and OTC were stubborn to hydrolysis and devise best method to get rid of them. The effect of heat during the drying of UF polymer should be studied carefully in order to determine its effect on antibiotic degradation. Finally, separate experiment for each antibiotic is needed to narrow the analysis and eliminate assumptions.

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APPENDICES

Appendix 1. Recovery percentage

The recovery of all experiment starting from 3-6, table 1 shows that.

Table 1. SPE Recovery

| SPE TRIAL RECOVERY RATE COMPARISON | | | | |
|------------------------------------|--------|--------|--------|--------|
| TRIAL no. | 3 | 4 | 5 | 6 |
| 0.05mg/l | | | | |
| OXY | 81.14 | 80.56 | 93.2 | 78.14 |
| ERX | 108.57 | 100.14 | 85.68 | 64.77 |
| SMX | 85.17 | 91.11 | 107.67 | 98.98 |
| Tylosin | 44.96 | 70.74 | 112.99 | 123.5 |
| | | | | |
| 0.25mg/l | | | | |
| OXY | 79.42 | 86.74 | 92.02 | 61.92 |
| ERX | 106.83 | 111.62 | 83.75 | 76.26 |
| SMX | 78.72 | 93.65 | 106.15 | 100.41 |
| Tylosin | 50.12 | 63.92 | 175.49 | 98.36 |
| | | | | |
| 2.5mg/l | | | | |
| OXY | 87.38 | 97.41 | 90.7 | 89.81 |
| ERX | 121.3 | 129.86 | 84.74 | 73.25 |
| SMX | 82.42 | 103.34 | 103.61 | 100.4 |
| Tylosin | 79.74 | 93.91 | 268.7 | 97.64 |

Appendix 2. Standard curve and peaks

The data from the experiment was well correlated and their reliability was guaranteed. Some of the standard curve as shown in figure 3, were obtained during recovery process

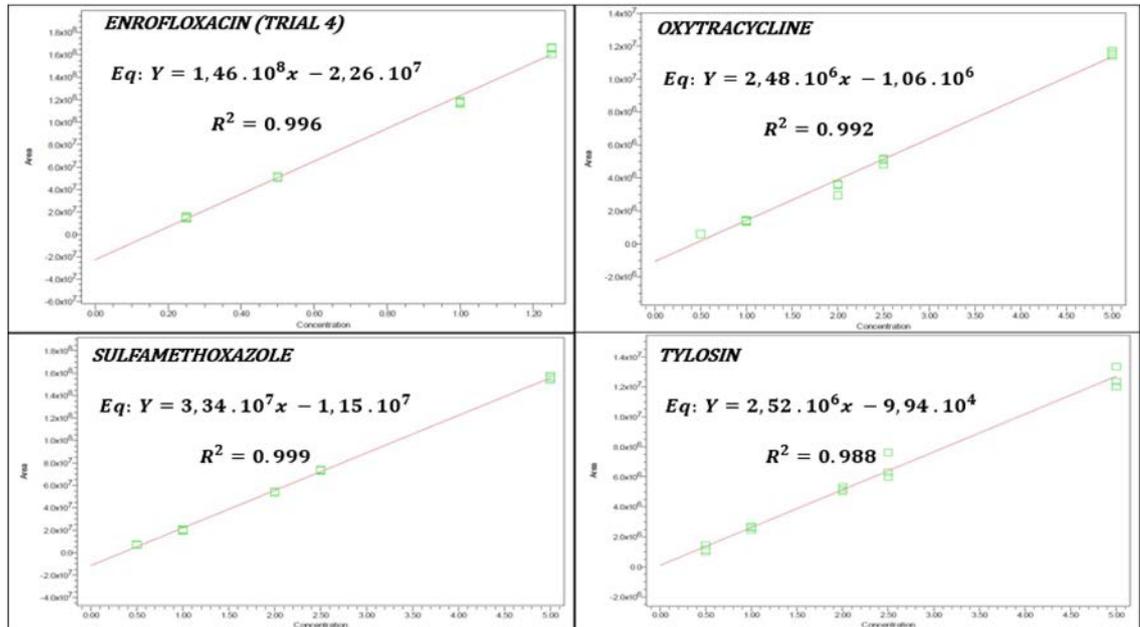


Figure 3. Data from recovery experiment

Figure 4, represent peaks obtained from recovery process

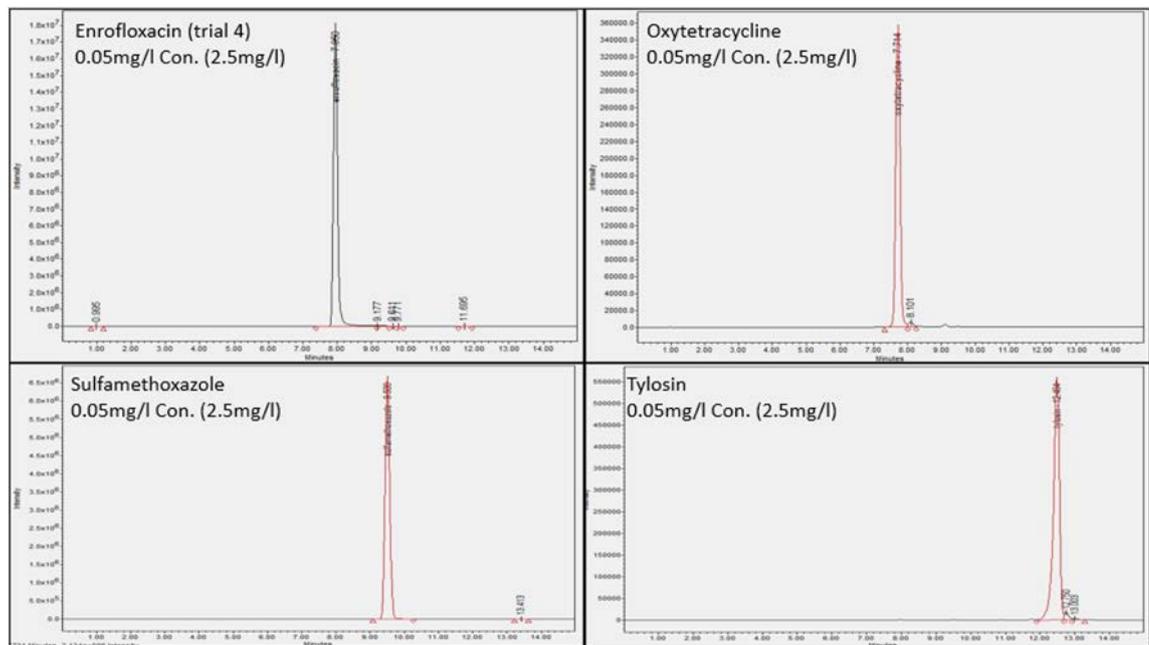


Figure 4. Shows the peaks obtained from the recovery process of OTC, ERX, SMX and Tylosin.