UNIVERSITY OF CAPE COAST

DEVELOPMENT OF A NEW ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD WITH FLUORESCENCE AND DAD DETECTION FOR EFFICIENT SEPARATION AND ANALYSIS OF ANTIPARASITIC (ANTIBIOTICS, PESTICIDES, ANTIOXIDANTS) AGENTS IN FISH

BY

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Thesis submitted to the Department of Chemistry of the School of Physical Sciences, College of Agriculture and Natural Sciences, University of Cape Coast, in partial fulfillment of the requirements for the award of Doctor of Philosophy degree in Chemistry

OCTOBER 2020

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidates Signature …….. Date…….

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Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

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ABSTRACT

The use of antiparasitic agents in aquaculture is of a major concern due to its adverse effect on human health. This research developed and validated a new rapid Ultra High Performance Liquid Chromatography (UHPLC) method using fluorescence and Diode Array (DAD) detection for identification and quantification of antiparasitic agents (antibiotics, pesticides antioxidants) in fish samples. This was done in accordance with the European Commission 2000 guidelines. Separation and identification of antiparasitic agents was achieved on a Syvchronis C18 analytical column (100 mm ×2.1 mm I.D., particle size 1.7 µm) with mobile phase acetonitrile/ acetic acid, magnesium nitrate at a flow rate of 0.6 mL min^{-1} and at 35°C. The detection wavelength of the detector was set at excitation wavelength (λ_{exc}) 350nm and emission wavelength (λ_{em}) 451nm for sarafloxacin and ethoxyquin, 251nm absorption wavelength (λ_{abs}) for diflubenzuron and teflubenzuron and finally, absorption wavelength (λ_{abs}) 596 nm for crystal violet and malachite green. Under the optimum chromatographic conditions, standard calibration curves were measured with good linearity (r^2 = 0.99991) for all the analytes (n = 10). The method detected lower concentrations of these antiparasitic agents studied in this research with the detection limit of 0.056, 0.0122, 5.080, 1.575, 0.2654, 0.3870 µ/kg for Sarafloxacin, Ethoxyquin, Diflubenzuron, Teflubenzuron, Crystal Violet and Malachite Green respectively. These detection limits were lower than the Maximum Residual Limit set by the European Union. Recoveries obtained for all the various type of antiparasitic agents in fish were between 80%-99.73%.

KEY WORDS

Antiparasitic Agents

Diode Array Detection

Fluorescence Detector

Optimization

Solid Phase Extraction

Ultra High Performance Liquid Chromatography

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Finally, I thank my mom, dad, siblings and all who in diverse ways helped in the completion of this work.

DEDICATION

To my lovely children and husband, Meshack Antwi-Adjei.

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ABBREVIATIONS

ACN	Acetonitrile
API	Active pharmaceutical Ingredient
BEH	Bridged Ethylene Hybrid
BG	Brilliant Green
CIP	Ciprofloxacin
CV	Crystal Violet
DAD	Diode array Detector
DM	1,8′-di(1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline
d-SPE	Dispersive Solid Phase Extraction
DFB	Diflubenzuron
EDTA	Ethylene diamine Ttra acetic acid
EC	European Commission
ENR	Enrofloxacin
EU	European Unioun
EQ	Ethoxyquin
FLR	Fluorescence
FLU	Flumequine
FDA	Food and Drugs Authority
HPLC	High Performance Liquid Chromatography
ICP-MS	Inductive Couple Plasma Mass Spectrometry

IL-DLLME	Ionic Liquid dispersive liquid-liquid Method Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
LCMS/MS	Leuco Malachite Green
LMG	Leuco Malachite Green
LLE	Liquid- Liquid Extraction
LC-EC	Liquid Chromatography Electron Capture
LCV	Leuco Crystal Violet
LC	Liquid Chromatography
MEPS	Micro Extraction Packed Sorbent
MG	Malachite Green
MS/MS	mass spectrometer (MS) and tandem mass spectrometer
MSDS	Material Safety and Data Sheet
MIP	Molecular Imprinted Polymer
MRL	Maximum Residual Limit
OXO	Oxolinic
NAL	Nalidixic
NMR	Neutron Magnetic Resonance
NPs	Nanoparticles
PDA	Photodiode Array
PAH	Polycyclic Aromatic Hydrocarbons

PFCs	Per fluorinated compounds
QI	2,6-dihydro-2,2,4-trimethyl-6-quinolone
QUECHERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
RSD	Relative Standard Deviation
SA	Sarafloxacin
SPE	Solid Phase Extraction
USA	United States of America
USFDA	United States Food and Drug Administration
TFB	Teflubenzuron
TPM	Triphenylmethane
UPLC	Ultra Performance Liquid Chromatography
UHPLC	Ultra High Performance Liquid Chromatography
UV	Ultra Violet

CHAPTER ONE

INTRODUCTION

Chapter one captures the introduction and the background of antiparasitic agents such as their definition, some analytical techniques used in identifying them as well as some existing analytical methods used in identifying and separating them. The problem statement gives insight into how the existing analytical methods quantitatively and qualitatively determine only one group of antiparasitic agent. The problem statement further indicates the need for having a single analytical method that is economical, save time and environmentally friendly.

The purpose of study and justification section under the chapter indicates some problems caused by antiparasitic agents and which calls for more research to be done to regularly check its presence in fish. When these antiparasitic are regularly checked international trade of fish would be enhanced and this would ensure the safety of fish to consumers. This chapter also raises some specific objectives and the hypothesis for the study.

Background to the Study

Pesticides, antibiotics, antioxidants are different types antiparasitic agents used in aquaculture. These are drugs which are purposed for preventing, curing, or controlling infestation as a result of helminths *(*Kappagoda, Shanthi, & Singh, 2011), amoeba (Molina *et al.*, 2002) and protozoa (Kappagoda *et al.*, 2011) and so on. Antiparasitic, target the parasitic agents of the infections by destroying them or inhibiting their growth (Purdue Research Foundation, 2015). Normally, they are effective against few parasites which are in the jurisdiction of a define group.

Diseases as a result of bacterial infections are normally cured with antibiotics. In most cases antibiotics are used in preventing bacterial infections in water or the fish rather than its use as curative measure (Alderman & Hastings, 1998; Christensen & Baun, 2006).

Other antiparasitic agents such as pesticides (Diflubenzuron (DFB) and Teflubenzuron (TFB) are some examples of pesticides that act by preventing the biological synthesis of chitin production. These pesticides are potent in controlling a variety of insects and infestation of the sea lice (Hormazabal & Yndestad, 1996). Lepeophtheirus salmonis and C'aligus elongatus are increasingly becoming a problem in aquaculture especially in salmonids aqua farming. These lice destroy the skin, causing thriftiness, and in critical cases, the lice may lead to the death of fish. The parasite can also transmit microbial pathogens, and infestations may affect wild salmonids. In comparing sea lice infestations between wild and farmed fish, there is a higher prevalence of infestations in farmed fish than wild fish (Hormazabal & Yndestad 1996). These scenarios explained above mostly may lead to fish's accumulation of these chemicals and exposes consumers to these chemicals. Indeed, the need to monitor occurrence of antiparasitic agent residues in fish samples is crucial to ensure food safety.

Currently, ethoxyquin is often added to feed for farmed fish, and also to pet food that have been canned. The essence of adding this additive to animal feed is to protect it against lipid peroxidation. Ethoxyquin is normally not added to edible food for humans only with the exception of spices. The route by which these additives get to human beings is through the feed of poultry which is also

transferred to the eggs and since human beings are at the end of the food chain they are exposed to these additives.

Apart from the fact that antiparasitic drugs have severe adverse reactions, it presence in the environment especially, water bodies may lead to drug resistance. A situation, which is rendering existing, drugs inactive. This means that, there is high demand for research and innovations in the management of neglected tropical diseases. For instance, there is a strong call for newer and safer drugs development for the management of Chagas disease, leishmaniasis, and trypanosomiasis (Lacerda, Pelegrini, Oliveria, Vasconcelos, & Sa, 2016). This is due to the fact that, the most effective drugs used for management of these diseases such as Mebendazole is associated with high failure rates in treating hookworm infections, and single-dose Albendazole is also associated with a low rate of cure for trichuriasis.

However, the need to monitor, detect and quantifying these agents in our environment is crucial and very necessary. This call for analytical techniques, which is reliable, sensitive and robust. Currently, there are several analytical methods available for such purposes but have few challenges, which need to be addressed. One of the challenges in monitoring and quantifying antiparasitic agents is unavailability of a dependable and sensitive single method for the effective analysis of different types of antiparasitic agents (antibiotics, dyes, pesticides, antioxidants). Some analytical techniques used in analyzing antiparasitic agents includes: Enzyme linked immunosorbent, Liquid chromatography with photometric and fluorescence detector, UHPLC with Electronspray Ionization

Tandem Mass Spectrometry (UHPLC/ES/MS), Liquid chromatography coupled with Tandem mass spectrometry (LC/MS), Liquid chromatography with visible and fluorescence detector (LC/FLD), High-performance liquid chromatography tandem mass spectrometry (HPLC/MS), Dispersive liquid-liquid micro-extraction using ionic liquid followed by zero crossing first derivative spectrophotometric analysis, Liquid chromatography-electrochemical, High performance liquid chromatographic method with diode array detector (HPLC/DAD), Reversed-phase high-performance liquid chromatography with fluorescence detector (LC/EC/HPLC), and among others. All the existing analytical methods available in identifying and determining antiparasitic agents only targeted one group of antiparasitic agents. For instance, high-performance liquid chromatographic method with fluorescence detector has been used for the screening and quantification of (antibiotics) oxolinic acid, flumequine and sarafloxacin in fish by Roudaut & Yorke (2002). This research only identified and separated antibiotic. Hormazhal &Yndestad (1996) and Luvizotto-Santos, Marques, Cordeiro, & Vieira, (2009) also used High performance liquid chromatography to identify and separated pesticides. Bohne, Hove & Hamre (2007) also used reversed-phase high-performance liquid chromatography with fluorescence detector for the detection of synthetic antioxidant (ethoxyquin) and its key metabolite in salmon.

A summary of an analytical method by Cañada-Cañada, Espinosa-Mansilla, Girón, & Muñoz (2012) is described as follows:

Cañada-Cañada *et al.* (2012), also developed a method based on the technique of chromatography. These researchers determined a number of different types of

antibiotics that were mainly fluoroquinolones. They detected these fluoroquinolones in fish with their method. The extracting solvent they employed in extracting the samples were m-phosphoric acid and acetonitrile, these organic solvents were in the ratio of 75:25 V/V. Purification of the extracts was conducted on the cartridge packed with ENV + isolute. A column packed with C 18 in a liquid chromatography process was used in the detection. The composition of the mobile phase was at a pH of 4.5 and it consist of methanol acetonitrile and 10 mM citrate buffer. At an optimized gradient program, the mobile phase was made to deliver at a flow rate of 1.5 ml/min these conditions contributed in the separation within 26 minutes. The fluoroquinolones were detected with both UV wavelengths (280 and 254 nm) and fluorescence (280/450, 280/495, 280/405 nm). The method achieved a lower limit of detection between 0.2-9.5 µg/kg and 0.7-32 µg/kg for limit of quantitation. At concentration of 50 and 100 µg/kg the fish were spiked and the average recoveries were between 50%-102%. (Cañada-Cañada *et al.*, 2012).

Some requirement of analytical method includes effectiveness, robustness, sensitivity and high resolution with minimal analysis time. The developed method in this research meets all the requirements and its application will improve the existing methods. However, in spite of the advances in chromatographic separations techniques, sample treatment is the most essential part of the analytical process and effective sample preparation is important for attaining better analytical results (Lucci, Pacetti, Núñez, & Natale, 2012). A perfect sample preparation method should be fast, precise, accurate and must keep sample intergrity. Solid phase extraction (SPE) has been chosen for the sample preparation because of its

high versatility in areas such as purification, trace enrichment, desalting, derivatization and class fractionation. Even though, SPE and liquid-liquid extraction (LLE) method have similar principles, SPE has been chosen over LLE because of the many problems associated with LLE. Some of the problems includes: incomplete phase separations (emulsion), low quantitative recoveries, use of expensive breakable, specialty glassware and disposal of large amount of organic solvents. A reversed phase (SPE) which is made up of polymeric bonded sorbent has been chosen for this new developed method because, it can withstand extreme pH, and thus it is more suitable for environmental applications for trapping organic compounds from acidified aqueous samples. In addition, polymeric sorbents have a higher potential of trapping aromatic compound, especially phenols (Masqué, Galia, Marce, & Borrull, 1999). Lastly, polymeric sorbents have a much higher surface area; hence, they have a higher loading capacity.

Statement of the Problem

The residual detection of these antiparasitic in samples of food origin is usually challenging due to the comparatively lower maximum residue limit (MRL) defined by the legalization and complexity of the food matrices composition. Hence, the use of effective pre-concentration methods and sensitive detection techniques are preferable tools widely used for antiparasitic determination in food samples (Stolker & Brinkman, 2005).

All the existing analytical methods developed for determining antiparasitic agents in fish (antibiotics, pesticides antioxidants) focused on either only antibiotics, (Roudaut & Yorke, 2002); (Cañada-Cañada *et al.*, 2012); (Guidia *et*

al., 2018); (Tyrpenou, Iossifidou, & Psomas, 2002); (Li *et al.*, 2009); (Lombardo Garcia Campana, Cruces-Blanco, & Gamiz-Gracia, 2015); (Ying *et al.*, 2011); (Dowling, Mulder, Duffy, Regan & Smyth, 2007); (Ashraful, Aktermst, Yeasmin, & Islam, 2015); **(**Sadeghi & Nasehi, 2018), pesticides (Hormazhal &Yndestad, 2006 and Luvizotto-Santos *et al.*, 2009) and antioxidants (Bohne *et al.*, 2007); (Rodríguez-Gómez, Marie, Zafra-Gómez, & Jean-Michel, 2018); (He & Ackman, 2000).

These methods developed though very good, they are not economical, do not save time and are environmentally unfriendly due to excessive use of chemicals. It would take several methods in order to analyze antiparasitic agents (antibiotics, pesticides antioxidants) in fish making the process expensive and tedious. Hence, there is the need to develop single analytical method that would be able to determine different forms of antiparasitic (antibiotics, pesticides antioxidants) agent in a single run.

Aims

The main trust of this work is to:

1. develop analytical method, which is of low detection limit, less expensive, robust, and efficient in quantifying, separating and detecting different forms of antiparasitic agents in a single run.
2. validate the analytical method on a representative matrix type (fish).

Specific Objectives

The specific objectives of this study is to:

1. develop analytical method, which is efficient in separation and detection of different forms of antiparasitic agents.
2. develop an analytical method of low detection limit, less expensive, robust for detection and quantifying antiparasitic agents in a single run.
3. optimise the pH of the buffer used in extraction and pH of the aqueous phase.
4. optimise the volume used in the SPE extraction and the concentration of the aqueous phase.
5. optimise the gradient time range.
6. optimise the flow rate and Injection volume.

Purpose of the Study

An appropriate analytical method is developed only after assessing the main and critical separation parameters of chromatography. Examples of UPLC/HPLC parameters are selection of diluent, wavelength, detector, stationary phase, column temperature, flow rate, solvent system, elution mode, and injection volume, etc.). This research seeks to present an analytical method that would help scientific research in chromatography to effectively, and efficiently monitor antiparasitic agents (antibiotics, dyes, pesticides and antioxidant) in a single run. The low levels of detection (μgkg^{-1}) established for these antiparasitic agents require sensitive and specific methods to monitor and determine unequivocally antiparasitic agents' residues in aquatic products. Based on this information, it is important to monitor the presence of antiparasitic agents in fish in order to allow international trade and to protect consumers from health hazards. The presence of such residues in food can be responsible for toxic effects, allergic reactions in individuals. This current

project seeks to contribute to the search for newer and more effective method of monitoring trace amount of environmental contaminant (antiparasitic agents). In addition, data from this study will provide a foundation for further chromatographic method development, may augment planning of policy makers, and serve as a data for reference purposes. The analytical method developed will provide a suitable developed analytical method that is fit for its intended use for the quantitative estimation of the targeted analyte present in biological samples especially, fish samples.

Justification

Globally the use of antiparasitic agent in aquaculture is of a major concern due to its adverse effect on human health. International bodies like European Union among others have set maximum residual limit to control some of these antiparasitics agents in foodstuff. To regulate some of these antiparasitic agents in food matrix, several scientific analytical methods have been developed. All these researches dwelt on only one type of antiparasitic agents. For instance, Cañada-Cañada *et al.* (2012) and Guidia *et al.* (2018), developed analytical method to analyse only antibiotics. Hormazhal &Yndestad (1996) and Luvizotto *et al.* (2009), also developed analytical method to analyse only pesticides. Bohne *et al.* (2007); Rodríguez-Gómez *et al.* (2018); He & Ackman (2000) also developed analytical method to analyse only antioxidants. All the methods available include laborious, procedures steps, this is because, it would require a method to analyse antibiotics another method to analyse pesticides, another method to analyse antioxidants. This would result in the consequences of more use of chemicals, generating large

amounts of residues in the environment and time wasting. In this context, there is a need for a single method that would be simple, fast and reliable quantitative analysis of antiparasitic agents in aquaculture fish.

Hypothesis

The study seeks to test the following hypothesis in attempt to achieving the objectives stated:

1. H_1: One single UHPLC method can identify and separate different groups of antiparasitic agents.
2. H_1: The LODs of the propose UHPLC method will be lower than literature methods and that set by European commission on Maximum Residual limit from food.
3. H_1: The recovery of antiparasitic agents in the present UHPLC method will be higher than the existing methods.

Delimitation

The fish samples used in this research were only obtained from Spain (Cordoba). The method was applied mainly to fish matrix and for other matrices optimization may be required

Limitation

Oxytetracycline was not included in the analytes studied since it was very difficult to separate it from sarafloxacin.

Organization of the Thesis

This thesis is divided into five chapters. Chapter one captures the introduction and the background of antiparasitic agents such as their definition, some analytical techniques used in identifying them as well as some existing

analytical methods used in identifying and separating them. The problem statement in chapter one states the actual problem of this study. The problem statement gives insight into how the existing analytical methods quantitatively and qualitatively determine only one group of antiparasitic agent. Other sections such as Aims, specific objectives and purpose of the study are also discussed in chapter one.

Chapter two is the Literature Review. Concepts such as health effect of the different group of antiparasitic agents, the maximum residual limit set by the European Union for this antiparasitic agent. Detailed explanation of one of the most useful analytical instrument was also captured (UHPLC). The Chapter further reviewed current methods used in analytical chemistry for the determination of these antiparasitic agent in fish. Chapter three of this study explains the detailed process of how the new analytical method was developed and validated.

Chapter four in this study highlights results and discussion. Results were compared to literature and MRL from the European Union. The new method was validated and compared to an already existing method.

Chapter five talks about Summary, Conclusion and Recommendations. Conclusions based on specific objectives of this study were made and summarized in a general conclusion based on the aims of the study. Some further studies were finally recommended.

Chapter Summary

Currently, there is no single analytical method reported in literature for the identification of different groups of antiparasitic agent in fish. Some existing methods used in analyzing them has been captured under this chapt

CHAPTER TWO

LITERATURE REVIEW

Introduction

Analytical instrumentation has become a necessary tool for monitoring and quantifying contamination in our environment. Food fraud and its effect to human health and the environment is having serious consequences leading to loss of life and economic loses. Many researches have been carried out to obtain analytical methods that can detect these antiparasitic in food. The European Commission and other Legal bodies have set Maximum Residual limits in order to monitor these contaminants in food matrix. There are many sample preparation techniques that are employed in the sample treatment. A sample preparation technique should be of high efficiency (Stolker & Brinkman, 2005).

Antiparasitic

Antiparasitic are a group of medications which are used for the treatment of parasitic diseases, such as those caused by helminths (Kappagoda *et al.*, 2011), amoeba (Molina *et al.*, 2002) and protozoa (Kappagoda *et al.*, 2011) among others. Antiparasitic agents attacks the parasitic agents of the infections by destroying them or preventing their growth. Antiparasitic agents comprises of antibiotics, pesticides and antioxidants.

Antibiotics

Antimicrobials destroy bacteria, fungi algae viruses and other microorganisms. Antibiotics are chemicals that are specifically made, to either kill or inhibit their growth. Antibiotics are designed to perform either by damaging the

cell membranes of bacteria (i.e. making them unable to function), or blocking protein or DNA synthesis, or interfering with the activities of certain enzymes unique to bacteria (Egorov, Ulyashova, & Rubtsova, 2018). Antibiotics are used to manage bacterial infections in aquaculture (Alderman & Hastings, 1998). In aquaculture, prevention of disease is paramount and this is usually done by regularly adding antibiotics to the water or feeding fish with medicated feed before the occurrence of disease. (Christensen & Baun, 2006). Although this method of preventing disease in aquaculture is profitable because it prevents loss and allows fish to grow more quickly, there are several disadvantages. Antibiotic resistant bacteria may be created in aquaculture because of excessive use of antibiotics. Antibiotic resistant bacteria can spontaneously occur when selective pressure to survive leads to changes in the DNA sequence of a bacterium allowing that bacterium to survive antibiotic treatments. Because most antibiotics that are used to treat fish are the same type for treating bacterial infection in human beings, the pathogenic bacteria that cause infection in human could result in antibiotics resistant because of the same treatment used in fish. (Egorov *et al.*, 2018; Marshall & Levy, 2011). Consequently, the excessive use of antibiotics in treatment of fish in aquaculture (among other agricultural uses) could create public health problems (Aly & Albutti, 2014; Gouvêa-Santos & Aquino, 2014). The most commonly used antibiotic for the treatment of fish in aquaculture is the quinolone.

Quinolones are large group of broad-spectrum bactericides (Anderson & MacGowan, 2003). They are used both in human and veterinary medicine to treat bacterial infections (Aly & Albutti, 2014). Almost all quinolone antibiotics in use

are fluoroquinolones, which contain a fluorine atom in their chemical structure and are they are effective against both Gram-negative and Gram-positive bacteria. Some examples of fluoroquinolones include ciprofloxacin, sarafloxacin among others. Below is an example of fluoroquinolone (Figure 1).

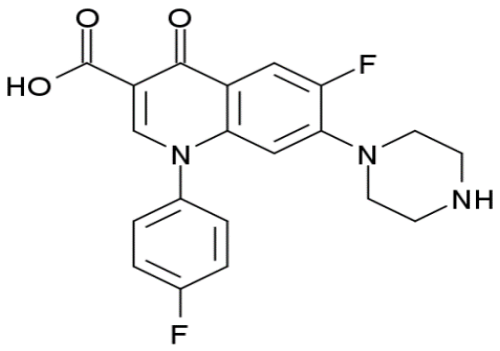


Figure 1: Structure of Sarafloxacin

Fluoroquinolones are one of the most widely used antibiotics worldwide (Anderson & MacGowan, 2003, Fletcher *et al.*, 2010). Currently, a law has been established to montor these contaminants in its applications. This law was established in order to protect human health from the dangers associated with its contaminations in food. In addition, the European Union has established some maximum residual limit to manage these contaminants (EC 2009).

Apart from antibiotics which are used in aquaculture to treat or prevent microbial infections the Triphenylmethane (TPMs) dyes also plays the same role (Figure 2). These dyes are not natural they are synthesized and are usually applied in quite a number of industries. (Ying-Jiang *et al.*, 2012). These dyes possess certain qualities like antibacterial, antifungal and antiparasitic and because they are inexpensive, they are most of the time used in fish farming. These dyes also have

adverse effect on human health, some effect includes gene mutation and cancer. These dyes are not allowed in both US and Europe and they have set lower limit of detection for these dyes as 1µg/kg for US FDA and 2 µg/kg for EU. These dyes are usually metabolized into it leucoforms so the lower limit of detection set by these international bodies is made up of the parent dye and it leucoform. As the parent dyes are rapidly metabolized to form their leuco-metabolites that predominate and persist in fish tissue, the detection limits are expressed as the sum of parent drug and corresponding leucoform. The figures below show some examples of dyes with their respective structures (Figures 2 & 3).

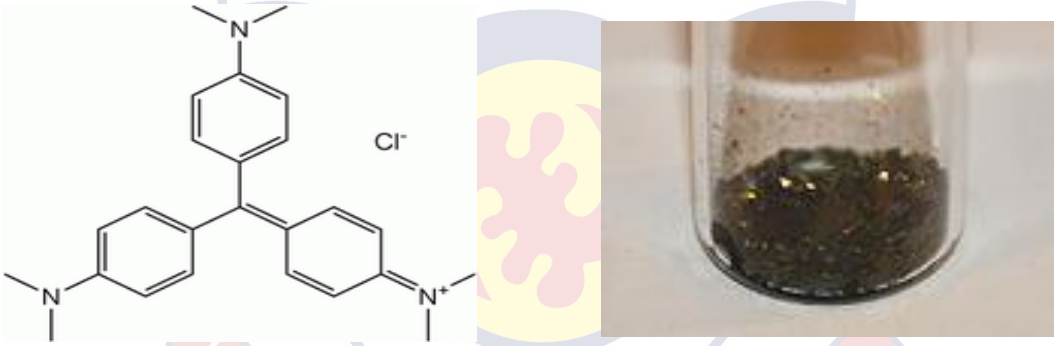


Figure 2: The Structure and Solid form of Triphenylmethane Dyes (TPMs).

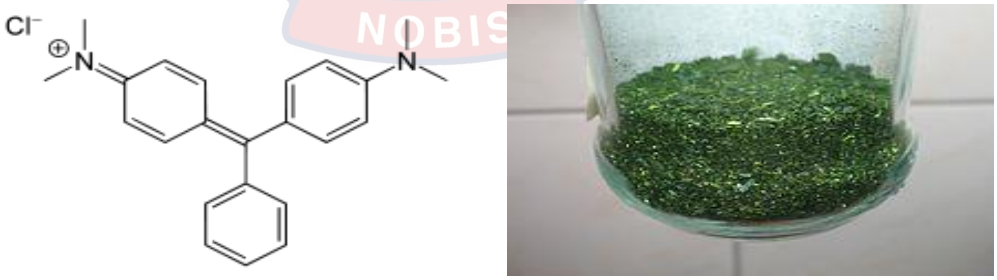


Figure 3: The Structure and Solid form of Malachite Green

Pesticides

Pesticides are substances used to control pests, including insects, waterweeds, and plant diseases. Naturally, occurring pesticides have been used for centuries, but widespread production and use of modern synthetic pesticides did not begin until the 1940s. Teflubenzuron (TFB) and Diflubenzuron (DFB) (Figure 4) are examples of insecticides that belong to a number of compounds that act by preventing biological synthesis of chitin. Their efficiency in the management of different types of insects is quite high. Infestation with sea lice is a major challenge in fish farming and it is on the increase with the infestation of Lepeophtheirus salmonis and C'aligus elongatus. These lice virtually attack the skin and finally destroys it. This effect result in causing thriftiness, and in worse situations leads to the death of the fish. The parasite can also transmit microbial pathogens and these infestations may also influence wild salmonids. In comparing sea lice infestations between wild and farmed fish, there is a higher prevalence of infestations in farmed fish than wild fish indicating enhanced transmission of sea lice under farm conditions (Hormazabal & Yndestad, 1996). A number of methods such as chemical, physical and biological have been introduced for controlling sea lice. Treatment with chemotherapeutics now includes the use of Pyrethrum, Trichlorfon, Carbaryl, dichlorvos, Ivermectin, Azamethiphos and hydrogen peroxide. Pesticides like DFB from Solvay Duphar and TFB from Cyanamid which can be given orally (in feed) to salmon are two of the recent drugs introduced in the treatment of sea lice in salmon. Methods for the determination of DFB in fish tissues in forestry substrates, environmental samples and residues in cabbage under sub-tropical field

conditions, have been described. Only one method for the determination of TFE_3 in fish tissues has been reported. However, none of the published methods appear to be applicable to medicated fish feed (Hormazabal & Yndestad, 1996). The figures 4 shows the structure of an example of pesticides.

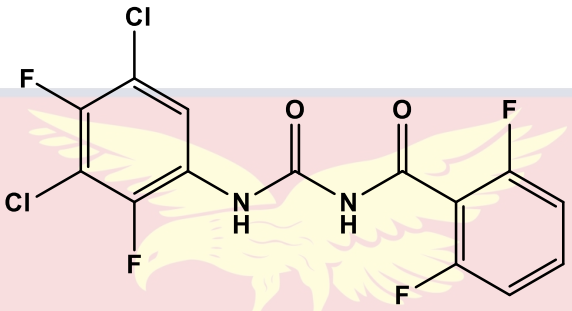


Figure 4: Structure of Teflubenzuron.

Antioxidants (Ethoxyquin)

The most popularly known antioxidants for domestic animal and fish is ethoxyquin. It has been reported that its contribution to a number of health-related issues in animals like dogs and even in humans is enormous (Rodríguez-Trabado, Miró, Balagué & Guspi, 2007). In 1977, FDA requested for optional reduction of the maximum level of EQ in complete dog foods from allowed 150 ppm (0.015%) to 75 ppm (0.0075%). New studies started at the same time by the Pet Food Institute to determine whether even lower EQ levels (between 30 and 60 ppm) would provide antioxidant protection for dog food (USFDA, 1997). A company (USA) taking into account its high antioxidant efficiency and stability including low costs of synthesis refined it later for use as a preservative in animal feeds since it protects against lipid peroxidation and stabilizes fat soluble vitamins (A, E). Ethoxyquin, currently is primarily used as an antioxidant in canned pet food and in feed intended

for poultry or farmed fish. The essence of adding Ethoxyquin (EQ, 6-ethoxy-1, 2-dihydro-2, 2, 4-trimethylquinoline) (Figure 5) to feed of animal, is to minimize lipid peroxidation. EQ is not allowed in any food for human consumption except spices (e.g., chili), but it can pass from feed to poultry, eggs and farmed fish, so human beings can be exposed to this antioxidant (Fig. 5).

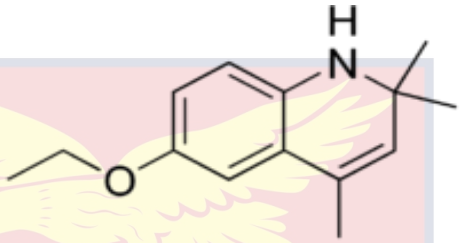


Figure 5: Structure of Ethoxyquin.

The Antiparasitic agents (Antibiotics, dyes, pesticides and antioxidants) mentioned above are some examples of pharmacologically active substances that are used in foodstuffs of animal origin. In order to protect public health, pharmacologically active substances, based on the scientific assessment of the safety of those substances, were classified by the European Commission 470/2009 laying down a community procedure for the establishment of maximum residue limits (MRL) of veterinary medicinal products in foodstuffs of animal origin. Table 1 summarizes some of the Maximum Residual limit set by the European Commission on monitoring of some of these antiparasitic agents in food stuffs of animal origin.

Tables 1: Some Maximum Residual Limit (MRL) Set by the European Commission on Monitoring of Antiparasitic Agents

Pharmacologically Active substance	Marker Residue	Animal Species	MRL (µg/kg)	Target Tissue
Albendazole	Sum of albendazole, sulphoxide, albendazole sulphone, and albendazole-2-amino sulphone, expressed as albendazole	All ruminants	100	Muscle
			100	Fat
			1000	Liver
			500	Kidney
			100	Milk
Amitraz	Sum of amitraz and all metabolites containing 2,4-DMA moiety expressed as amitraz	Bovine	200	Fat
			200	Liver
			200	Kidney
				Milk
Diflubenzuron	Diflubenzuron	Salmonidae	1000	Muscle and skin in natural proportions
Eprinomectin	Eprinomectin B1a	Bovine	50	Muscle
			250	Fat
			1500	Liver
			300	Kidney
			20	Milk
Flumethrin	Flumethrin (sum of trans-Z-isomers	Bovine	10	Muscle
			150	Fat
			20	Liver
			10	Kidney
			30	Milk

Cont'd

Oxytetracycline	Sum of parent drug and its 4-epimers	All food processing species	100 300 600 100 200	Muscle Liver Kidney Milk Eggs
Sarafloxacin	Sarafloxacin	Salmonaide	30	Muscle and skin in natural proportions
Teflubenzuron	Teflubenzuron	Teflubenzuron	500	Muscle and skin in natural proportions

Source: European Union 2010

Analytical Instrument used in Detecting these Antiparasitic Agents

There are many scientific analytical instruments used in the determination of antiparasitic agents, some of which includes High Performance Liquid Chromatography (HPLC), Ultra High-Performance Liquid Chromatography (UHPLC), Gas Chromatography, liquid chromatography-tandem mass spectrometry (LC-MS/MS) and among others. In this research, the main analytical instrument used was the UHPLC (Figure 6).

Figure 6: Ultra High Performance Liquid Chromatography (UHPLC).

Instrumentation

The UHPLC system basic principle and instrumentation remains the same as that of HPLC but only differs in upgrading instrumentation and hardware. The UHPLC system consists of a binary solvent system, sample manager, column manager and detector. The solvent manager uses two flow pumps to deliver a parallel binary gradient, which is mixed under high pressure (Swartz, 2005). Degassing system degasses the mobile phase, which is selected by valve up to four solvents. UHPLC system can withstand pressure of about 15,000 psi (about 1000 bar) to take full advantage of the sub-2-mm particles (Wu, Naijun, & Thompson, 2006). The sample manager also has advance technology where sample

temperature can be taken to as low as 0°C (Wales *et al.*, 2008) whereas column manger can manage the column temperature up to 90°C using high temperature. In ultra-high-performance liquid chromatography (UHPLC), it is possible to drastically decrease the analysis time without loss in efficiency (Nguyen *et al.*, 2007) (Fig. 7).

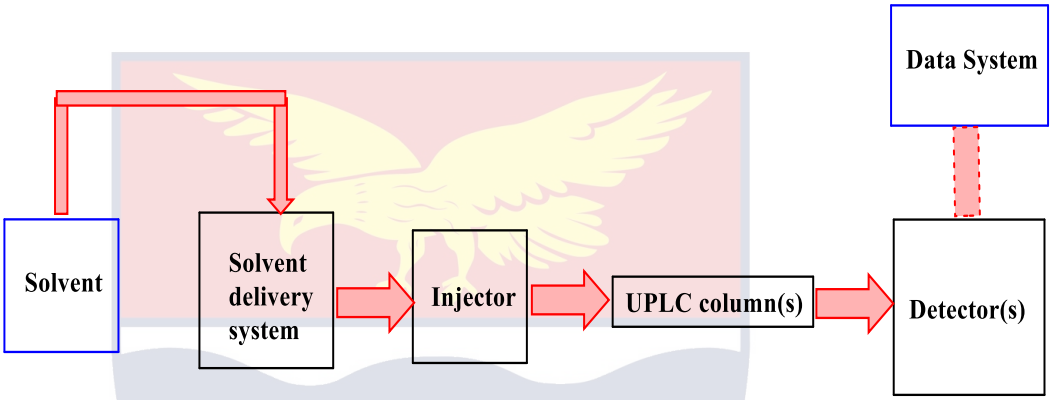


Figure 7: Schematic Diagram of UHPLC.

Detector and Hyphenation of UPLC

Detectors such as UV/Visible, Photodiode array (PDA), Evaporative light scattering (ELS), Refractive index (RI) and Fluorescence (FLR) are commonly used with UPHLC. Beside these detectors, capability of UPHLC can be greatly increased by hyphenating instrument with other technique such as mass spectrometer, ion chromatograph, nuclear magnetic resonance spectrometer, inductive coupled plasma-mass spectrometer and Infrared spectrometer (Shaikh & Hussain, 2016). Ultra-violet/ visible (UV) detector is used for organic compound which absorb the light in the range of 190 to 800 nm. This detector can be tuned to specific wavelengths in UV or Visible range for detection. It provides performance benefits for both routine and complex analyses in Pharmaceutical, life science,

environmental, agricultural and petrochemical applications (Shaikh & Hussain, 2016).

Photodiode Array (PDA) Detector

This detector offers simultaneous advanced optical detection in the range of 190 to 800 nm. It provides unprecedented trace impurity detection and quantitation with spectral analysis capabilities. Definitive compound identification and co-elution detection with simultaneous 2D and 3D operation. This detector finds major application in drug discovery and pharmaceutical development (Shaikh & Hussain, 2016).

Fluorescence (FLR) and Refractive Index (RI) Detector

For sensitivity and selectivity to fluorescence-based applications, this detector is used. It extends the benefits of UHPLC technology for the analysis of polynuclear aromatic hydrocarbons (PAHs), drugs of abuse, and vitamins and any component with chemiluminescent properties, such as fluorescence or phosphorescence (Shaikh & Hussain, 2016).

RI is a universal detector which is used where chemical is having no or limited UV absorbance. These include alcohols, sugars, fatty acids, excipients, raw material and pharmaceutical drug products. Beside this characterization of low molecular weight polymers also finds application on UPHLC. Main disadvantage of this detector is that it lacks sensitivity. Mass detector (MS), UPHLC can be coupled with mass spectrometer (MS) and tandem mass spectrometer (MS-MS) detector that find application in various fields and is used for identification, quantitation and mass analysis of materials. In addition, structural elucidation of unknown molecule can be found out by fragmentation. This detector has various

mass analyzers depending upon their application, some of the analyzer are Single quadrupole, Ion trap, Triple quadrupoles (Tandem) and Time of flight (TOF). These detectors provide very high sensitivity, selectivity and time resolution. Beside these detector many other detectors can be hyphenated to UHPLC such as Infrared (IR), Inductive Coupled plasma mass spectrometry (ICP-MS), nuclear magnetic resonance (NMR) and Evaporative light scattering detector (ELSD), Electrochemical detector (EC) (Shaikh & Hussain, 2016).

Advantage of UHPLC Over HPLC

UHPLC has many advantages over conventional HPLC of which major advantages are speed, quality and cost of analysis. Compound separated on UHPLC are more resolved as compared to conventional HPLC also peak capacity is also increased (number of peaks resolved per unit time). Sensitivity of the method is increased up to 3-5-fold. Band spreading is reduced due to the population of analyte molecule is more concentrated in UHPLC resulting in a higher plate count. The time spent in optimizing and validating new methods is greatly reduced with the use of UHPLC. Mobile phase solvent consumption for the analysis is greatly reduced due to low flow rate and short run time. Reduces process cycle times, so that more product can be produced with existing resources. Real time analysis by UHPLC reduced the cost of failure of product and process control (Shaikh & Hussain, 2016). UHPLC system can be hyphenated with various techniques for which it finds application in vast areas. Conventional HPLC are nowadays are replaced with UHPLC considering greater commercial benefit, superior sensitivity, resolution, speed and sample Throughput (Shaikh & Hussain, 2016).

Applications of UHPLC

Pharmaceutical Analysis

UHPLC finds major application in pharmaceutical analysis. Methods use in drug substances and drug product analysis should be well developed and validated and these processes are much time consuming with conventional HPLC. In drug development impurity, profiling is major activity where detecting and quantifying impurities in drug substance and drug product can be done by UHPLC as it has good resolving power, reproducibility, efficiency and short time. As UHPLC provides faster analysis of samples, this advantage can be used to monitor the process and real time samples of reaction monitoring where very short time is required to control the processes thus saving the cost of failure. UHPLC also finds application in dissolution testing, which is one of very important test in the formulation of drug product. This test requires uniformity reliability and batch-to-batch reproducibility. Metabolites studies are required in the new drug development process where main metabolite is determined and identified quickly enough in the drug discovery phase. UHPLC is capable of determining and identifying metabolite and biomarker structure elucidation (Shaikh & Hussain, 2016).

Environmental Analysis

Environmental sample requires innovative techniques to detect and identify the chemical contamination. UHPLC provides the analysis of these samples with less time, cost and more information about sample content. Some applications of UHPLC are analysis of organic component in soil, air, hazardous wastes, drinking

water, wastewater, pesticide residue and per fluorinated compounds (PFCs) analysis (Shaikh & Hussain, 2016).

Food Analysis

Food manufacturer are always in search of a compressive solution for food testing thus UHPLC decrease operation cost, increase productivity and provide identification of diverse chemicals in a food sample, thus providing public safety. Beside this, it also offers quality and consistency of the product. UHPLC is also applied for food profiling, identification of natural toxins, vitamins and pesticide residue in food product (Shaikh & Hussain, 2016).

Forensic and Toxicological

UHPLC finds good application in the identification of drug of abuse from blood, urine and oral samples. Several cannabinoids, opioids, barbiturates can be identified and analysed by UHPLC. The combination of UHPLC with various instruments gives the unique benefit of drug screening with excellent sensitivity and accuracy at trace level (Shaikh & Hussain, 2016).

Method Development

There are many publications on HPLC and UHPLC method development strategies (Fekete & Fekete, 2012).

1. Collecting information on sample
2. Mobile phases and scouting of columns.
3. Analyzing scouting results.
4. Selecting of separating conditions.
5. Optimization.

6. Validation

Sample Information Gathering and Method Goals

Gathering information about a sample is crucial since information gathered throws more light into how the sample was made and characteristics such as it reactivity, toxicity, chemical composition, its solubility, density, molecular mass and among others. Some impurities in a sample might be known while other impurities in a sample might not be certain. Drugs are produced as a result of combining API with excipients. Mostly, key constituents of the sample are certain but the breakdown of the product might not be certain. Furthermore, understanding of the physical nature, chemical reactivity and structure of sample is helpful in the development of the method. The structure of the chemicals gives further information like the functional groups of the chemical and this can help in knowing the pKa solubility chromophores among others.

Mobile Phases and Scouting of Columns

A suitable UHPLC mode can be selected after gathering sample information and defining goals. There are four major separation modes in UHPLC, these include reversed phase, normal phase, ion exchange, and size exclusion (http://www.ir.vub.ac.be/chis/KineticPlot/). However, there are only two UHPLC modes which are normal-phase and reversed-phase UHPLC (Grittli & Guiochon, 2006). Reversed-Phase UHPLC are mostly used to separate pharmaceutical compounds. Several UHPLC columns available are packed with silica gel and the disadvantage of this type is the fact that it is unstable at alkaline pH with less tailed

peaks. (Borges, 2015). Other UHPLC packing columns are core-shell particles C18, C8, C4, phenyl, and polar-embedded C18 bonded phases for BEH packing material.

Sample diluent must be appropriately selected when preparing sample solution. It must be able to dissolve all components in a sample. Furthermore, all sample components must be stable in the selected diluent. The selected diluent also does not interfere with separation. The mobile phase typically consists of aqueous mobile phase or a mixture of aqueous mobile phase and organic mobile phases. A scouting experiment can be run when columns, mobile phase organic solvents, buffers, method of detection are selected and the sample is prepared. There are many UHPLC method strategies published (Fekete & Fekete, 2012; Gritti & Guiochon, 2010).

Analyze Scouting Results and Select Separation Conditions

Analyze Scouting Results

Scouting results are immediately analyzed when all the test run are done. An analyst needs to analyze the scouting results after a sample set run is complete. Based on how difficult the sample may be, there could be a lot of steps with several conditions that may be employed to obtain preferred separations. In certain instances, after trying a number of steps and conditions, separation would not be realized. When a situation like this arises, the analyst has to understand the results and find out what factors could work out in the separation.

Selecting Separating Conditions

Once separation is achieved, for all peaks of interest, a condition needs to be selected from many potential conditions. The aims of a method must be considered against candidate conditions. Resolution and tailing factor at this point must be evaluated for candidate conditions.

Optimization

After requirements like buffer, column, organic solvent are determined the method needs to be optimize in terms of the flow rate, mobile phase organic solvent gradient, gradient time etc. These are normally done in ensuring good resolution, robustness and shorter run time.

Validation

The method must be validated when separating conditions has been duly optimized. The aim is to identify the crucial parameters and to establish acceptance criteria for method system suitability.

The International Organization for Standardization (ISO) defined validation as "verification, where the specified requirements are adequate for an intended use", where the term verification is defined as "provision of objective evidence that a given item fulfills specified requirements" (International Organization for Standardization, 2007). Other validation parameters include linearity, accuracy, precision, LOD, LOQ and selectivity or specificity.

Linearity

The linearity of an analytical procedure is defined as the capability to have test results that is within a given range, that are directly proportional to the

concentration of the analyte in the sample (Krier *et al.*, 2011; Chan, Leo, & Lam, 2004). It is very important to detect the useful range at which the instrumental response is proportional to the analyte concentration. The evidence of an acceptable fit of the data to the regression line is to have a value of correlation coefficient (r) > 0.998 (Chitturi *et. al.*, 2008). At 95% confidence limits for the intercept generally is the significance of the deviation that are obtained from the intercept of the calibration line that passes through the origin (Shabir, Lough, Arain, & Bradshaw, 2007; Miller & Miller, 2005). Linearity is evaluated by injecting three (3) or more standards. A plot of the peak areas on the y-axis against the concentration on the x-axis and its linearity is determined from the coefficient of correlation.

Accuracy

ISO explains accuracy to be the "closeness of agreement between a measured quantity value and a true quantity value". This measure is not a quantitative property that can be expressed with a numerical value. These characteristics, has an inverse relation to both random and systematic errors, where higher accuracy means lower errors (Mowafy, Alanazi & Maghraby, 2012). It is determined as a result of analysis of several analytes whose concentrations differ. Samples are spiked with concentration of the analyte that are exact and known and the samples are prepared in triplicate.

Precision

Precision expresses how close or the level of distribution that exist within measurements made serially that were obtained in a number of similar samples under requisite conditions. Is usually checked at different levels of three these comprises repeatability, intermediate precision and reproducibility (International

Conference on Harmonization, 2005). Repeatability is the extent at which the results are coherent, when all the experimental requirement are duly met. It is usually presented in terms of the relative standard deviation. Intermediate precision is defined by ICH as the variations within the measurements in the longer term and is usually assessed by comparing the results obtained from a method run within a single laboratory within a period of time. This is referred to as intraday precision (Tranfo, Enrico, Renata, & Daniela, 2008).

Selectivity and Specificity

ISO (International Organization for Standardization) define selectivity of an analytical method to be a "property of a measuring system, used with a specified measurement procedure, whereby it provides measured quantity values for one or more measured such that the values of each measured are independent of other measured or other quantities in the phenomenon, body, or substance being investigated". It is the potential to estimate without a doubt the component of interest in the presence of other components that could be present. By comparing test results from an analysis of samples containing impurities, degradation products, or placebo ingredients with those obtained from an analysis of samples without impurities, degradation products, or placebo ingredients, the specificity of a test method is determined.

Lower Limit of Detection and Lower Limit of Quantitation

Limit of detection (LOD) of an analytical procedure is the lowest concentration of an analyte in a sample which can be detected but not necessarily quantitated as an exact value whiles limit of quantitation (LOQ) is the lowest

amount of analyte in a sample which can be quantitatively determined with appropriate precision and accuracy. ICH guidelines has described three methods for determining LOD and LOQ that consist the following:

Visual Evaluation

Visual evaluation may be used for both instrumental and non-instrumental methods. The LOD and LOQ are estimated by analysing samples with known concentrations of analyte and establishing the minimum level at which the analyte can be reliably identified or quantified with acceptable accuracy and precision respectively.

Signal to Noise Ratio Approach

This type of system used in determining the lower limit of detection is usually applied when there is baseline noise in the analytical method. In this system, the LODs is determined by comparing signals that have been measured in a samples that are of minimal concentration with that of blank samples. This confirms the minimal concentration the analyte can be convincingly determined. It is mathematically defined as S/N ratio of 3:1 and this is usually used in the assessment and credible accounting for the LODs with relative standard deviation lesser than or equal to 10. The LOQ is also mathematically defines as S/N ratio of 10:1 and this is appropriate with relative standard deviation less than or equal to 3%. The LODs and LOQs are calculated as 3 times the ratio of the standard error of the Y intercept to the slope of the curve, and 10 times the ratio of the standard error to the slope of the curve respectively. The slopes is obtained from the plotted calibration curves while the standard deviation is obtained from the standard deviations of

background from analytical feedback of a required number of blank samples. In other terms it can be referred to as deviation from residues of standard deviation of the y-intercept when regression lines obtained from samples contain an analytes in the range of LOQ and LOD.

Sample Treatment

Basically, sample treatment is necessary in analytical procedures. Some characteristics of a good sample treatment include; the quickness of the sample treatment, the precision and how intact the sample treatment keeps the composition of the sample, (Lucci, Pacetti, Núñez, & Natale, 2012). There are a number of sample treatments procedures, some of which includes solid phase extraction, molecular imprinted polymer and among others.

Solid Phase Extraction (SPE)

SPE is a widely known sample preparation technique. It is applied in the analysis of environmental and food samples. Its flexible use makes it more applicable in several areas like trace enrichment purification and so on. SPE and LLE (Liquid-Liquid Extraction) have similar principles. The Principle entails distribution between a liquid sample matrix or solvent with analytes and a solid sorbent phase. LLE is faced with challenges such as phases that are unable to completely separate, low recoveries, increase in waste production and costly apparatus. These challenges of LLE can be overcome by employing the use of SPE (Lucci *et al.*, 2012). The SPE process comprises of four various steps: conditioning, sample addition, washing and elution.

The first step in the SPE process is the selection of the suitable solid sorbent. In SPE, the sorbent selectivity depends on the attractive forces between the analytes and the functional groups on the sorbent surface. The sorbent can interact with analytes by hydrophilic interactions (polar-polar, hydrogen bonding, dipole-dipole, dipole-induced dipole) and hydrophobic interactions (non-polar non-polar, Van der Waals), cationic-anionic and selective antigen antibody interactions. Basically, there are different types of sorbents for the packaging of SPE and are grouped according to the following; reverse phase, normal phase ion exchange and immuno-affinity adsorbents (Lucci *et al.*, 2012).

The next step is conditioning with a suitable solvent. For the functional groups to be able to interact with the sample, they are solvated during the conditioning process. After the conditioning process, the sample is concentrated onto the SPE cartridge. In the cause of this process, the analytes including part of the matrix constituents are withheld on the SPE sorbent. Consequently, interference separation together with the analytes can be obtained by the following means: selective extraction, selective washing or selective elution. When the SPE technique is used to eliminate the interfering constituents (trace enrichment), selective extraction is done. From this process, only selected constituents are kept. Since the target analytes together with the impurities are kept on the sorbent bed, selective washing is achieved. Furthermore, the impurities are rinsed through with stronger wash solutions to be able to eliminate them, but weak sufficiently to retain the analytes. In a different manner, selective elution contains the elution of the absorbed components of interest by a solvent that completely leaves the retained

impurities behind. When SPE is used so as to carry out the class compound fractionation, the elution of target analytes may involve different solvents. The diagrammatical example of SPE manifold is shown (Figure 8).

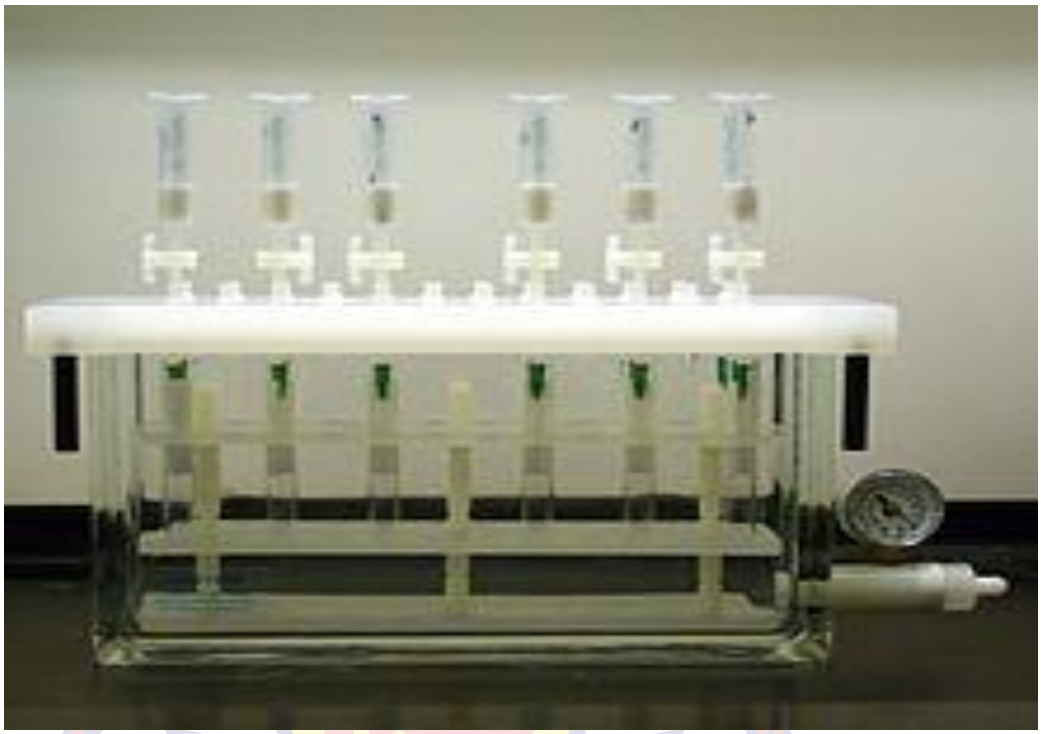


Figure 8: Diagram of the SPE Manifold.

Types of SPE

Reversed Phase (SPE)

In reversed phase SPE, analytes are separated based on their polarity. The cartridges of reversed stationary phase SPE are derivatized with hydrocarbon chains which retain compounds of mild to low polarity based on the hydrophobic effect. By washing the cartridges with a non-polar solvent, the analytes could be eluted which breaks the bonds holding the analytes together with the stationary phase (Poole & Poole, 2012). Silicon with carbon chains is mostly used as stationary phase. Depending solely on hydrophobic interactions (non-polar), only

very weakly polar or non-polar compounds will adsorb to the surface (Poole & Poole, 2012).

Ion Exchange (SPE)

Depending on the electrostatic forces holding the analytes which are of concerned and the positively or negatively charged groups on the stationary phase, the analytes are separated via ion exchange sorbents. When the sample pH and the stationary phase at a point are both charged, ion exchange occurs (Poole & Poole 2012).

Anion Exchange

Strong anion exchange sorbents contain quaternary ammonium groups that have a permanent positive charge in aqueous solutions, and weak anion exchange sorbents use amine groups which are charged when the pH is below 9. Strong anion exchange sorbents are functional because any strongly acidic impurities in the sample will bind to the sorbent and normally will not be eluted with the analyte of interest. In order to recover a strong acid, a weak anion exchange cartridge should be used. To elute the analyte from either the weak or strong sorbent, the stationary phase is rinsed with a solvent that neutralizes the charge of either the analyte, the stationary phase, or both. Once the charge is neutralized, the electrostatic interaction between the analyte and the stationary phase no longer exists and the analyte will elute from the cartridge (Poole & Poole, 2012).

Cation Exchange

Sorbents that are derivatized with functional groups that interact and retain positively charged cations like bases are cation exchange sorbents. Strong cation

exchange sorbents have aliphatic sulfonic acid groups that are always negatively charged in aqueous solution, and weak cation exchange sorbents have aliphatic carboxylic acids, which are charged when the pH is above about 5. Strong cation exchange sorbents are functional because any strongly basic impurities in the sample will bind to the sorbent and often will not be eluted with the analyte of interest. In order to recover a strong base, a weak cation exchange cartridge should be used. To elute the analyte from either the weak or strong sorbent, the stationary phase is washed with a solvent that neutralizes ionic interaction between the analyte and the stationary phase (Poole & Poole, 2012).

Cartridges

The stationary phase are in the form of a packed syringe-shaped cartridge, a 96 well plate, a 47 or 90 mm flat disk, or a micro extraction by packed sorbent (MEPS) device, a SPE method that uses a packed sorbent material in a liquid handling syringe (Abdel-Rehim & Zeneca, 2003; Abdel-Rehim, 2011). These are usually mounted on specific types of extraction manifolds. By keeping many SPE media in place and permitting an equal number of samples to pass through them at the same time, the manifold allows multiple samples to be processed. After analytes have passed through the stationary phase, they are mostly collected in sample tubes below or inside the manifold. Solid phase extraction cartridges and disks are accessible with diverse stationary phases, each of which can separate analytes based on different chemical properties. Many stationary phases are made of silica that has been linked to a specific functional group. Examples of these functional groups include quaternary ammonium or amino groups (for anion exchange), sulfonic acid

or carboxyl groups (for cation exchange) and hydrocarbon chains of variable lengths (for reversed phase SPE) (Poole & Poole, 2012).

Sorbents and Modes of Interaction

The sorbents used for SPE are similar to those used in liquid chromatography, including normal phase, reversed phase, size exclusion, and ion exchange. Normal-phase sorbents consist of a stationary phase which is more polar than the solvent or sample matrix that is applied to the SPE sorbent. This means that water is not usually a solvent in normal-phase SPE because of its high polarity. When the sample containing the analyte of interest is in the organic form, normal phase sorbents are used. Polar interactions, like dipole–dipole interactions and hydrogen bonding are the basic mechanisms for solute retention (Poole & Poole, 2012).

Packing materials in reversed phase sorbents are more hydrophobic than the sample. To use reversed phase sorbents in SPE, aqueous samples are mostly involved. The mechanism of interaction is non-polar or hydrophobic (i.e. Van der Waals forces), or reversed-phase interactions and sometimes secondary interactions including dipole-dipole and hydrogen bonding. Size-exclusion sorbents uses a separation process based on the molecular size of the analyte. This method presently employed in SPE is mostly coupled with reversed phase or ion exchange. Based on the ionic state of the molecule, either anionic or cationic, the ion exchange sorbents isolate the analytes where the analyte exchanges for another charged analyte that is already sorbed to the ion exchange resin. In this case, SPE applications are mostly identical to classical ion exchange. Hence, the mechanisms

of interaction involve hydrogen bonding and dipole–dipole forces (polar interactions), Van der Waals forces (non-polar or hydrophobic interactions), size exclusion, and cation and anion exchange. For stronger selectivity, some sorbents combine variety of interactions. The extensive line of sorbent chemical structures facilitates one of the most powerful aspects of SPE, which is selectivity. The degree to which an extraction method can separate the analyte from interferences in the original sample is called selectivity. Selectivity is facilitated by a number potential interactions between the analyte and the solid phase (Poole & Poole, 2012).

Molecularly Imprinted Polymers (MIPs)

Molecularly imprinted polymers (MIPs) are artificially produced from cross-linked, synthetic polymers with three dimensional structure capable of rebinding to an analyte of interest (Valérie & Karsten, 2007). The synthesis of MIPs is comparatively cheap and easy for the purification process of natural antibodies. These have resulted in a favourably increased of interest in the use of MIPs in many analytical techniques and applications. Applications of MIPs as stationary phase on chemical sensing, capillary electro chromatography, micro-extraction, immunoassay determinations, liquid chromatography and solid-phase extraction have been successful in last 15 years with a rapid increase in the number of publications (Valérie & Karsten, 2007). The table 2 summarizes the results in terms of LOD_S, LOQs and % recoveries as nanomaterials were used in the extraction of some antiparasitic agents.

Table 2: Summary Results of Some Nanomaterials used as Sample Treatment

LOD	Nano-material	Sample treatment	Technique	Analyte	Matrix	% Recovery	Reference
0.71 ug/ Kg n=6	Fe_3O_4@ MIP (50mg)	m-dSPE	HPLC/ UV	Malachite Green	fish	82.7%-111%	Zheng-zhong, Hong-yuan, & Zhi-yong, 2006.
	MIP(150m)	SPE	LC/MS/MS	Malachite Green	Fish	98%	Jie *et al.*, 2013
0.05 ug/ ml	Fe3O4@ Mel (0.3g)	d-MSPE	Spectrometry	Malachite Green	Fish		Mirzajani & Ahamadi, 2015.
		SPE	HPLC/DAD	Malachite	Rainbow trout	70.2-84.8%	Fallah & Barani, 2014.
	Alumina		LC/VIS	MG LMG	salmon	94.6%	Kaplan *et al.*, 2014.
0.31 ug/ Kg	IAC		ELISA	MG	Fish		Bilandzic, Varenina, Kolanovic, Oraic, & Zrnic, 2012.
0.1-4.7 ug/ Kg			UHPLC fluoresce	Quinolone	Fish	72-108%	Lambardo-Agui *et al.*, 2015.
	Fe_3O_4(SDS	d-MSPE	UV-VIS	MGLMG	Water	93.7-104.5%	Dallegrave *et al.*, 2016.
	PAN-co-AA	d-MSPE	UV-VIS	MG	Water	95.83-103.3%	Sabzroo *et al.*, 2017.

QuEChERS Method

Anastassiades and co-workers introduced another method known as QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe). QuEChERS techniques need only little reagents, and sample identification clean-ups are carried out in a single step as compared to the traditional pesticides analysis which involves quite a number of time-consuming solvent extraction procedures. The extracting solvent used is acetonitrile fuse with dispersive solid-phase extraction (d-SPE) which uses primary, secondary amine or other sorbents for purifying the extract. The technique is environmentally friendly because it gives less toxic acetonitrile extract instead of using organo-chlorine solvents. It was initially designed for a multi class residue method for determining pesticides in fruits and vegetables (Anastassiades, Lehotay, Tajnbaher, & Schenck, 2003).

To date, QuEChERS has commonly been used for analysing foods from plant origin that have been contaminated. Also, pesticides in various fruits and vegetables (Wilkowska & Biziuk, 2011), acrylamide in potato chips, chocolate and peanut butter (Mastovska & Lehotay, 2006) and toxins in noodles (Sirhan, Tan, & Wong, 2011) have been detected. The problems to resolve for the successful analysis of xenobiotics include the presence of fat and milk as well as the complex nature of animal tissues. Even though, many matrices specific methods are reported here, a simple, modular QuEChERS method is significant to be developed for analysing pesticides in animal products with different amount of fat, to obtain adequate recoveries that could be a better option for routine sample screening. This process consists of hexane extraction in the presence of a QuEChERS salt and

clean-up by SPE (C18) following the extraction of the sample in acetonitrile (Lichtmannegger Fischer, Steemann, Unterluggauer, & Masselter, 2015). Many Scientific Research have been carried out in the determination of these antiparasitic agents in food and as a result, many scientific analytical methods have been developed.

Summary of Some Existing Developed Methods Indicating their LODs, LOQs % Recoveries and Among Others

Roudaut & Yorke (2002) adapted and used an earlier work on a chromatographic method that was used in identifying contaminant of quinolones from muscles of meat (porcine, chicken, bovine and ovine). Roudaut & Yorke used the same method on the analysis of tissues of fish samples. In the process, three different quinolones were identified (flumequine, oxolinic acid and sarafloxacin). Acetonitrile basic solution was employed as the main extracting solvent. This was carried out as analytes were subjected to extraction from muscles that were homogenized. Extracts were subjected to centrifugation after which evaporation was done partially. Clean-up was done using hexane and finally, injected directly into the chromatographic unit. A column packed with PLRP-S together with fluorescence detector was employed for the separation. The various conditions were duly optimized. Spiked tissue samples were employed in attaining of the recoveries, linearity, accuracy and precision. This was achieved using concentrations ranging from 15 to 120 μkg^{-1} for sarafloxacin and 75 to 600 μkg^{-1} for oxolinic acid and flumequine as directed by EU maximum residual limit.

Recoveries of quinolones in the fish were between 56.9 to 71.0% (Roudaut & Yorke, 2002).

Cañada-Cañada *et al.* (2012), also developed a method based on the technique of chromatography. These researchers determined a number of different types of antibiotics that were mainly fluoroquinolones. They detected these fluoroquinolones in fish with their method. The extracting solvent they employed in extracting the samples were m-phosphoric acid and acetonitrile in the ratio of 75:25 V/V. Purification of the extracts was conducted on the cartridge packed with ENV + isolute. A column packed with C18 in a liquid chromatography process was used in the detection. The composition of the mobile phase was at a pH of 4.5 and comprised methanol acetonitrile and 10mM citrate buffer. At an optimized gradient program, the mobile phase was made to deliver at a flow rate of 1.5 ml/min and these conditions contributed in the separation within 26 minutes. The fluoroquinolones were detected with both UV wavelengths (280 and 254 nm) and fluorescence (280/450, 280/495, 280/405nm). The method achieved a lower limit of detection between 0.2-9.5 µg/kg and 0.7-32 µg/kg for limit of quantitation. At concentration of 50 and 100 µg/kg, the fish were spiked and the average recoveries were between 50%-102% (Cañada-Cañada *et al.*, 2012).

In 2018, Guidia *et al* also developed a chromatographic method which was used for assessing a number of antimicrobials from the family of quinolones and tetracycline. The method was use to quantify these contaminants in fish and was duly validated. Extraction of these contaminants were done using trichloroacetic acid. A column packed with C18 with gradient elution was employed in the

chromatographic separation. EC, 2002 was followed duly in the validating process. Very good linear graphs were obtained in the matrix as the coefficients of the detection were greater than or equal to 0.98. In the application of the method to rainbow trout and Nile tilapia, the results revealed the content of enrofloxacin was 14% with concentrations more than the limit of quantitation (12.53-19.01 µg/kg) and somewhat, the levels were lower than the MRL (Guidia *et al.,* 2018).

Another chromatographic technique was employed to develop a new method. This method mainly determined a number of quinolones in fish tissues. A column that is made of a perfect Sil ODS-2 with the size 250nm x 4nm x 5µm was used as the stationary phase for the separation of these antibiotics. The separation was carried out using a gradient time program and a mobile phase of pH 1. The mobile phase comprised 0.1% trifluoroacetic acid, acetonitrile and methanol. At the temperature of 25°C and within 22 minutes, the detection of the antibiotics were done with a DAD detector. Fish samples were spiked with the antibiotics and were extracted with citrate buffer of pH 4.7. Purification of the extract was achieved on SPE cartridge. In the validation process, the linearity, accuracy, precision and selectivity were conducted in accordance to EC 2002 (BCR® 725).

In 2009, Li *et al.* employed chromatographic method coupled with mass spectrometry in determining a number of antibiotics in muscle of pig and tissue of fish. Extraction of the antibiotics were successfully carried out with acetonitrile. A column packed with C18 was used as the stationary phase and a mobile phase composed of 0.1% formic acid and methanol with an optimized gradient elution, the antibiotics were detected. Correlation coefficient obtained were greater than

0.9956 and with a concentration linear range between 0.3-50 µg/kg. The results revealed that the detection limit was 0.1 µkg^{-}1 and the average recoveries for pig was between 75.3% to 96.3% and 79.7% to 94.2% for fish. The relative standard deviations for both samples were below 10% (Li *et al.*, 2009).

Lombardo-Agui *et al.* (2015) proposed a simple but sensitive method for the simultaneous determination of Quinolones (flumequine, ciprofloxacin, danofloxacin, difloxacin, marbofloxacin and oxolinic acid) in different fish samples. An ultra-high liquid chromatography using a partially C18 column coupled to a fluorescence detector with a wavelength of excitation/ emission program was used. The sample preparation consisted of extraction and clean-up using the Quenchers. The methodology showed high efficiency without interferences in the chromatography detection. The method was characterized in fish tissue in terms of linearity, precision, trueness, limit of detection and quantification. Limit of detection obtained was between 0.1 and 4.7 µg/Kg with recoveries between 72 and 108%. The proposed method has been tested in bass trout, panga and sturgeon showing its simplicity, sensitivity and suitability for routine analysis in that complex matrix (Lombardo-Agui *et al.*, 2015).

Ying-Jiang *et al.* (2009), described a chromatographic method in conjunction with electron spray ionization tandem mass spectrometry which was used to detect dyes such as crystal violet, malachite green, azure A, azure B and C. Extraction of these dyes were carried out using acetonitrile and ammonium acetate buffer. Purification was achieved using dichloromethane on SPE cartridge. The volume of the extract was reduced with nitrogen at a temperature of 45 ^{o}C. A

column packed with C18 was the stationary phase employed in the separation. Separation was carried out with a mobile phase composed of acetonitrile and ammonium acetate containing 0.1% formic acid. Detection was done with a mass spectrometry system. Crystal violet, malachite green, leucocrystal violet, leuco malachite green obtained lower limit of detection of 0.15 µg/kg and lower limit of quantitation of 0.50 µg/kg. The mean recoveries obtained after samples were spiked were above 75%. The relative standard deviations obtained were all less than 15%. On the other hand, azure A, B and C obtained lower limit of detection of 0.3 µg/kg, lower limit of quantitation of 1.0 µg/kg. Recoveries obtained for azure A, B and C was above 70% and relative standard deviation less than 15% was obtained. The method was described as very simple and rapid in it use and also, its sensitivity was high in determining analytes of aquatic origin (Ying-Jiang *et al.*, 2011).

Another method has also been developed for the analysis of crystal violet (CV), leucocrystal violet (LCV), malachite green (MG) and leucomalachite green (LMG) residues in salmon. Salmon samples were extracted with acetonitrile, Mcllvaine pH 3 buffer (90:10 v/v) and sample extracts were purified on a baker bond strong cation exchange solid phase extraction cartridge. Aliquots of the extracts were analysed by LC–MS/MS. The method was validated in salmon according to the criteria defined in Commission Decision 2002/657/EC. The decision limit (CC_α) was 0.17, 0.15, 0.35 and 0.17 μgkg^{-1}, respectively, for MG, LMG, CV and LCV, and for the detection capability (CC_β) values of 0.30, 0.35, 0.80 and 0.32 μgkg^{-1} were obtained respectively. Fortified salmon samples (n=6) in three separate assays showed the accuracy to be between 77 and 113% for LCV,

CV, MG and LMG. The precision of the method, expressed as RSD values for the within-laboratory reproducibility, for MG, LMG and LCV at the three levels of fortification (1, 1.5 and 2.0 μgkg^{-1}), was less than 13%. For CV, a more variable precision was obtained with RSD values ranging between 20 and 25% (Dowling *et al.*, 2007).

A confirmatory method has been developed to analyse malachite green (MG), leucomalachite green (LMG), crystal violet (CV) and leucocrystal violet (LCV) residues in Rui fish (Labeorohita) and Bagda shrimp (Penaeusmonodon). The samples were extracted with dichloromethane using liquid-liquid extraction process and reconstituted with 80% of acetonitrile with water. Aliquots of the extracts were analysed by LC-MS/MS with electrospray ionization in positive mode using multiple reaction monitoring. This method was validated in fish and shrimp matrices according to the criteria defined in Commission Decision 2002/657/EC. In the case of fish and shrimp sample, the decision limit (CCα) ranged between 0.75-0.92 µg/kg and detection capability (CCβ) was in the range of 1.28-1.57 µg/kg. Fortified samples (n=7) in three separate assays showed the accuracy between 93% and 108%. The precision of the method, expressed as RSD values for the within-laboratory reproducibility, at the six levels of fortification (0.5, 1, 1.5, 2.0, 3.0 and 4.0 µg/kg), was less than 11%. The capacity to simultaneously quantify residues of LMG, MG, CV and LCV, and to confirm the chemical structure of a marker residue using LC-MS/MS, this suggests that the procedure may be helpful in monitoring the food supply for the unauthorized use of these dyes in aquaculture (Ashraful, Aktermst, Yeasmin, & Serajul, 2015).

Bin Zheng *et al.*, (2007), developed a new method for the determination of MG and gentian violet (GV) residues in Anguilla japonica using high performance liquid chromatography (HPLC) equipped with fluorescence detector. The sample was extracted with acetonitrile buffer mixture followed by partitioning with dichloromethane. Clean-up and isolation were performed on PRS solid phase extraction (SPE) column. Afterwards, the extract was analysed using a HPLC system incorporating purospher C18 column, fluorescence detector and external standard. Linearity was demonstrated with standards over the range of 1-600 µg/L with relative coefficient of 0.9999. Limit of quantification was 0.4 µg/kg and recoveries of MG and GV from sample tissues fortified at 0.4-100 µg/kg were 70.0%-90.6% and 73.0%-87.2% respectively. The RSDs were less than 10% (Bin Zheng *et al.*, 2007).

Sadeghi & Nasehi in (2018), developed a spectrophotometric method for detecting dyes (crystal violet and brilliant green). In the extraction of the dyes, ionic liquid (IL-DLLME) in conjunction with zero-crossed which was under a first derivative spectrophotometric was employed. The dyes were monitored at 670 and 532 nm for brilliant green and crystal violet respectively. Conditions like pH, extracting solvent and among others were duly assessed. Optimization was duly done and the curves obtained from the calibration were linear. The lower limits of detection obtained were 1.4 µg/L and 2.7 µg/L for crystal violet and brilliant green respectively. The relative standard deviations obtained were all less than 15%. Sample extraction technique employed was QuECHERS (Sadeghi & Nasehi, 2018).

In 1996, Hormazhal & Yndestad designed a chromatographic method for detecting Diflubenzuron and Teflubenzuron. This method was described as very simple in it use. In this method, fish feed was analysed for the contaminants (DFB and TFB) and the extraction of these contaminants was carried out using acetone tetrahydrofurane. The analysis on the extract was done using HPLC with DAD detection. Concentrations of 0.25 and 0.4 g/kg for diflubenzuron and teflubenzuron in feed of fish were obtained as the limit of quantitation respectively (Hormazhal & Yndestad, 1996).

Luvizotto-Santos *et al.* (2009), developed a method for the determination of Diflubenzuron (DFB) accumulation in tilapia (Oreochromisniloticus) filet. Liquid-chromatography (LC) with diode array detection method and C18 solid-phase extraction clean-up was used. This method exhibited no significant matrix effect as verified by the recovery efficiency. Limits of detection and quantification were 32 µg/kg and 110 µg/kg respectively. LC-tandem mass spectrometry analysis confirmed the presence of DFB in filet of tilapia exposed to this pesticide. This method was successfully applied for the analysis of fish captured in three different fishing farms during two seasons. In addition, the analysis of fish was also done in an experimental pond (subjected to Dimilin exposition) and depuration tank during different time intervals (Luvizotto-Santos *et al.*, 2009).

Bohne *et al.* (2007) developed a method for simultaneous quantitative determination of ethoxyquin (EQ) and its major metabolite in Atlantic salmon tissues, ethoxyquin dimer (EQ dimer). The separation was obtained on tandem coupled phenyl-hexyl and C18 columns by 2-phase gradient elution with

acetonitrile-ascorbic, acetic acid, diethylamine acid organized in a 23.5 min sequence. Extraction of compounds was done with hexane from samples saponified in NaOH, ethanol and protected from air- and light-mediated oxidation by addition of saturated ethylenediaminetetraacetic acid, ascorbic acid, and pyrogallol. The identity of peaks was confirmed by spiking samples with standards verified by proton nuclear magnetic resonance spectrometry, mass spectrometry, and high-performance liquid chromatography. Detection limit (at 358/433 nm) of matrix-spiked EQ was 0.02 and 0.06 µ/L for EQ dimer, with 0.5 g sample weighed and resuspended in 0.5 mL hexane. The linearity ranged between 0.2-175 µ/L for EQ and 0.3-5100 µ/L for EQ dimer. Another two more ubiquitous compounds were identified as de-ethylated EQ and quinone imine. Altogether, 14 peaks sharing spectral properties of EQ were separated in a single run, including a major peak present in all muscle samples, labelled unknown metabolite of EQ (UMEQ). The concentrations of EQ, EQ dimer, and de-ethylated EQ, including concentrations of UMEQ (in arbitrary units), in the muscle were correlated to the amount of EQ fed to the salmon, thus specifying their possible metabolic origin. The pattern of 14 peaks in the muscle indicated high specificity could be used to discriminate between wild salmon and salmon fed EQ-supplemented feed. This method will be a beneficial tool for studying EQ metabolism and kinetics, and for the routine surveillance of residual levels of dietary EQ in farmed Atlantic salmon (Bohne *et al.*, 2007).

In another development, Rodríguez-Gómez *et al*. (2018), designed a diamond electrode that was doped with boron in conjunction with liquid

chromatographic in the analysis of ethoxyquin and it dimer. The skin of pear and salmon was analysed with the designed method. The sample treatment used in the extraction was SPE. The designed method was duly optimized and also it was validated as described by European Commission. Calibration graphs were made and also samples were spiked to carry out the recovery process. LODs obtained ranged from 0.05 to 0.1 $mgkg^{-1}$. The RSD which defined the precision was less than 15% and percentage recoveries obtained were nearly 100% (Rodríguez-Gómez *et al.,* 2018).

In 2000, He and Ackman designed a new method. In this method ethoxyquin and its dimers were determined in meals of fish. Hexane was initially employed for the extraction. Acetonitrile was also employed in the extraction of the hexane extract that was oily. A column packed with C18 in a reverse phase HPLC with ultra-violet detection was employed in the analysis. The UV detector was measured at $\lambda = 280$ nm and the mobile phase system used in running the analytes comprised acetonitrile, 0.01M ammonium acetate (80:20 v/v). The recoveries ranged between 90-100%, 75-85% and 90-100% for EQ, QI and DM respectively. Comparatively, ethoxyquin was lost more quickly to its dimers and in the process, it led to oxidation of unknown compounds. In view of this, their levels during storage process were not ascertained (He & Ackman, 2000).

Halme *et al.* (2004) developed another chromatographic method coupled with mass spectrometry. The method was used to detect malachite green and leucomalachite green in muscle of rainbow trout. Extraction was carried out using acetonitrile acetate buffer and methylene chloride was used for partitioning. Clean-

up of the methylene chloride extract was carried out using SPE. The SPE was packed with alumina and propyl sulfonic acid. Separation of malachite green and leucomalachite green was carried out on column packed with chromosphere 5 B with a mobile phase composed of acetonitrile acetate buffer. The lower limit of detection obtained for malachite green and leucomalachite green was 0.8µg/kg and 0.6µg/kg respectively. The average recoveries obtained after spiking with malachite green and leucomalachite green were 65% and 74% respectively. Relative standard deviations obtained for malachite green and leucomalachite green were 16.1% and 11.4% respectively (Halme *et al.*, 2004).

Le *et al.* (2010) described the determination of malachite green and crystal violet analysis in fish products that are processed. Extraction was carried out on samples that were homogenised using ammonium acetate buffer and acetonitrile. The extract containing the contaminants was made to be partitioned into dichloromethane. The extract was cleaned-up using SPE. The SPE was packed with neutral alumina and propyl sulfonic acid. Malachite green and Crystal violet were monitored with UV visible detector at 618 nm and 588 nm respectively. Recoveries obtained ranged 74.8-83.8% for malachite green and 68.6-73.9% for crystal violet respectively (Le *et al.*, 2010).

A new method for the simultaneous analysis of malachite green (MG) and crystal violet (CV) in fish samples has been developed. In the presence of thiocyanate, the second order data of MG or CV obtained from the spectra of MG or CV in a series of water-ethanol mixed solvents with various ethanol volume fractions could be expressed as the combination of two bilinear data matrices. With

the bilinear model, the second order spectra data of mixtures containing MG and CV those coexist with interferents could be analysed using the second order calibration algorithms, and the simultaneous determination of MG and CV in fish samples could be achieved. The algorithms were based on residual bilinearisation (RBL) and unfolded partial least-squares/ residual bilinearisation (U-PLS/RBL) respectively. The method has been successfully used to determine simultaneously MG and CV in some fish samples with satisfactory results (Liao *et al.,* 2009).

Wang *et al.,* 2018 developed a method which made use of electrophoresis and DAD detection. The method was described as very simple and very fast. It was used in the analysis of antibiotics (sarafloxacin and difloxacin) in beef. Systematically, conditions that contributed to the development of the method were duly optimized. These conditions were the wavelengths taken for the monitoring of the antibiotics, the pH and the buffer system that was chosen, temperature at which separation was carried out. With a composition of mobile phase comprising of $H_3BO_3/Na_2B_4O_7$ pH of 8.8 with temperature of 22 degrees Celsius, the antibiotics were determined within 4 minutes. The lower limit of detection was 0.8 µg/mL for sarafloxacin and lower limit of 0.3 µg/mL for difloxacin. The relative standard deviations the peaks were 4.8% (intra-day) and 7.8% (inter-day) (Wang *et al.,* 2018).

Chapter Summary

This Chapter focused on the properties and health effect of the different group of antiparasitic agents studied in this work. The maximum residual limit set by the European Union for this antiparasitic agents were enumerated. The chapter

also focuses on the steps for developing analytical method and also the different sample treatment methods available. Detailed explanation of one of the most useful analytical instrument was also captured (UHPLC). The Chapter further reviewed current methods used in analytical chemistry for the determination of these antiparasitic agent in fish.

CHAPTER THREE

METHODOLOGY

Introduction

This Chapter explains how the new analytical method was developed. In summary, it shows the origin of analytes and preparation of standards. The different spectral analysis of the analyte, different choice of mobile phase, calibration curves, how the LC time programme was made, optimization of buffer, flow rate, SPE Extraction of analyte, SPE Extraction of spiked fish etc. In addition, the method was compared with an already existing one.

Materials and Chemicals

The Standard used for the research work were Sarafloxacin, Ethoxyquin, Diflubenzuron Teflubenzuron, Malachite green and Crystal violet (all purchased from Sigma Aldrich, USA). Acetonitrile, Methanol (both of HPLC-gradient grade) were supplied by Sigma-Aldrich (Germany) Magnesium Nitrate hexahydrate and Acetic acid (HPLC grade) were purchased from Panreac AppliChem (Germany). Ethylene diaminetetraacetic acid disodium salt Fluka Chemika Switzerland, Disodium hydrogen phosphate anhydrous, Citric acid purchased from Panreac Quimica (Spain), SPE catridges (HLB, 3 ml tubes) purchased from Supelco Sigma-Aldrich (Germany), 0.2 µm nylon membrane, Nylon syringe filer Sigma-Aldrich (Germany). Hydrated iron chloride ($FeCl_2.4H_2O$) terbium nitrate Ammonium hydroxide (NH_4OH) Ethanol (EtOH) Polyethyleneglycol (PEG) and ammonium acetate were all purchased from Sigma-Aldrich (Germany).

Quality Assurance and Quality Control

To guarantee the validity of the analytical results, a number of quality assurance and quality control (QA/QC) measures were performed. All the standards were of analytical Grade and were kept under optimum conditions. Stock solutions of 1000 ppm of all the standards were stored at 4^{o}C for all except Diflubenzuron and Teflubenzuron which were stored at room temperature. Intermediate standards were prepared by diluting the stock in Milli Q water for every week. Mobile phase procedural blanks were injected to check for background contamination. Mobile phase was always filtered using micro filtration apparatus and they were freshly prepared every day. Standards, blanks, were all injected in triplicate, and blanks were processed in a similar manner as the samples. Blanks and standards were all run at the same time under the same conditions.

Instruments

Lambda 35 spectrometer, UV-$_{VIS}$ spectrometer purchased from Perkin Elmer, Fluoromax-4 spectrofluorometer (USA). UHPLC (Shimadzu, Kyoto, Japan) T25 digital Ultra-Turrax, Centrifuge, Vortex mixer (Germany). The UHPLC system employed comprises a pump that is of higher pressure (LC-30DAD), an auto sampler (SIL-30AC) a degasser (DGU-20A5), coupled to a sample pot that is cold with a temperature of 8^{o}C, an oven which contain the column (CTO-10AS vp) and a fluorescence detector (RF-20AXs) (Shimadzu, Kyoto, Japan). For the separation, a Syvchronis C18 analytical column (100 mm × 2.1 mm I.D, particle size 1.7 µm; Thermo Scientific. Inc; Walthan, MA) equipped with a guard cartridge

of the same type (seguirity guard ULTRA cartridge UHPLC C18: Phenomenex) was used as stationary phase.

Preparation of Stock and Intermediate Standards

Stock standards of 1000 ppm were prepared by accurately weighing out 10 mg of each standard and dissolving with solvent (methanol) in 10 ml different volumetric flask and finally each standard was homogenized and stored in the refrigerator. The intermediate standards 20 and 10 ppm were prepared by dissolving 200 µl and 100 µl respectively, of the stock in 10 ml volumetric flask containing Milli Q water. Finally, each standard was homogenized and stored in the refrigerator at 4℃ except for Diflubenzuron and Teflubenzuron that were kept at normal room temperature).

Preparation of Standards

Sarafloxacin standard of 2 ppm was prepared by dissolving 200 µl of the intermediate standards (20 ppm) in Milli Q water in 2 ml volumetric flask. Similarly, Ethoxyquin, Diflubenzuron Teflubenzuron, Malachite green and Crystal violet standards were also prepared and stored under 4^0C for further analysis.

Preparation of Mcllvaine Buffer 1 and 2

Citric acid (9.36 g) and 1.55 g of disodium hydrogen phosphate were weighed and dissolved in 450 ml Milli Q water in 500 ml beaker. The source of the Milli Q water was from the Ultra-pure water treatment equipment in the Analytical laboratory (Sigma Aldrich, Germany). The pH was adjusted to 2.5 using 0.5 M NaOH. Finally, the solution was transferred into a 500 ml volumetric flask, topped to the mark and homogenised as buffer 1. Similarly, buffer 2 was prepared by

weighing 9.36 g of citric acid and 1.55 g of disodium hydrogen phosphate. The pH was adjusted to 3.0 and labeled as buffer 2.

Preparation of 0.1 M EDTA

Exactly 1.8612 g of EDTA was weighed and was dissolved with 400 ml Milli Q water in 500 ml beaker. The pH was adjusted to 8.0 using 0.5 M NaOH. Finally, the solution was transferred into 500 ml volumetric flask and was topped up to the mark.

Method Development

Spectrophotometric Analysis of Standards

The UV Spectrophotometer was used to determine spectral information of all the standards. In addition, blank samples were also run just as the standards. Preparation of 1 ppm of each standard for the spectral analysis was done by pipetting 10 µl of the standard (1000 ppm) into 10 ml volumetric flask and topping it up to the mark with Milli Q water. The blanks were also prepared by pipetting 10 µl of the solvent in 10 ml volumetric flask and treated similarly. This was transferred into a cuvette and the spectral analysis determined using the spectrophotometer. All the possible absorption wavelengths including the lamda max of each standard were noted.

Spectrofluorometric Analysis of Standards

The spectrofluorometre determined the excitation and emission spectrum of all the standard. The excited wavelength on the spectrum was used to check which excitation wavelength does maximum fluorescence emission occurs. Standard of 2 ppm was placed in the cuvette with a slit width of 5 nm (this slit width gave better

resolution) and excitation set between 200-600 nm. This was done for each of the standards and also to investigate which of the standards exhibits fluorescence.

Selection of Mobile Phase

Many combinations of organic solvents and chemicals were employed to ascertain which combination was best for the identification separation, quantification of standards. Initially, 50% methanol and a 0.05 M ammonium acetate, 0.2 M magnesium nitrate was prepared and used as the mobile phase followed by varying combinations of other reagents and concentration listed in Table 3. All the different combinations of the aqueous phase were prepared and after every preparation of the aqueous phase, it was filtered using the microfiltration apparatus together with 0.2 micro filter prior to UHPLC analysis.

Table 3: Combinations of Solvent and Reagents of Mobile Phase

Meth.	Organic Phase (A)	Aqueous phase (B)	
1	50%methanol	0.050 ammonium acetate	0.200 magnesium nitrate
2	50 % methanol	0.080 ammonium acetate	0.200 magnesium nitrate
3	62 % methanol	0.050 ammonium acetate	0.200 magnesium nitrate
5	Acetonitrile	0.050 ammonium acetate	0.200 magnesium nitrate
6	Acetonitrile	0.050 ammonium acetate	0.200 calcium nitrate
7	Acetonitrile	0.050 ammonium acetate	0.200 magnesium nitrate
8	Acetonitrile	0.080 ammonium acetate	0.200 magnesium nitrate
9	Acetonitrile	0.030 acetic acid	0.200 magnesium nitrate
10	Acetonitrile	0.050 acetic acid	0.200 magnesium nitrate
11	Acetonitrile	0.010 acetic acid	0.200 magnesium nitrate
12	Acetonitrile	0.025 acetic acid	0.200 magnesium nitrate
13	Acetonitrile	0.005 acetic acid	0.200 magnesium nitrate
14	Acetonitrile	0.010 acetic acid	0.100 magnesium nitrate
15	Acetonitrile	0.010 acetic acid	0.025 magnesium nitrate
16	Acetonitrile	0.010 acetic acid	0.040 magnesium nitrate
17	Acetonitrile	0.010 acetic acid	0.050 magnesium nitrate

Source: Laboratory Analysis (2017-2019)

Gradient Method Development

In the gradient method development, the composition of the mobile phase varies. In this type of separation, the mobile phase composition changes as the

separation progresses with time. The mobile phase consists of two components, A and B where A is the organic solvent while B is the aqueous buffer (Table 4-18).

Table 4: Gradient Method 1 Organic Phase (A) as 8% with Aqueous Phase (B) as 92% with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	92	8
1.50	92	8
3.50	30	70
5.00	30	70
9.00	90	10
10.00	90	10
11.00	92	8

Source: Laboratory Analysis (2017-2019)

Table 5: Gradient Method 2 Presenting the Organic Phase (A) as 25% with Aqueous Phase (B) as 75% with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	75	25
1.00	75	25
5.00	35	65
6.00	35	65
9.00	10	90
10	10	90
11	75	25

Source: Laboratory Analysis (2017- 2019)

Table 6: Gradient Method 3 Showing the Organic Phase (A) as 25% with Aqueous Phase (B) as 75% with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	75	25
2.00	75	25
5.00	35	65
6.00	35	65
10.00	10	90
11.00	10	90
12.00	75	25

Source: Laboratory Analysis (2017- 2019)

Table 7: Gradient Method 4 Organic Phase (A) as 18% with Aqueous Phase (B) as 82% with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	82	18
1.00	40	60
3.00	40	60
5.00	10	90
9.00	10	90
10.00	82	18
11.00	82	18

Source: Laboratory Analysis (2017- 2019)

Table 8: Gradient Method 5 Organic Phase (A) as 12% with Aqueous Phase (B) as 88% with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	88	12
1.00	88	12
3.00	30	70
5.00	30	70
9.00	10	90
10.00	10	90
11.00	88	12

Source: Laboratory Analysis (2017- 2019)

Table 9: Gradient Method 6 Organic Phase (A) as 8% with Aqueous Phase (B) as 92% with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	92	8
1.50	92	8
3.50	30	70
5.00	30	70
9.00	90	10
10.00	90	10
11.00	92	8

Source: Laboratory Analysis (2017- 2019)

Table 10: Gradient Method 7 Showing the Organic Phase (A) as 3% with Aqueous Phase (B) as 97% with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	97	3
2.50	97	3
4.50	35	65
6.00	35	65
10.00	97	3
11.00	97	3
12.00	97	3

Source: Laboratory Analysis (2017- 2019)

Table 11: Gradient Method 8 Showing the Organic Phase (A) as 25% with Aqueous Phase (B) as 75% with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	100	0
1.50	100	0
3.50	38	62
5.00	38	62
9.50	90	10
10.50	90	10
11.00	100	0

Source: Laboratory Analysis (2017- 2019)

Table 12: Gradient Method 9 Organic Phase (A) as 5% with Aqueous Phase (B) as 95% with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	95	5
2.50	95	5
4.00	33	67
6.00	33	67
7.00	10	90
8.00	10	90
10.00	95	5

Source: Laboratory Analysis (2017- 2019)

Table 13: Gradient Method 10 Organic Phase (A) as 5% with Aqueous Phase (B) as 95% with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	95	5
2.50	95	5
6.50	40	60
7.50	40	60
8.50	10	90
9.50	10	90
10.50	95	5

Source: Laboratory Analysis (2017- 2019)

Table 14: Gradient Method 11 Organic Phase (A) as 5% with Aqueous Phase (B) as 95 % with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	95	5
2.50	95	5
4.50	33	67
6.00	33	67
10.00	95	5
11.00	95	5
12.00	95	5

Source: Laboratory Analysis (2017- 2019)

Table 15: Gradient Method 12 Showing the Organic Phase as (A) 65% with Aqueous Phase (B) as 35% with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	35	65
2.50	35	65
4.00	20	80
6.00	20	80
7.00	10	90
8.00	10	90
10.00	35	65

Source: Laboratory Analysis (2017- 2019)

Table 16: Gradient Method 13 Organic Phase A as 5% with Aqueous Phase (B) as 95% with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	95	5
2.50	95	5
4.00	40	60
6.50	40	60
7.50	10	90
8.50	10	90
10.50	95	5

Source: Laboratory Analysis (2017- 2019)

Table 17: Gradient Method 14 Organic Phase A as 5% with Aqueous Phase (B) as 95% with it Corresponding Time (min)

Time (min)	Percentage of B	Percentage of A
0.01	95	5
2.50	95	5
6.00	40	60
7.00	40	60
8.00	10	90
9.00	10	90
10.00	95	5

Source: Laboratory Analysis (2017- 2019)

Table 18: Gradient Method 15: Organic Phase A as 5% with Aqueous Phase (B) as 95% with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
0.01	95	5
2.00	95	5
4.00	33	67
5.50	33	67
9.50	90	10
10.50	90	10
11.50	95	5

Source: Laboratory Analysis (2017- 2019)

Analysis of the Individual Standards and Mixture of Standards.

The six standards were run and identified individually using the above different combinations of mobile phase tabulated in Table 3 *via* gradient method with the following time and ratios of solvents program tabulated in Tables 4-18. Finally, all the six standards were mixed in a 2 ml volumetric flask. Briefly, 200 µl of each intermediate standards (20 ppm) were measured into a 2 ml volumetric flask and diluted to the mark with Milli Q water (2 ppm). Blank was also prepared by dissolving 200 µl of the working solution (20 ppm) in 2 ml volumetric flask and diluted with Milli Q water to the mark. The mixture was injected into the chromatographic system.

Optimization of the Method

In the optimization of the method, gradient method 14, the aqueous phase concentration, pH, flow rate injection volume were all adjusted to attain the best condition. Briefly, a number of concentrations (0.01 M, 0.05 M, and 0.075 M) of

the acetic acid were prepared by measuring 5.7, 28.6 and 43.0 ml in 1 litter respectively. In addition, different concentrations (0.2, 0.1, 0.05, 0.04, 0.025 M) of the hydrated magnesium nitrate were prepared by measuring 51.282, 25.64, 12.820, 10.256, 6.41025 g respectively and dissolved in 1 liter of distilled water. These concentrations with initial pH about 2.7 were adjusted to 3.5, 3.8, 3.9 and 4.0 using 0.5 M NaOH solution. Flow rate of different volumes (0.3, 0.5 and 0.6 ml/min) of each prepared concentration were manually adjusted and was run using gradient method 14 for the mixture of standards.

Preparation of Final Aqueous Phase for the Work

HPLC grade acetic acid (0.01 M) 5.74 ml was measured into a 1 L beaker containing a little Milli Q water and 10.2564 g of Magnesium nitrate hexahydrate (0.04 M) was added. Milli Q water was added to the 850 ml mark and finally homogenized using the magnetic stirrer. The pH of the homogenized solution (around 2.7 depending on the temperature) was checked and was then adjusted to 3.5 with drops of 0.5 M NaOH with continuous magnetic stirring. The adjusted pH solution was then transferred into 1L volumetric flask, the beaker was rinsed with a little Milli Q water to remove all traces of the solution from the beaker and was added to the solution in the volumetric flask. The solution in the volumetric flask was corked and finally homogenized. The homogenized solution was then filtered using 0.2 µm filter.

Calibration of Method

The optimized method was calibrated by preparing different concentrations of the standard mixture. These mixtures were prepared by pipetting specific amount

listed in the table 16 and 17 above in 2 ml volumetric flask. The following prepared standards were injected into the chromatographic unit and run using conditions stipulated in gradient method 14 with acetonitrile, 0.01 M acetic acid and 0.04 M magnesium nitrate as mobile phase. Higher concentrations were prepared from 1000-ppm stock solution and the lower concentrations were prepared from 20 ppm intermediate standards except for 0.01 M, which was prepared from 10 ppm intermediate standards. The following tables (Table 19-20) show the volumes taken to prepare the lower concentration and higher concentrations.

Table 19: Lower Concentrations with it Corresponding Volumes of Standards used in Calibrating Gradient Method 14

Lower concentrations of standards (µg/ml)	Volume of standards pippeted (µl)
0.01	2
0.05	5
0.07	7
0.10	10
0.50	50
1.50	150

Source: Laboratory Analysis (2017- 2019)

Table 20: Higher Concentrations with It Corresponding Volumes of Standards Administered for Calibration of Gradient Method 14

Higher concentrations of standards (µg/ml)	Volume of standards pippeted (µl)
3.00	6
5.00	10
8.00	16
10.00	20
15.00	30
20.00	40

Source: Laboratory Analysis (2017- 2019)

Repeatability and Reproducibility

Prepared standards were administered to check the repeatability of results and this was done by preparing 10 different standards of 15 ppm for a number of weeks. Also, a concentration of 15 ppm was injected in triplicate for five different days.

Recovery Studies (A) of the Analytes (Eluting with 0.5 ml, 1 ml with Buffer 1 of pH 2.5)

Each standard (0.3 ppm) was dissolved with Mcllvaine buffer 1 in 25 ml volumetric flask. SPE cartridges were mounted and conditioned first with 10 ml methanol and followed with 10 ml Mcllvaine buffer 1 of pH 2.5. Standard was concentrated onto the cartridge after which it was left to dry. SPE cartridge was eluted with 0.5 ml 20% EDTA and 80% ACN. The extract was poured out into UHPLC vials, concentration of 2ppm of the mixture was also prepared and poured out into another flask and these were run using acetonitrile, 0.01 M acetic acid, and

0.04 M magnesium nitrate with gradient method 14. The procedure was repeated with 1 ml eluent.

Recovery Studies B of the Analyte (Eluting with 0 .5 ml, 1ml, Buffer 2 of pH 3.0)

The recovery studies procedure A was repeated for buffer 2 with pH 3.

Preparation of Fish Samples

Fish were bought from the supermarket (Mercadona Spain). Bones and fish skin were removed from smoked fish samples after which it was homogenized using the blender T25 digital Ultra Turrax (IKA, Germany. The homogenized fish was then kept in the fridge for further analysis.

Extraction of Spiked Fish Samples (Using Buffer 1)

Extraction Method 1

Homogenized fish samples of 2 g was spiked with a known concentration (0.08 ppm) with all the standards. The spiked fish samples were stored in the fridge for 25 min this was done so that the sample matrix and the standards would be equilibrated. To start the extraction, 25 ml 50v/50v ACN Mcllvaine buffer 1was homogenized and divided into two. The first part was added to the spiked fish samples and this was vortex within 10 minutes with the vortex mixer. After centrifugation the supernatant was collected into a new centrifuge tube and was kept. The extraction procedure was repeated with the second part of ACN/ Mcllvaine buffer 1 and this was vortex again within 10 minutes. After it was centrifuged, the supernatant was added to the earlier one. The supernatant was kept in the freezer for 20 min. at 4^{o}C. After which it was centrifuge at 3000 within 5

min. Fat deposits were remove from the top layer of the extract. After which the whole extract was concentrated on the SPE cartridge. Blank fish were also extracted in the same way alongside the spiked fish.

SPE Extraction (Eluting with 1 ml)

HLB cartridges were conditioned first, with 10 ml, methanol and followed with 10 ml Mcllvaine buffer. Extracts was concentrated onto the cartridge after which it was left to dry. SPE cartridge was eluted with 1ml 20% EDTA and 80% ACN. The final concentration in the cleanup extract was calculated using dilution formula $C_1V_1 = C_2V_2$. The extract was poured out into UHPLC vials also concentration of 2ppm of the mixture was also prepared and poured out into another flask and these were run using acetonitrile, 0.01 M acetic acid, and 0.04 M magnesium nitrate with gradient method 14.

Extraction Method 2

SPE extraction of spiked fish using 2 ml acetonitrile (ACN) and concentrating the whole extract. Homogenized fish samples of 2 g was spiked with a known concentration of 0.08 µg/ml of the standards. The spiked fish samples were stored in the fridge for 25 min this was done so that the sample matrix and the standards would be equilibrated. To start the extraction, 2 ml ACN and 23 ml Mcllvaine buffer 1 was homogenized and divided into two. The first part was added to the spiked fish samples and this was vortex within 10 minutes. After it was centrifuged, the supernatant was collected into a new centrifuge tube and kept. The extraction procedure was repeated with the second part of ACN/ Mcllvaine buffer 1 and this was vortex within 10 min. After it was centrifuged, the supernatant was added to

the earlier one. The supernatant was kept in the freezer for 20 min. at 4°C after which it was centrifuge at 3000 rpm within 5 min. Fat deposits were remove from the top layer of the extract and the whole extract was concentrated on the SPE cartridge. Blank fish were also extracted in the same way alongside the spiked fish. SPE extraction and UHPLC analysis procedures were repeated as in extraction method 1.

Extraction Method 3

In this method, 2 g of fish samples were spiked with 5 times (0.08 ppm) concentration of standards using low ACN (2 ml). The spiked fish samples were stored in the fridge for 25 min, this was done so that the sample matrix and the standards would be equilibrated. To start the extraction, 2 ml ACN and 23 ml Mcllvaine buffer 1 was homogenized and divided into two. The first part was added to the spiked fish samples and this was vortex within 10 minutes. After it was centrifuged, the supernatant was collected into a new centrifuge tube and kept. The extraction procedure was repeated with the second part of ACN/ Mcllvaine buffer and this was vortex within 10 minutes. After centrifuging, the supernatant was added to the earlier one. The supernatant was kept in the freezer for 20 minutes at 4°C after which it was centrifuge at 3000 rpm within 5 min. Fat deposits were remove from the top layer of the extract. After which 5 ml of the extract was taken and diluted to 25 ml with Mcllvaine buffer 1. Blank fish were also extracted in the same way alongside the spiked fish. SPE extraction and UHPLC analysis procedures were repeated as in extraction method 1.

Method Validation

Fish samples were spiked with different concentration of the MRL of each standard. The spiking of fish samples were done at the following level (0.5 × MRL, MRL and 2× MRL) for five different days. MRL for sarafloxacin, diflubenzuron and teflubenzuron, crystal violet and malachite green are 30, 3000, 1000 and 500, 2 µg/kg, 2 µg/kg respectively. In addition, the linearity, accuracy, sensitivity and selectivity were conducted.

Method of an Already Existing Analytical Method Castillo-García *et al.* (2015).

Synthesis of Tb-Fe_3O_4 NPs

In Castillo-García *et al.* (2015) method, terbium nanomaterial was synthesized. This was done by accurately weighing 0.5 g of $FeCl_2.4H_2O$ dissolved in a 30 ml of distilled water. The solution was stirred using the magnetic stirrer. After 5 ml of 0.067 mol/L PEG solution and a 35 mL of a 10 mmol/L terbium nitrate solution were added to the $FeCl_2$ solution. The mixture was stirred again with the magnetic stirrer. After, the mixture was put in a mechanical stirrer at 279 rpm and 2.5 mL of NH_4OH was added drop wise. The initial colour of white was turned to black when the first drop of ammonium hydroxide solution was added solution. After the addition of the 10 ml ammonium hydroxide, the dispersion was left for 10 min. as it was undergoing mechanical stirring. Dispersion was later poured into a hydrothermal reactor and left in an oven for 90 min at 125ºC. After 90 minutes, the solution was allowed to cool down at room temperature. The cooled solution was washed a number of times with ethanol and distilled water. The

washed product was dried for 12 hours at 80 ºC after which it was calcinated for 1 hour at 650 ºC. The initial colour of black turned to reddish.

Extraction of Analytes from Spiked Fish using Terbium Coated Magnetic Nanoparticles (DSPE)

T25 digital Ultra-Turrax (Sigma Aldrich Germany) was used to homogenise fish samples. Fish samples of 0.3 g were spiked at of 0.5, 1 and twice the MRL which has been specified by the EU (2009). All the spiked samples in an eppendorf tubes were placed in the dark at 4ºC for 1 hour and this was done so that the sample matrix as well as the standards would be equilibrated. In the extraction process, a volume of 1 ml acetonitrile was added to the tubes containing the spiked samples. The mixture was vortex and centrifuge for 5 min at 10,000 rpm. The supernatants was collected and the pellet was re-extracted, with ACN. The two supernatant were combined and the extract was adjusted to pH 8.3 before the DSPE extraction. The dispersive Solid Phase extraction (DSPE) was made by adding 12 mg of the sorbent to the supernatants for 10 mins. The mixture was vortexed and an external magnet was used to remove the nano-composites and the supernatants discarded. A buffer, ammonium acetate with volume of 500 µl was used to rinse the nano-composite. This was done to take away matrix components. After adding 0.5 ml magnesium nitrate (0.1 $molL^{-1}$) with pH 9.8, mixture was continuously stirred within 30 min. A magnet was used to separate the eluates and the nanomaterial. To the mixture, a volume of 0.5 ml (75 $mmolL^{-1}$) EDTA was poured into it and stirring continued within 30 min. The eluates were all put together and finally, neutralised and was filtered with a filter known as (regenerated cellulose membrane) of pore size 0.22

µl. 15 µl volume of the sample extract that contain the analytes at concentration in it corresponding dynamic range was injected onto the chromatographic system. Components in the extract was separated by a reverse phase chromatographic system operating under gradient mode with flow rate 0.5 ml/min. varying LC gradient time (Table 21).

Table 21: Gradient Method by Castillo-García: A (organic) with B (Aqueous Phase) with it Corresponding Time (min.)

Time (min)	Percentage of B	Percentage of A
2.50	88	12
6.50	40	60
7.50	88	12
12.50	88	12

Source: Laboratory Analysis (2017- 2019)

The oven temperature was maintained at 32^{o}C for the column and the extract was assayed in triplicate. Absorption and emission wavelength was set at 255 nm and 360 nm respectively for chromatographic detection. The temperature was kept in the column oven and the extract was assayed in triplicate. Chromatogram were recorded at 255 nm for absorption wavelength and 360 nm for emission.

Chapter Summary

This chapter focused on analytical method development. The Spectral analysis of the standards were done. Different combination of mobile phase were investigated. Also, the gradient LC time programme was also investigated. In addition, the right choice of buffer, volume of eluent, and ratio of acetonitrile Mcllvaine buffer was investigated and sample treatment.

CHAPTER FOUR

RESULTS AND DISCUSSION

Introduction

This chapter discusses the results obtained from the various steps outlined in the chapter 3 in order to obtain the new analytical method. Results were compared to literature and MRL from the European Union. The new method was validated and compared to an already existing method.

Method Development

Determination of Wavelength and Spectral Analysis of Antiparasitic Agents

In the electromagnetic spectrum, the UV region covers the wavelength range 100-400 nm. The UV is further divided into three bands which includes 315-400 nm UVA, 280-315 nm UVB and 100-280 nm UVC. The visible light has a wavelength range from ~400 nm to ~700 nm. The standards studied in this work were Ethoxyquin, Sarafloxacin, Diflubenzuron, Teflubenzuron Crystal violet and Malachite green. The results from the spectral analysis revealed that Ethoxyquin, Sarafloxacin, Diflubenzuron and Teflubenzuron had their wavelength mainly in the ultra-violet region (Table 22, Fig. 9-12). Crystal violet and Malachite green had their wavelength from the ultra-violet through to the visible region with their maximum absorption at the visible region (596 and 622 nm) respectively.

Table 22: Wavelengths of the Standards

Standards	λ_{max} (nm)	λ 1 (nm)	λ 2 (nm)	λ 3 (nm)
Crystal violet	596	255	308	211
Malachite green	622	321	432	258
Diflubenzuron	260	207	-	-
Teflubenzuron	254	208	-	-
Ethoxyquin	358	232	-	-
Sarafloxacin	334	282	323	209

Where λ is absorption wavelength. Source: Laboratory Analysis (2017- 2019)

Among all the analytes studied for this work, crystal violet and malachite green had the longest wavelengths (λ_{max} 596 and 622 nm) respectively. This was because compounds that have many conjugations in their structure have longer wavelengths. Actually, in the ultraviolet and visible regions absorption is as a result of electronic transitions. The term transition is known as moving of an electron form one position to the other. Comparatively, the movement of π electrons from a compound that have conjugations in it system occurs more frequent than σ electrons that make up the molecular frameworks (Nakahara, 2002). When there is collision as a result of a π electron and a photon, π electron quickly changes it motion to a different one. In cases where the amount of energy of the photon is small, the π electrons still changes its motion this is because the change in motion of the π electron does not depend on the amount of energy the photon is carrying. Conjugated systems are most likely to be affected by photons of lower energy this is because they contain a higher number of π electrons and this π electrons can be affected by the smallest amount of energy. Consequently, transition expresses the

way the electrons absorb the energy from the photon (Nakahara, 2002). If a photon has a relatively small amount of energy, the value of hc/λ for that photon is relatively small, and therefore the value of λ is relatively large. λ is observed as the absorption wavelength and so, if there is a conjugated system, peaks tend to appear in regions where λ is large i.e., the long wavelength region (Nakahara, 2002).

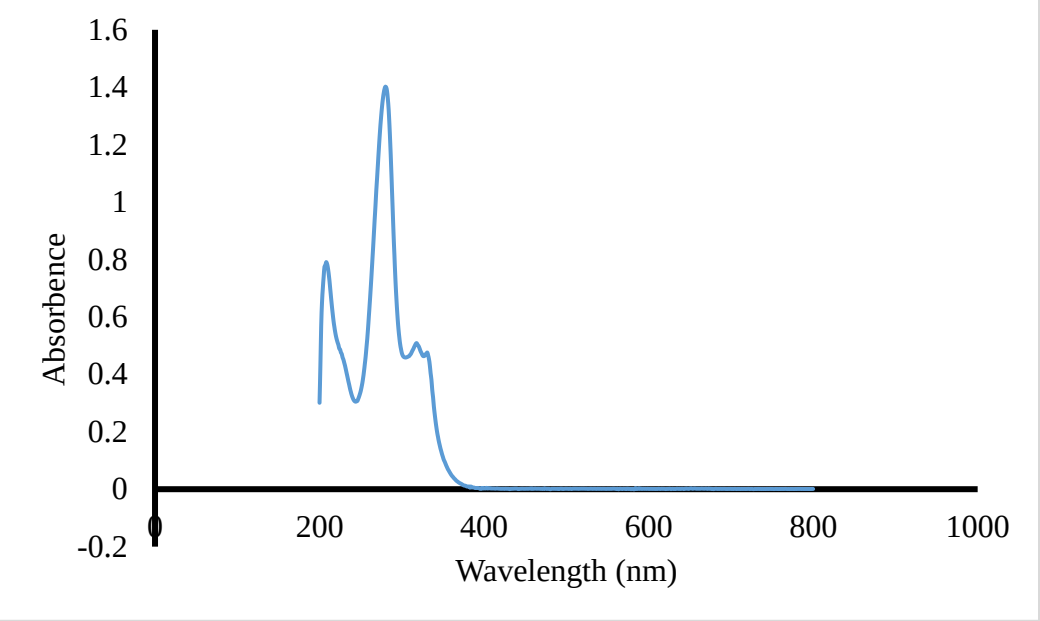


Figure 9: UV-Vis Spectrum of Sarafloxacin.

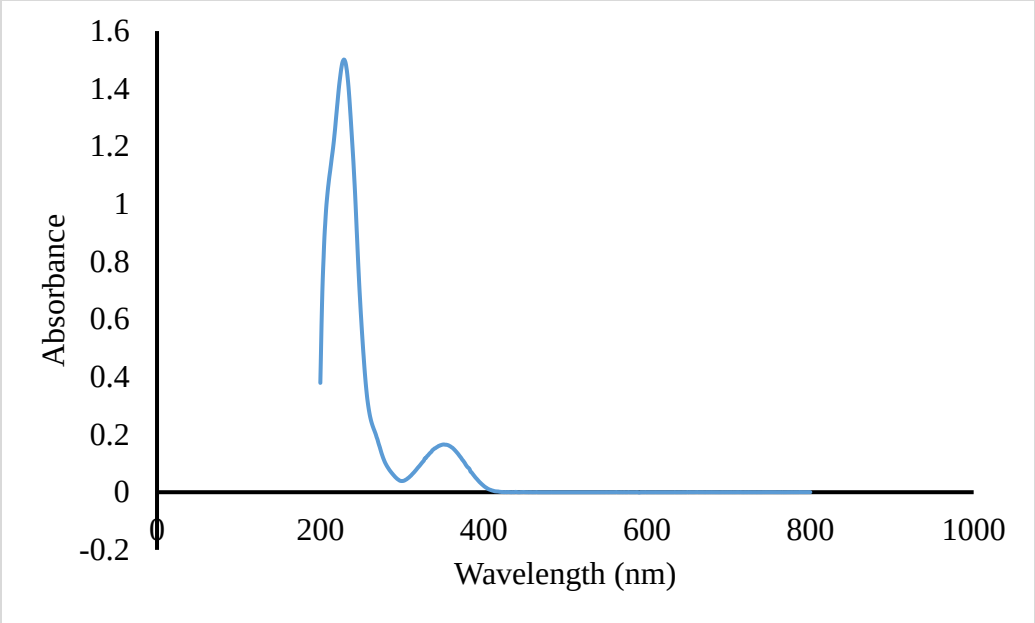


Figure 10: UV-Vis Spectrum of Ethoxyquin.

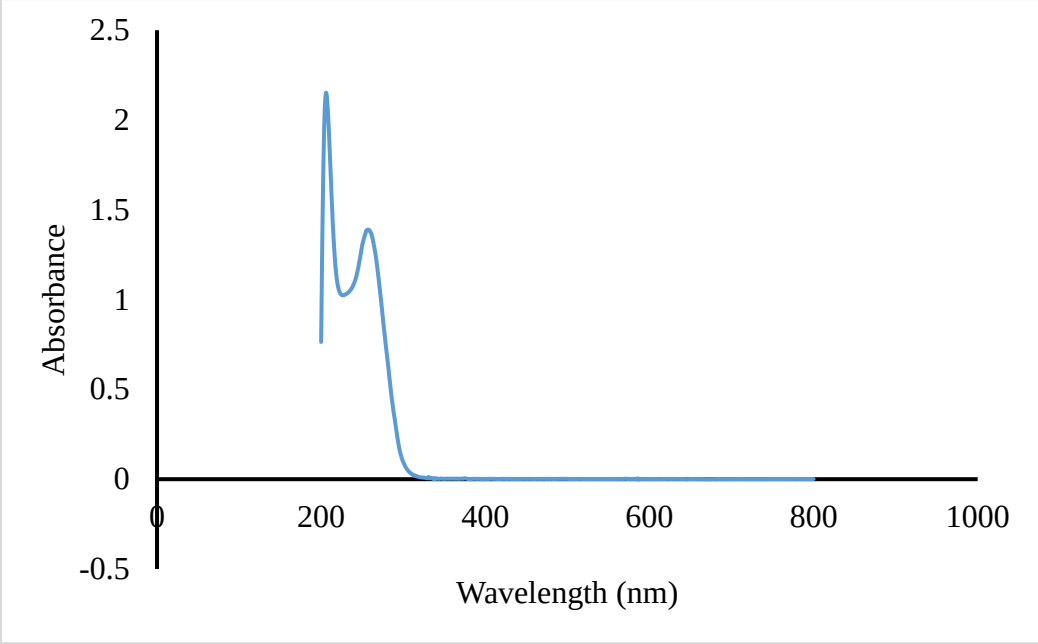


Figure 11: UV-Vis Spectrum of Diflubenzuron.

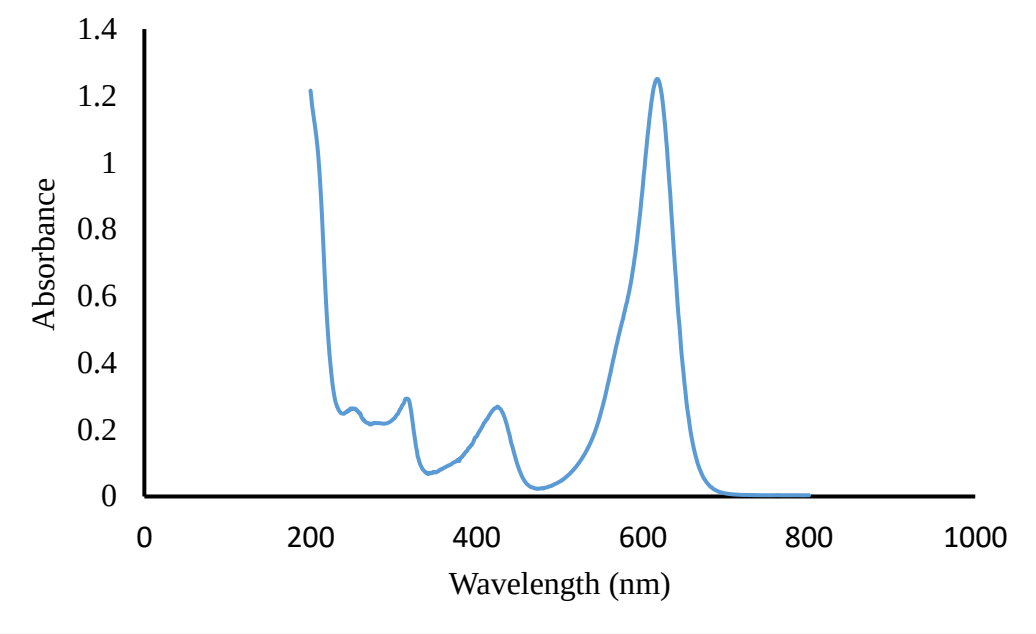


Figure 12: UV-Vis Spectrum of Malachite Green.

Diflubenzuron, Teflubenzuron, Malachite green and Crystal violet on the other hand showed weak fluorescence emission. Hence, they were better monitored using their absorption wavelengths (Table 22). Referring to table 22 all the wavelengths obtained from the spectrophotometer were used in detecting these compounds (Diflubenzuron, Teflubenzuron, Crystal violet, Malachite green). It was after that the wavelengths in table 24 were chosen as the wavelengths for the individual standards. These wavelengths were chosen because at these wavelengths detection of the compounds were very high comparatively.

Fluorescence Emission of Standards

For a compound to fluoresce, firstly, the molecule must get into the excited state, this usually occurs by absorbing a photon of light. Molecules start out in the ground electronic state (because of a Boltzmann's distribution). The ground state will usually be a singlet state. Then a photon of light can be absorbed if the energy

of that photon (E = hf) matches the energy difference between a higher molecular state and the ground state. The probability of a photon being absorbed is based on the Born-Oppenheimer approximation and Franck-Condon principle. If the photon is absorbed, the molecule will find itself in a higher singlet electronic state. For a molecule to fluoresce, it will first relax to the lowest-energy singlet-excited state (Kasha's rule). Then again, according to the Born-Oppenheimer approximation and Franck-Condon principle, it will relax to the ground electronic state, with concomitant emission of a photon, which is known as fluorescence. Sarafloxacin and Ethoxyquin were the only standards that showed maximum fluorescence emission. Referring to the Table 23, the emission spectrum comparatively was very intense when sarafloxacin was excited at 337 nm while ethoxyquin had the highest intensity when it was excited at 319 nm. This was the reason 337 and 319 nm were chosen over the other wavelengths (Fig. 13 &14). Fluorescence emission from the other standards were very small or insignificant. Since Sarafloxacin and Ethoxyquin exhibited maximum fluorescence, they were monitored using fluorescence emission.

Table 23: Excitation and Emission Wavelengths of Standard Compounds

Standards	Excitation (nm)	Emission (nm)
Sarafloxacin	282	455
	317	455
	337	450
	448	456
Ethoxyquin	319	454
	352	453
	368	453

Source: Laboratory Analysis (2017- 2019)

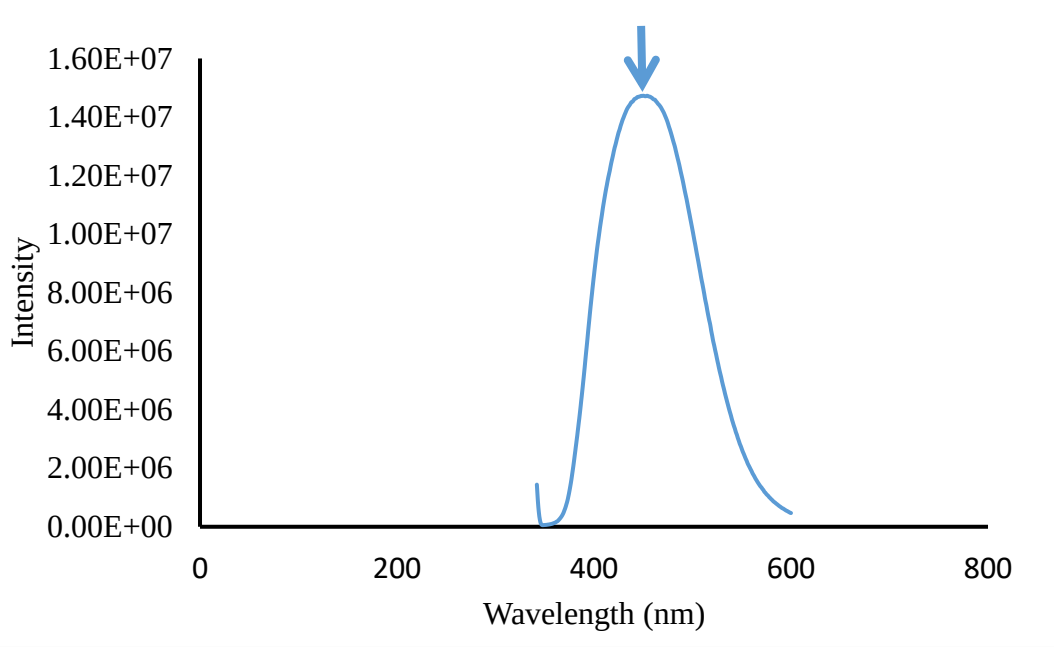


Figure 13: Emission Spectrum of Sarafloxacin.

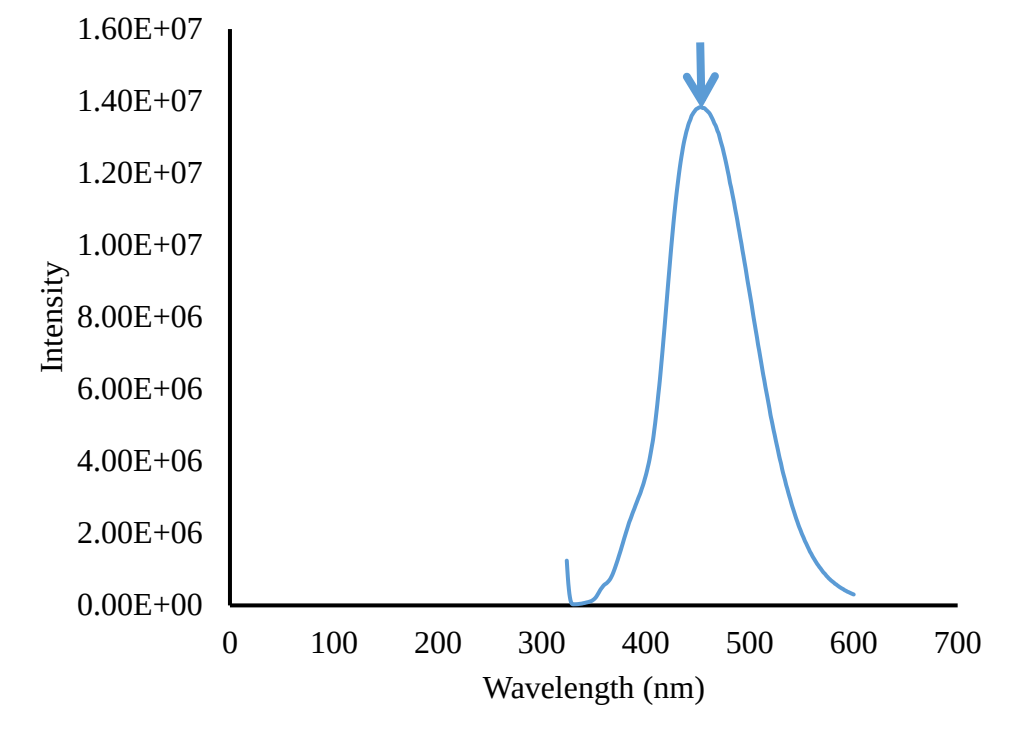


Figure 14: Emission Spectrum of Ethoxyquin.

In the selection of the appropriate wavelengths, it was ensured that there were no interferences from the solvents (blanks) used in dissolving the standards. This was achieved by checking the absorption, excitation and emission spectral of the solvents (solvent used in preparing standards) and later exciting the blanks (solvent) with the excitation wavelengths of the standards to check if there would be little or no emission from the blanks. In the case of ethoxyquin and sarafloxacin, they were monitored *via* florescence emission with excitation and emission wavelength detected at 337 and 319 nm as well as 450 and 451 nm respectively (Table 24-25).

Table 24: Absorption Wavelengths used for the Individual Standards

Standards	Absorption wavelength (nm)
Diflubenzuron	251
Teflubenzuron	247
Crystal Violet	596
Malachite Green	434

Source: Laboratory Analysis (2017- 2019).

Table 25: Excitation and Emission Wavelengths used for the Individual Standards

Standards	Excitation wavelength (nm)	Emission wavelength (nm)
Sarafloxacin	337	450
Ethoxyquin	319	451

Source: Laboratory Analysis (2017- 2019)

Choice of Solvents for Mobile Phase with Gradient Method

Gradient elution is the separation process in which the mobile phase composition does not remain constant. The mobile phase composition gradually varies with time as the separation progresses (Snyder & Dolan, 2006). The two components of the mobile phase are typically termed "*A*" and "*B*"; *B* is the aqueous buffer solvent which allows the solute to elute only slowly, while *A* is the "organic solvent which rapidly elutes the solutes from the column. In reversed-phase chromatography, solvent *B* is often water or an aqueous buffer, while *A* is an organic solvent miscible with water, such as acetonitrile, methanol, THF, or

isopropanol (Snyder & Dolan, 2006). The gradient elution process was chosen over isocratic elution for this work because of the following advantages. It decreases the retention of the later-eluting components so that they elute faster, giving narrower (and taller) peaks for most components. This also improves the peak shape for tailed peaks, as the increasing concentration of the organic eluent pushes the tailing part of a peak forward. This also increases the peak height (the peak looks "sharper"), which is important in trace analysis. The gradient program may include sudden "step" increases in the percentage of the organic component, or different slopes at different times all according to the desire for optimum separation in minimum time (Snyder & Dolan, 2006).

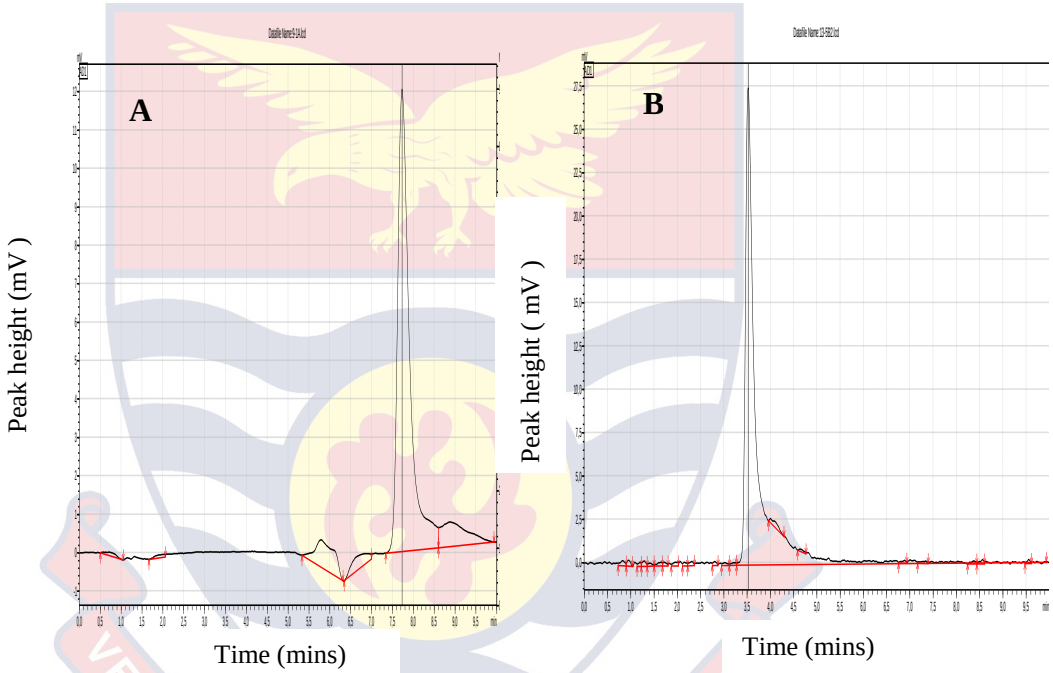
The pH of analyte retention, type of buffer to be use and its concentration, solubility in the organic modifier and its effect on detection were some of the factors that were considered in choosing the buffer for the mobile phase. These were considered because inaccurate preference of buffer in terms of buffering species, and pH, can result in poor or irreproducible retention and tailing in reverse-phase separation of polar and ionisable compounds. In addition, challenges like partial ionization of the analyte and strong interaction between analytes and residual silanoles or other active sites on the stationary phases can also be overcome by proper mobile phase buffering (maintaining the pH within a narrow range) (McMaster, 1994; Temesi & Law, 1999; www.sigma-aldrich.com/analytix). In reverse phase with silica-based packing normally the pH range is between 2 to 8. The choice of buffer is dependent on the pH. It is necessary that the buffer has a pKa close to the needed pH since buffers control pH best at their pKa (Temesi &

Law, 1999). Most of the chromatogram results obtained from the various combinations of mobile phase in Table 3 of chapter 3 can be seen in the appendix (A-L) of this work.

Using mobile phase combination of 62% methanol with 0.05M ammonium acetate and magnesium nitrate with gradient method 11, the chromatogram of these dyes (crystal violet, malachite green) showed tailing peaks (Fig.15). The pesticides (diflubenzuron and teflubenzuron) did not show any good prominent peaks .The antioxidant (ethoxyquin) did not show one prominent peak the antibiotics (sarafloxacin) on the other hand gave one prominent peak. In decreasing the methanol to 50%, the peaks obtained were poorly shaped for almost all the analytes, the chromatograms can be viewed at the appendix B. The use of methanol as the organic phase generated an increased pressure in the UHPLC system. This increased in the pressure is due to the fact that mixing of the aqueous phase with the methanol which is also polar create a lot of increased hydrogen bonding in the mobile phase. In view of this methanol was substituted with acetonitrile, which is less polar character.

Using mobile phase combination of acetonitrile, 0.05 M ammonium acetate and 0.2 M calcium nitrate with gradient method 11. All the standards showed distinct peaks except the dyes (Crystal violet and Malachite green) which did not show any distinct peaks (Fig. 16). In view of this, magnesium nitrate was maintained in the preparation of the subsequence mobile phase preparation. This implies that methanol, ammonium acetate with calcium nitrate were not good choice for the mobile phase composition. When acetonitrile, 0.01M acetic acid and

0.04M magnesium nitrate were used in gradient method 14, the chromatogram results of all the standards were identified and better resolved as compared to the above mobile phase combinations with it corresponding gradient method used (Fig. 17). Chromatograms of some the standards with their respective gradient method and combination of mobile phase used can be viewed at the appendix A-L of this work.



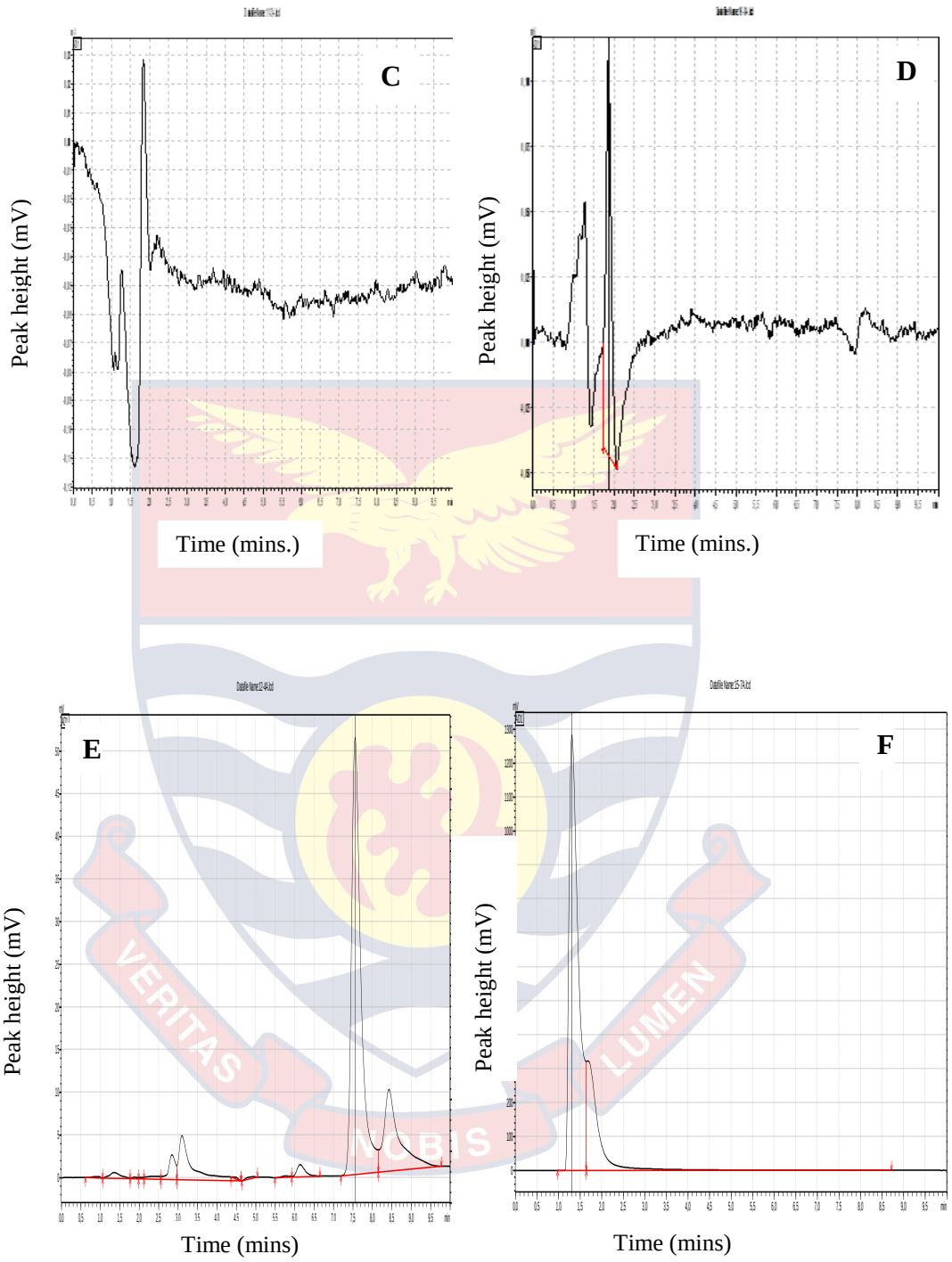


Figure 15: UHPLC Spectra of (A) Crystal violet (B) Malachite green, (C) Diflubenzuron, (D) Teflubenzuron, (E) Ethoxyquin, (F) Sarafloxacin using Gradient Method 11 with Mobile Phase Combination of 62% Methanol with 0.05M Ammonium Acetate and Magnesium Nitrate.

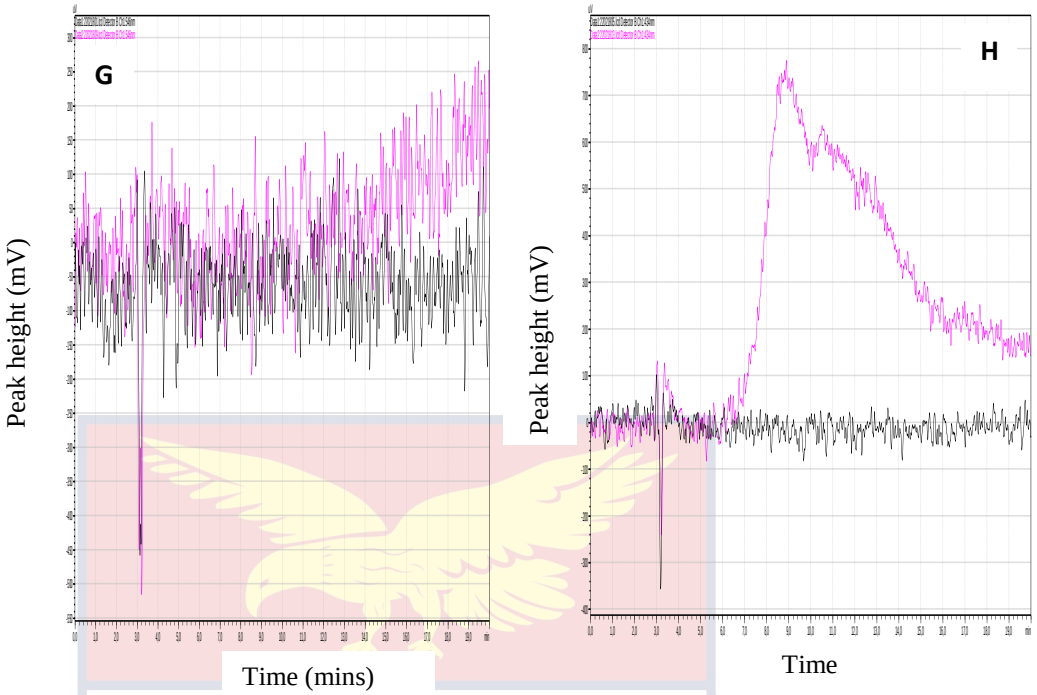


Figure 16: UHPLC Spectra of (G) Crystal violet and (H) Malachite Green using Gradient Method 11 with Mobile Phase Combination of Acetonitrile, 0.05 M Ammonium Acetate and 0.2 M Calcium Nitrate.

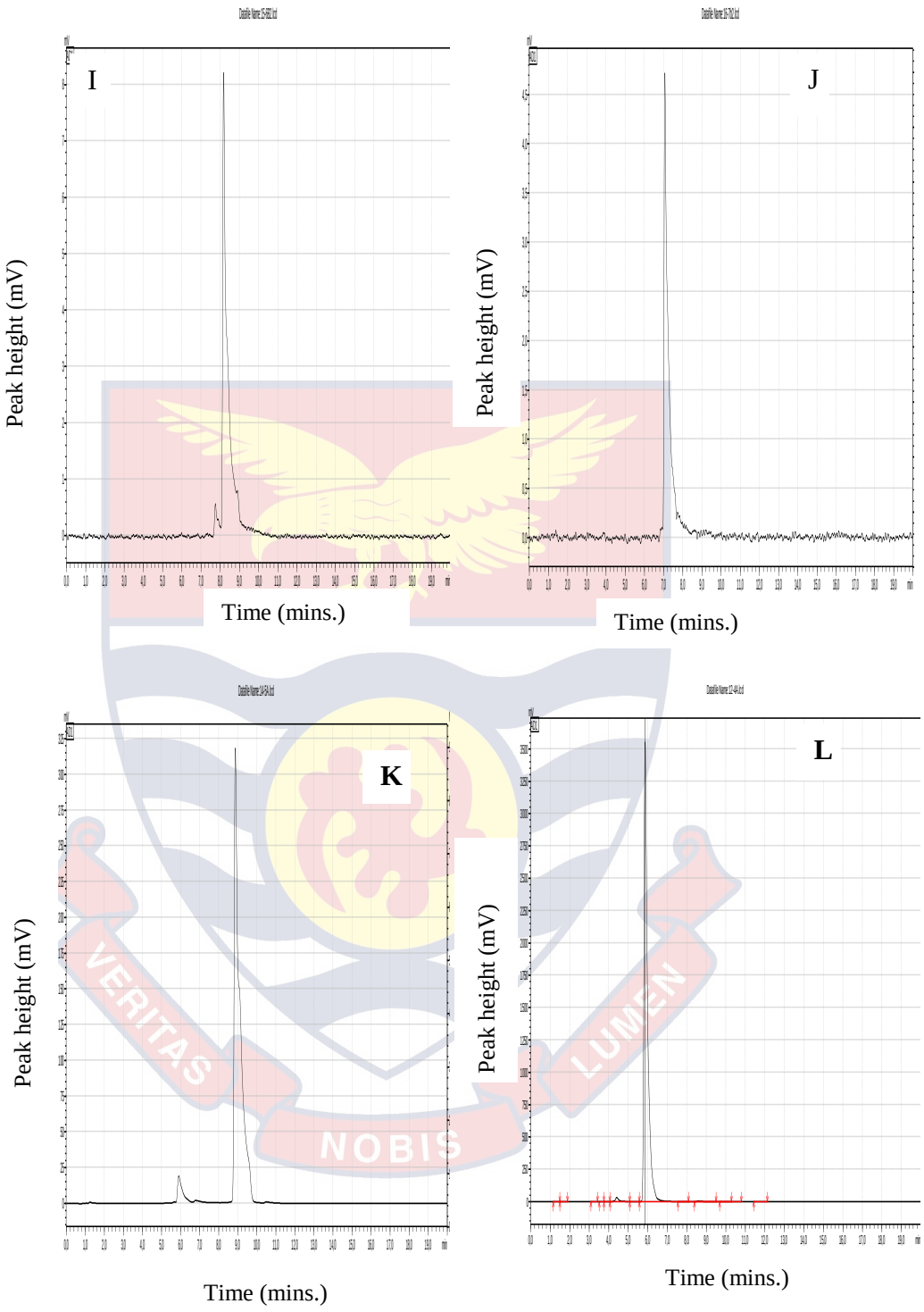


Figure 17: UHPLC Spectra of (I) Crystal violet, (J) Malachite green (K) Ethoxyquin and (L) Sarafloxacin using Gradient Method 14 with Mobile Phase Combination of Acetonitrile, 0.01M Acetic Acid and 0.04M Magnesium Nitrate.

The final choice of mobile phase composition (acetonitrile, 0.01M acetic acid, 0.04 M hydrated magnesium nitrate) chosen for this work is novel since no single mobile phase composition has been used to identify and separate antibiotics (sarafloxacin, crystal violet and malachite green), pesticides (diflubenzuron and teflubenzuron) and antioxidants (ethoxyquin) at the same time. For instance, Cañada et al (2012), used methanol-acetonitrile-10 mM citrate buffer as mobile phase to determine antibiotics in fish. Halme *et al.* (2004), used acetonitrile–acetate buffer as mobile phase to determine malachite green in fish. Hormdbal, &Yndestad (1997) on the other hand, used acetonitrile-water-tetrahydrofurane as mobile phase to determine pesticides in fish. Bohne *et al.* (2007) used solid ascorbic acid in acetonitrile as mobile phase for the determination of ethoxyquin.

Characteristics like the structure of the analyte molecule plays a major function in its retaining properties. Generally, an analyte with a larger hydrophobic surface area (C–H, C–C, and non-polar atomic bonds like S-S) is retained longer (increase retention time) since it does not interact with the water molecule due to hydrogen bonding and therefore delay elution time. This means that the compound interacts favorably with the stationary phase and however, increasing it retention time rate. Analytes with higher polar surface area (having polar groups, like OH, -NH_2, COO^- or -NH_3^+ in their structure) are less retained on the surface of stationary phase as they are better integrated into water mainly due to hydrogen bonding. Such interactions are subject to steric effects in that very large molecules may have only restricted access to the pores of the stationary phase, where the interactions with surface ligands (alkyl chains) take place. Such surface hindrance typically results

in less retention time (Gerber *et al.*, 2004). Retention time increases with hydrophobic (non-polar) surface area. Similarly, organic compounds with single C-C bonds elute slower than those with a C=C or C-C triple bond, as the double or triple bond is shorter than a single C-C bond (Gerber *et al.*, 2004). Referring to the structures of the compound (Fig. 1 to Fig. 6) studied in this work, all the compounds have C=C bonds as well as having different degrees of higher polar surface area conferred by the presence of polar groups, such as -OH, $-NH_2$, COO^- or $-NH_3^+$. Considering the structural and different degrees of higher polar surface areas of the compounds studied in this work, different gradient methods (as tabulated in chapter 3 of this work) were made to ascertain which best separated these compounds. After running several analyses with the various generated gradient methods, gradient method 14 was chosen as the best gradient method for the separation of the compounds. The addition of a mobile phase modifier affects the analyte retention time. For instance, the addition of inorganic salts (magnesium nitrate) causes a moderate linear increase in the surface tension of aqueous solutions and because the entropy of the analyte-solvent interface is controlled by surface tension, the addition of salts tend to increase the retention time (Gerber *et al.*, 2004).

The UHPLC system was the reverse type, meaning the stationary phase is non-polar while the mobile is phase is polar. In the identification and detection of the various standards, it was realised that starting the LC programme with 100% aqueous phase did not give good detection of the peaks. In addition, starting the LC program with 92%, 88% and 75% aqueous phase also did not give well resolved and separated peaks. Starting with 95% aqueous phase with 5% organic phase

(Method 14) and holding the time between 2.5 to 6 min. and gradual decrease of the aqueous phase to 40% made Sarafloxacin to be eluted at 5.658. Sarafloxacin was the first to be eluted because among all the compounds it has the highest polar surface area (containing polar groups, like OH, $-NH_2$, COO^- in it structure) which makes it less retained on the surface of stationary phase as they were better integrated into water mainly due to hydrogen bonding. In the gradient method 14, as the time approaches between 8-9 min, the percentage of aqueous phase had decreased to 10%, the organic phase had increased to 90%, and that was where Ethoxyquin, Diflubenzuron, Teflubenzuron and Crystal green were eluted at retention times of 8.926, 8.264, 9.541, 8.336 min respectively (Table 26). These compounds were held much more strongly to the stationary phase as compared to sarafloxacin, hence, required more organic phase for them to be eluted (Gerber *et al.*, 2004). The addition of magnesium nitrate to the aqueous phase improved the chromatographic peaks separation and resolution. The reason for choosing magnesium over other divalent cations was the fact that magnesium nitrate had a higher solubility in water.

Table 26: The Retention Times of the Standards using Gradient Method 14

Standards	Retention time (min)
Sarafloxacin	5.658
Malachite Green	7.741
Diflubenzuron	8.264
Crystal Violet	8.336
Ethoxyquin	8.926
Teflubenzuron	9.541

Source: Laboratory Analysis (2017- 2019)

Diflubenzuron and Crystal violet almost had the same retention times. In order to check whether their wavelengths do not interfere, diflubenzuron was detected at the wavelength of crystal violet and crystal violet was detected at diflubenzuron wavelength. Results indicated that there was no interferences in the absorption wavelength (Fig. 18 & Fig. 19). Ethoxyquin and diflubenzuron also revealed no interference between their wavelengths. This was achieved by measuring Ethoxyquin at diflubenzuron wavelength and measuring diflubenzuron at ethoxyquin wavelength (Fig. 20 & Fig. 21).

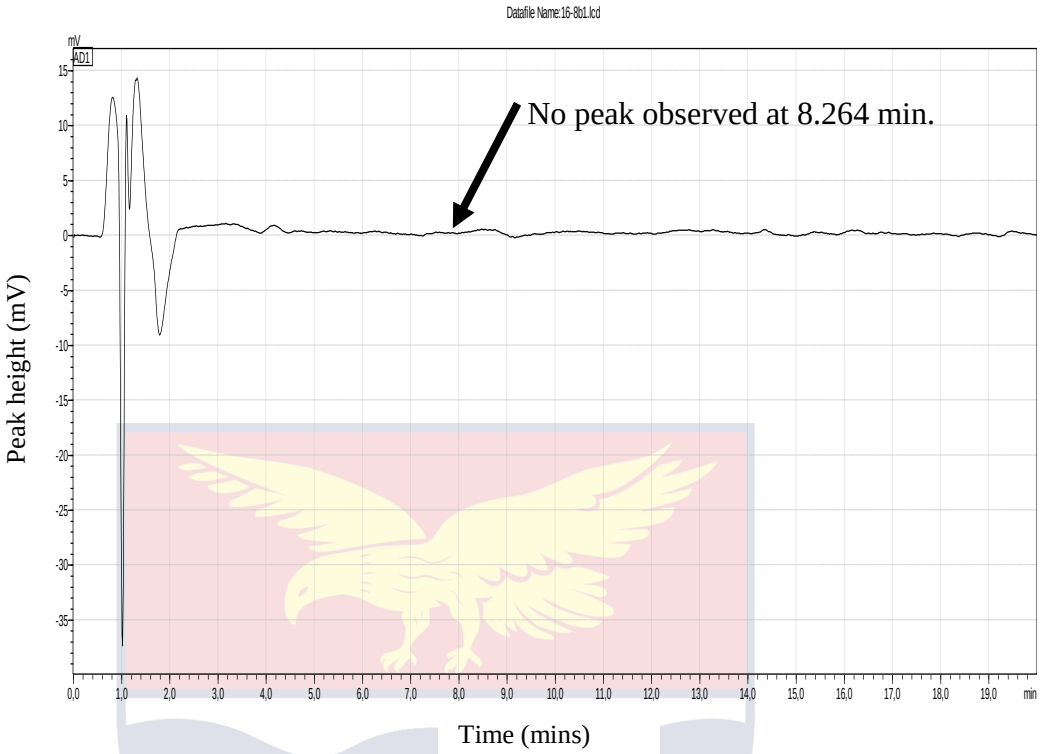


Figure 18: Diflubenzuron with Crystal Violet Wavelength shows Spectrum with no Peak appearing.

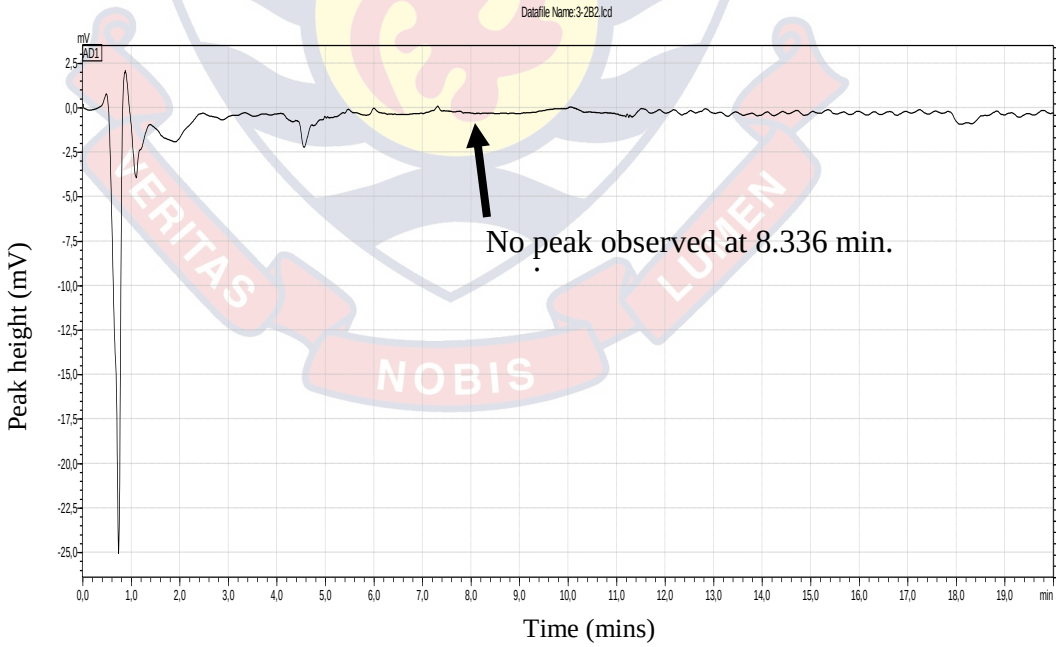


Figure 19: Crystal Violet with Diflubenzuron Wavelength shows Spectrum with no Peak appearing.

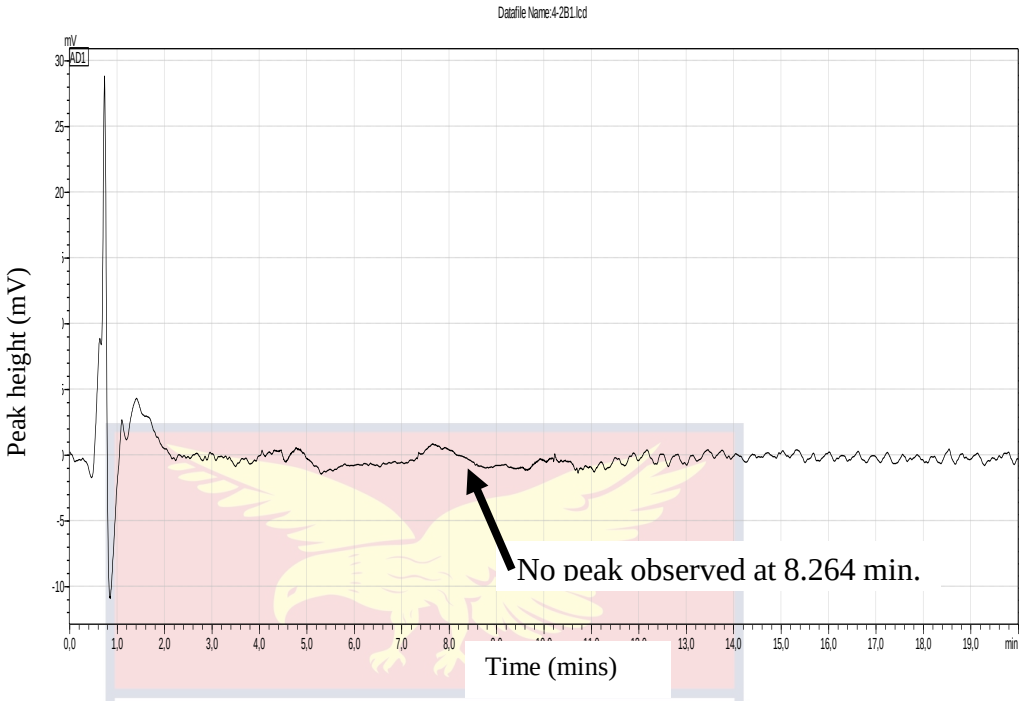


Figure 20: Diflubenzuron with Ethoxyquin Wavelength with no Peak appearing.

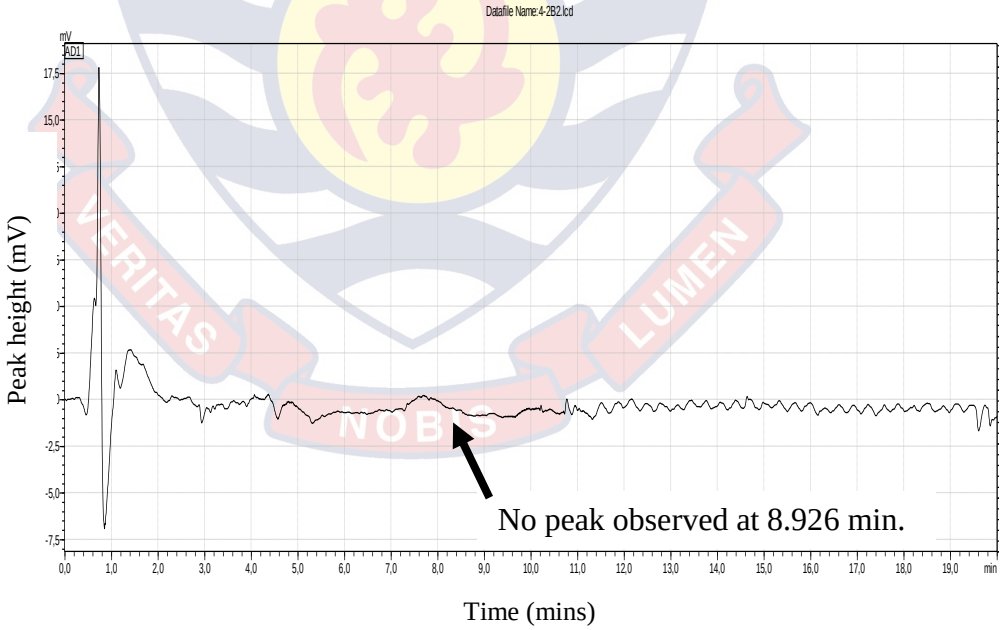


Figure 21: Ethoxyquin with Diflubenzuron Wavelength with no Peak appearing.

Choice of Common Wavelength for the Mixture (all the Standards together)

Based on the individual wavelengths, several wavelengths were tried in order to get common wavelengths for the standards. This was achieved by scanning the wavelength a few nanometers forward and backwards. For instance, ethoxyquin (368 nm for excitation and 453 nm for emission), Sarafloxacin (337nm for excitation and 451 nm for emission), had their individual wavelengths a bit similar (Table 26). Between the excitation wavelengths (337-368 nm) and emission wavelengths (451-453 nm) analysis were run in order to ascertain at which wavelengths would sarafloxacin and ethoxyquin have a common wavelength in their detection. The same was done for diflubenzuron, teflubenzuron between the wavelengths of 247-251 nm and for malachite green and crystal violet wavelengths between 434-596 nm. Finally, three common wavelengths were chosen. Sarafloxacin and ethoxyquin had a common wavelength, Diflubenzuron, Teflubenzuron also had a common wavelength, and finally Malachite green and Crystal violet also had a common wavelength (Table 27, Fig. 25-27). The measurements, which demonstrated high maximum fluorescence emission ($\lambda_{em.}$ 451 nm) was excited at 350 nm (λ_{exc}) for Sarafloxacin and Ethoxyquin. This excitation and emission differ from the wavelengths reported by Bohne *et al.* (2007) and Lombardo-Agüí *et al.* (2015). Also diflubenzuron, Teflubenzuron, malachite green and crystal violet were monitored via absorption wavelength (λ_{abs}) 251 nm, 596 nm respectively. This demonstrated high maximum absorption which differed from the wavelengths reported by Hormdzbal, & Yndestad (1996); Bajc *et al.* (2007) and Sadeghi & Nasehi (2018) respectively.

The UHPLC have two detectors, these are diode array detector (DAD) and fluorescence detector (Table 27). The fluorescence detector was used to monitor sarafloxacin and ethoxyquin (Fig.22). The diode array detector (DAD) have two wavelength channels, so one of the channels was used to monitor diflubenzuron and teflubenzuron (Fig. 23) and the other channel was used to monitor Malachite green and Crystal violet (Fig. 24).

Table 27: Absorption, Excitation and Emission Wavelengths used for Mixture of the Standards

	Wavelengths (nm)		
Standards	Absorption	Excitation	Emission
Diflubenzuron	251	-	-
Teflubenzuron	251	-	-
Crystal Violet	596	-	-
Malachite Green	596	-	-
Sarafloxacin	-	350	451
Ethoxyquin	-	350	451

Source: Laboratory Analysis (2017- 2019)

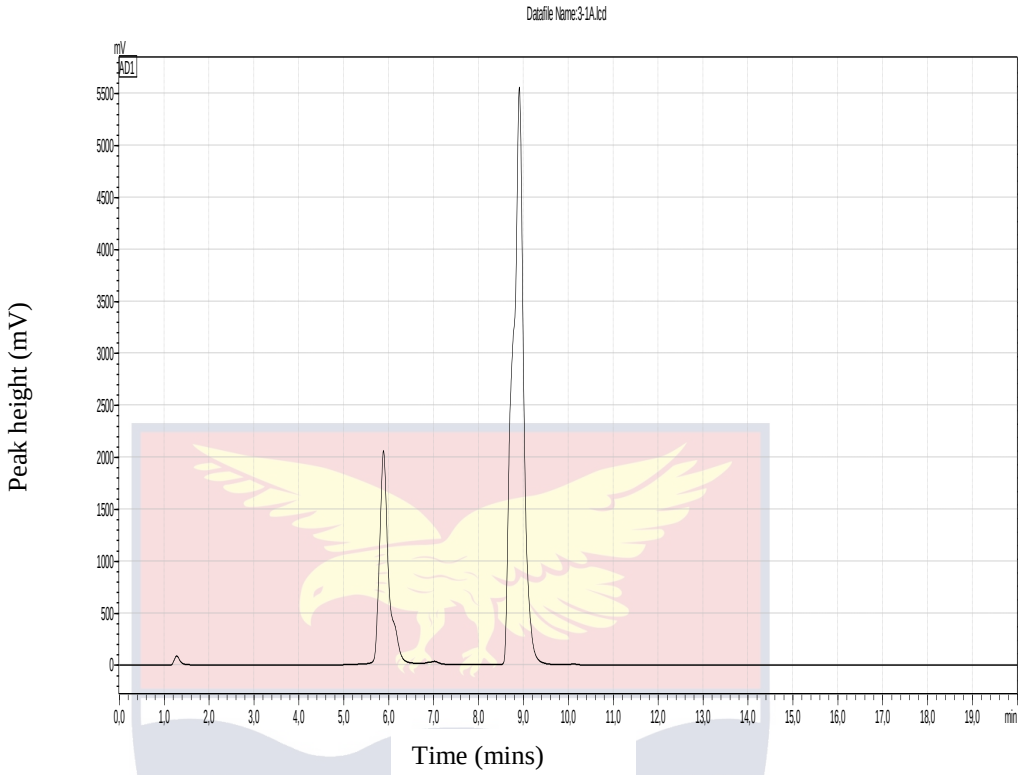


Figure 22: Sarafloxacin and Ethoxyquin respectively (Fluorescence Detector).

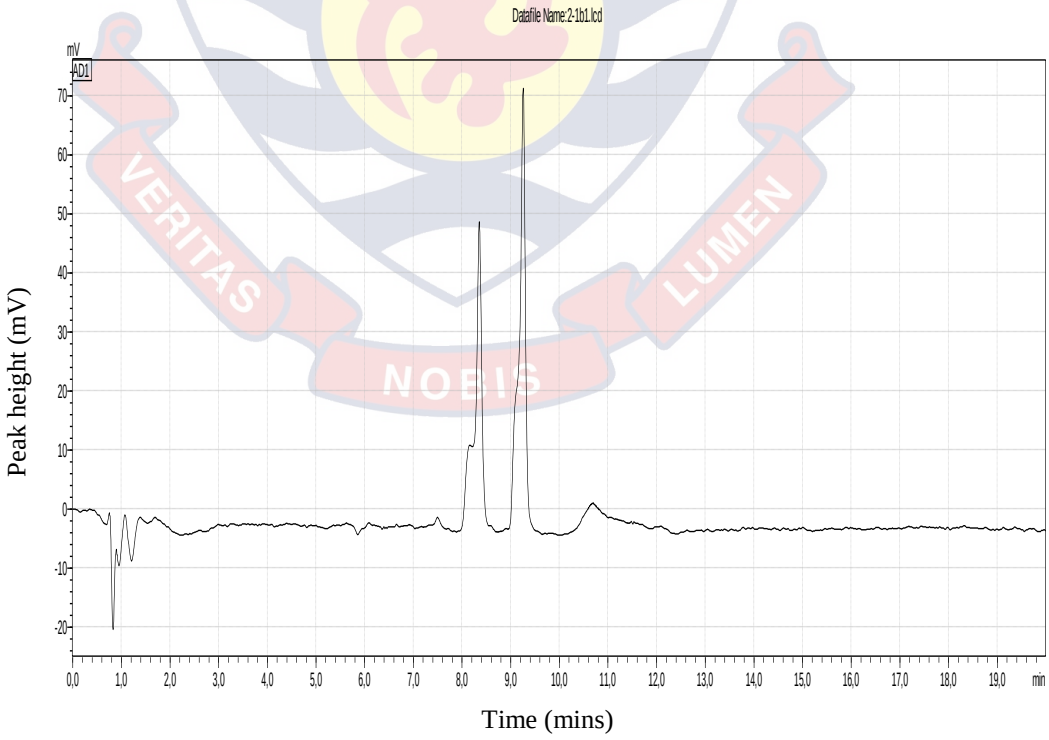


Figure 23: Diflubenzuron and Teflubenzuron DAD Channel 1.

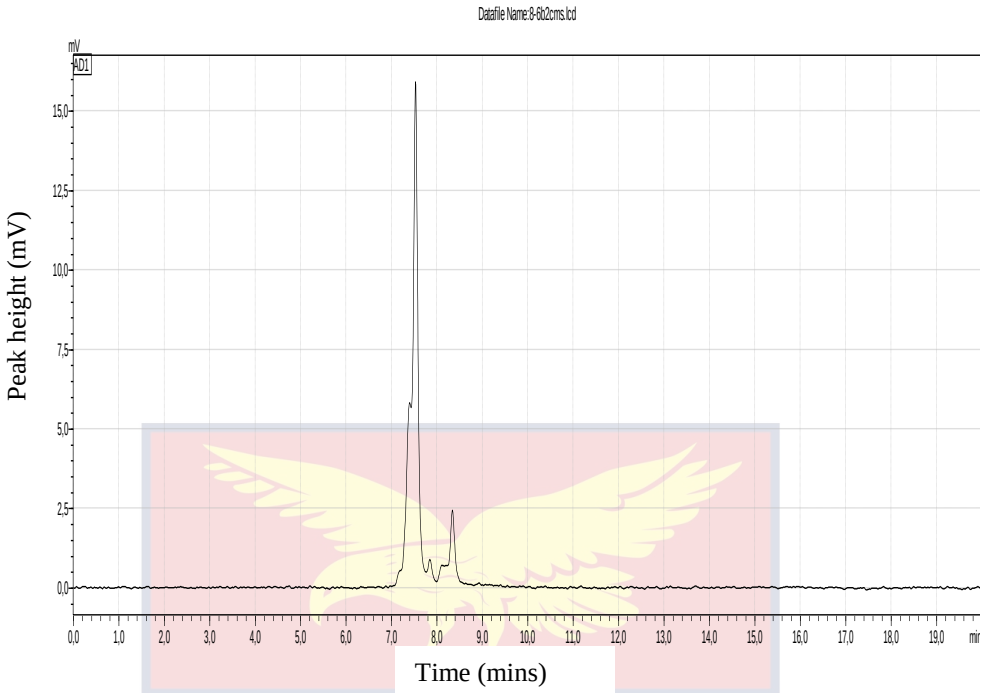


Figure 24: Malachite Green and Crystal Violet DAD channel 2.

Optimization of Chromatographic Separation

Optimization of pH

Another important factor that improve chromatographic separation and resolution is the mobile phase pH, since it can change the hydrophobic character of the analyte. For this purpose, buffering agent, such as sodium phosphate, acetic acid among others can be used to regulate the pH. Buffers are used for several purposes such as to check pH, neutralize the charge on the silica surface of the stationary phase and act as ion pairing agents to neutralize analyte charge. Applications of buffers and acids vary in their actions but generally improve chromatographic resolution (Gerber *et al.*, 2004). However, different pH of the buffers were studied (Table 28).

Table 28: Retention Time of the Standard at different pH of Mobile Phase Using Method 14

pH	3.5	3.8	3.9	4.0
Standards				
Sarafloxacin	5.658	4.666	4.650	4.636
Ethoxyquin	8.926	6.503	6.473	6.323
Diflubenzuron	8.264	4.913	4.902	4.843
Teflubenzuron	9.540	5.323	5.239	5.210
Crystal violet	7.741	5.621	5.612	5.606
Malachite Green	8.336	5.379	5.234	5.155

Source: Laboratory Analysis (2017- 2019)

From the table 28 above, it could be seen that gradual increasing of the pH of the aqueous phase from 3.5 to 4.0 resulted in analytes having close retention times. The standards were better separated and resolved at pH 3.5, At pH 3.5 challenges, in terms of analytes undergoing partial ionization, analytes and residual silanols having high interaction or between analytes and active sites on the stationary phases were over came at this pH (McMaster, 1994; Temesi & Law, 1999).

Concentration Optimization

In the optimization of the method, different concentrations of the acetic acid was studied. Table 28 below shows some of the areas obtained by the standards at different concentrations. Increasing the concentration of the acetic acid resulted in decreased in peak areas. 0.01 M was chosen to be the concentration for the acetic

acid. This is because at this level of concentration of the buffer the analyte detection was maximized (Table 29).

Table 29: Peak Areas for the Concentration of the Acetic Acid

Conc. of acetic acid (M)	0.01	0.05	0.075
Standards			
Sarafloxacin	6105860	5182504	3367730
Ethoxyquin	728343	615679	436272
Diflubenzuron	49856	44244	34568
Teflubenzuron	90556	89832	68579
Crystal violet	90556	89832	68579
Malachite Green	130059	118325	105103

Source: Laboratory Analysis (2017- 2019)

Injection Volume Optimization

Different volumes of the injection volumes were studied (10, 20, 30, 40 and 50 µl) in order to ascertain the optimize volume of injection (Fig. 25 and Appendix Fig. T6-8). Comparing the results from all the injection volumes investigated, it was clearly seen that as injection volume increases (from 10-50 µl) peak height also increased. Injection volume of 50 µl was chosen as the optimized injection volume for the method (Fig. 25). When large injection volumes are used, the peak height reaches its maximum and the sample concentration remain constant until the tail of the injection band is reached. Precaution was taken not to over increase the injection volume. This is because, when too large sample volume is injected, the peak rises normally, then is flat-topped, then drops back to the baseline with the normal

Gaussian tail (Dolan 2014; Snyder *et al.*, 2010). In addition, the injection volume should be in proportion to the size of the UHPLC column used (Mills *et al.*, 1997).

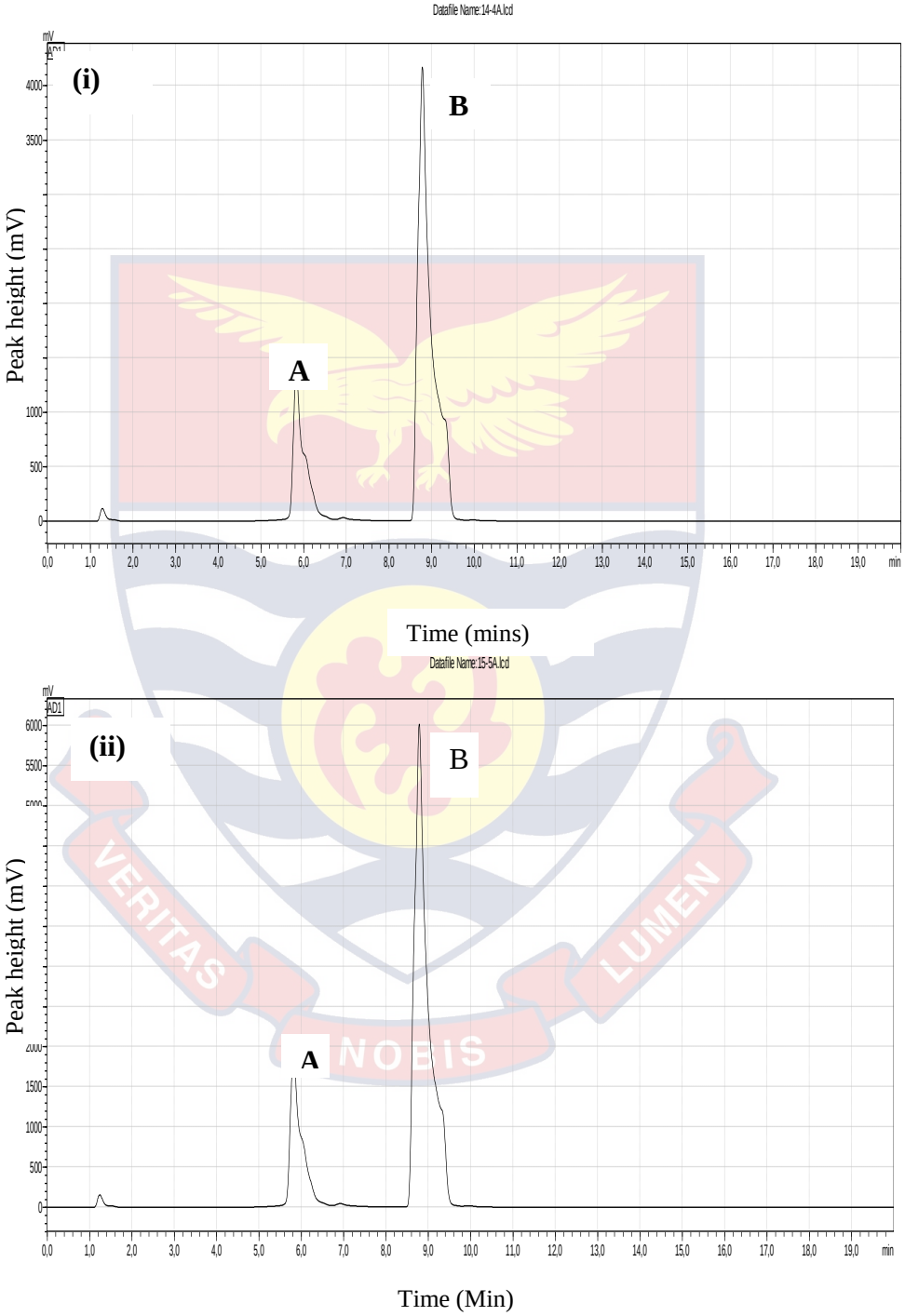


Figure 25: Chromatogram of Injection Volumes (i) 40 µl and (ii) 50 µl: where A and B are Sarafloxacin and Ethoxyquin respectively.

Flow Rate Optimization

A very high unusual flow rate may adversely affect the quality of the chromatography since higher flow rate do not allow analyte enough time to interact with the stationary phase. However, at high flow rates the adsorption of the analyte to the stationary phase results in some of the sample lagging behind. If flow rates are set too low or too high, band broadening could occur. Different flow rates were investigated (0.3 ml, 0.5 ml and 0.6 ml Appendix) to ascertain the optimised flow rate. The flow rate of 0.6 $mlmin^{-1}$ produced proper peak shape and resolution without exceeding the limit of column pressure as instructed by the manufacturer. (Fig. 24-26). Flow rates in already existing methods ranged between 0.3 to 1.5 ml/ min (Cañada-Cañada *et al.,* 2012; Roudaut & Yorke, 2002). The present method had similar flow rate of 0.6 ml/min. A column temperature of 35^{o}C was chosen since higher temperatures affected peak resolution negatively. Finally, after obtaining the required procedure and conditions necessary for separation and good resolution, calibration curve was made.

Calibration of Method

The rationale for performing a calibration of an analytical method is to obtain a valid relationship between the signals produced by the analyte and its concentration. A plot of the signal produced by different concentration of the standard against concentrations of the standards gives the calibration curve. The calibration graph helps the analyst to access quantitative data on the analyte in a sample of unknown analyte concentration. The developed analytical method was calibrated using the concentrations in tables 18 and 19 of Chapter 3. A plot of the

signal obtained from the different concentrations against it respective peak intensity was linear graph (Figs. 26-31).

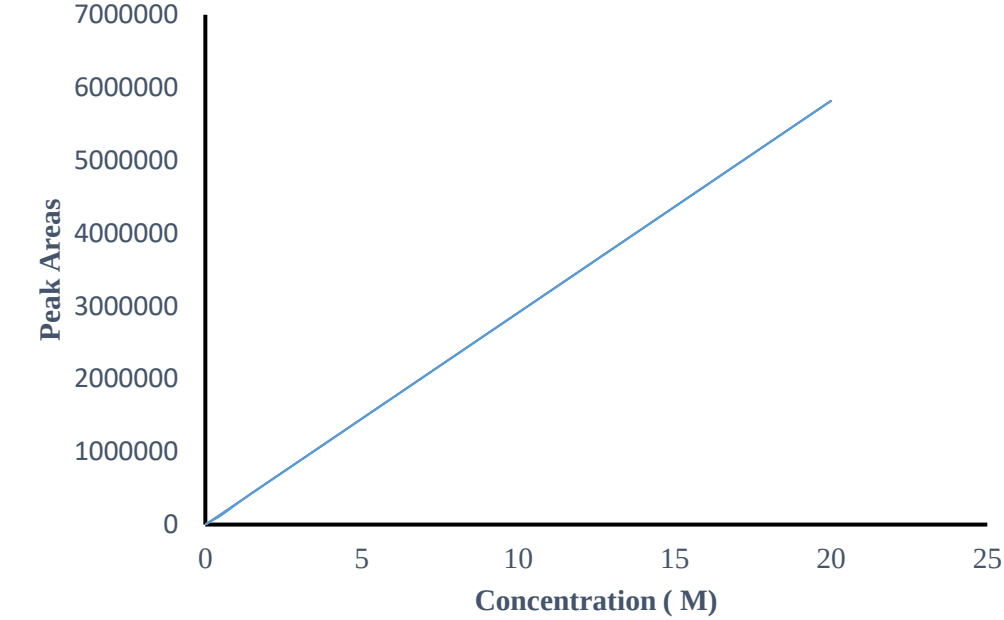


Figure 26: Calibration Curve of Sarafloxacin.

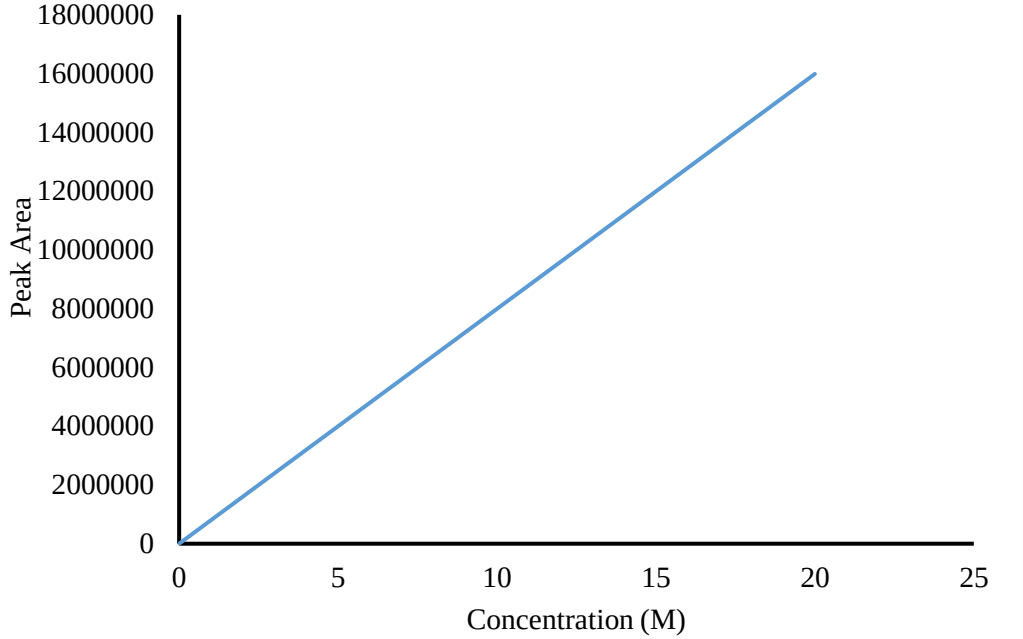


Figure 27: Calibration Curve of Ethoxyquin.

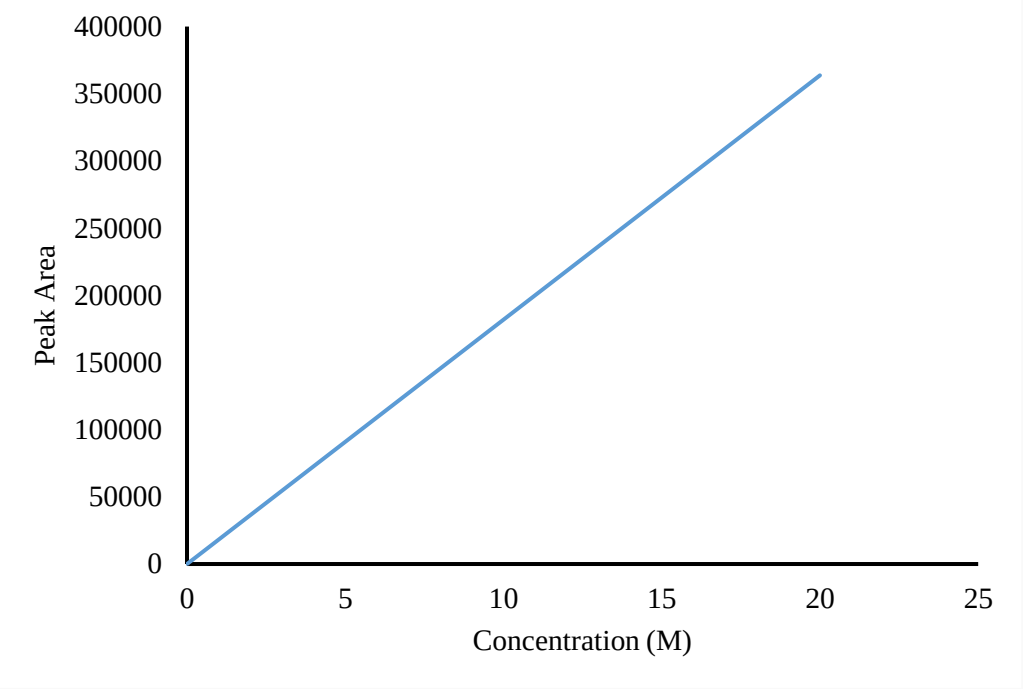


Figure 28: Calibration Curve of Malachite Green.

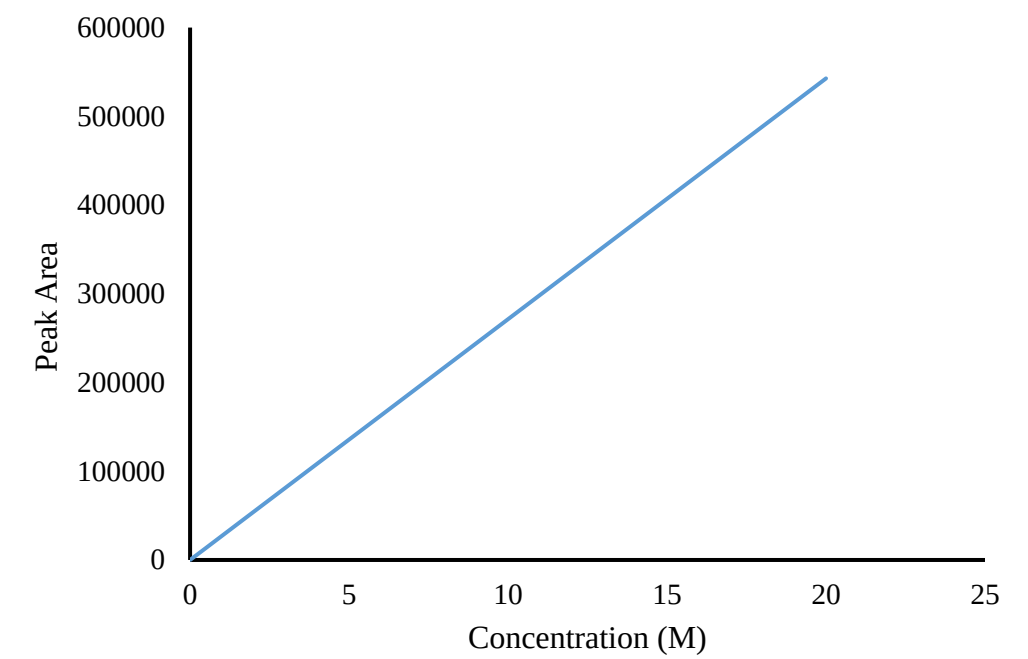


Figure 29: Calibration Curve of Crystal Violet.

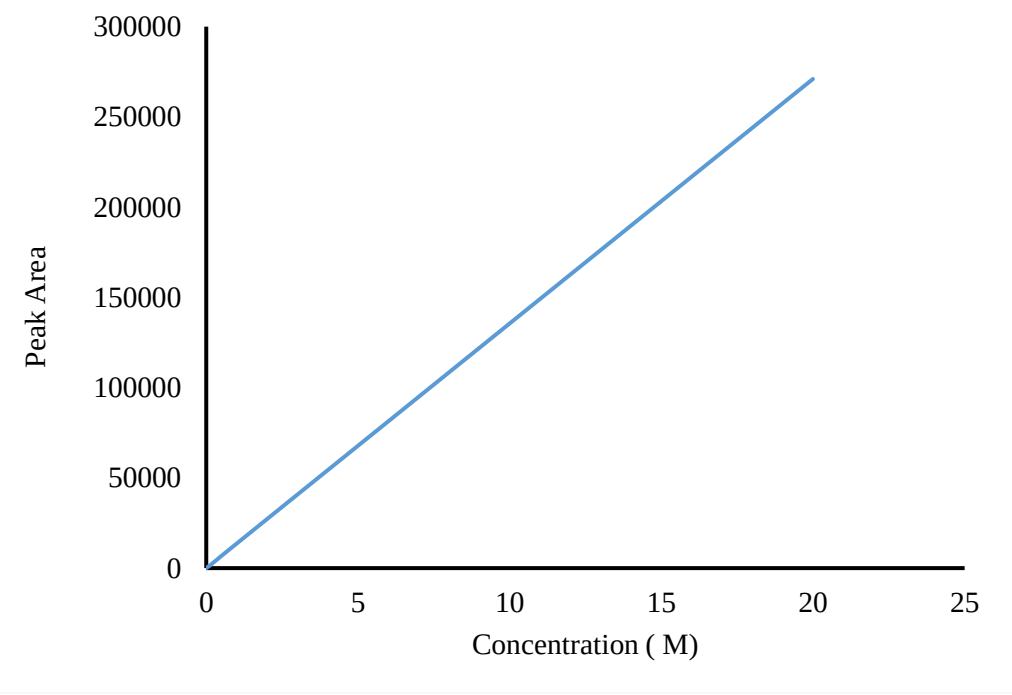


Figure 30: Calibration Curve of Diflubenzuron.

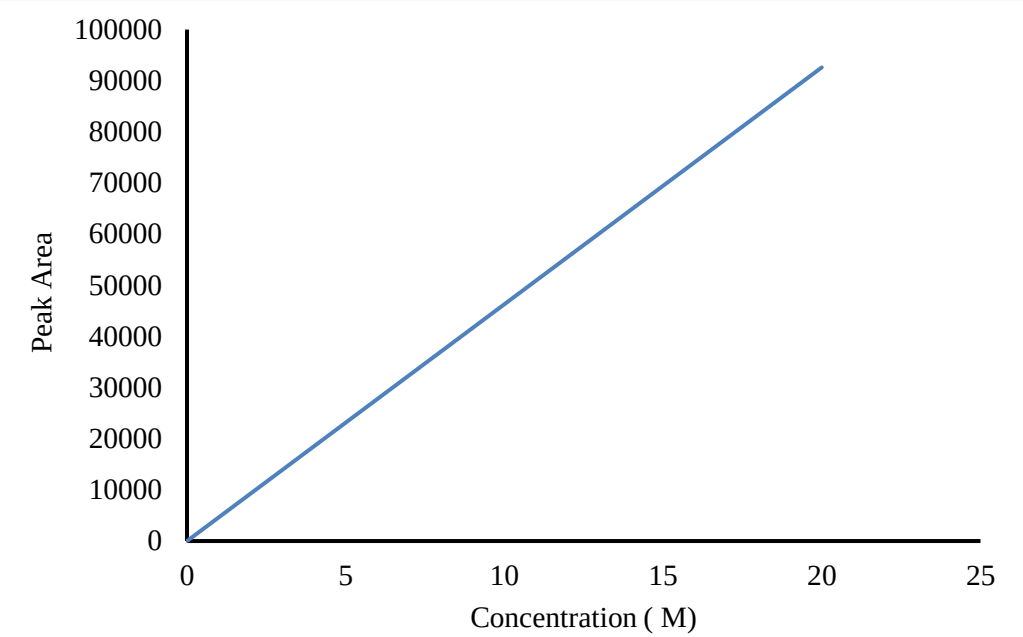


Figure 31: Calibration Curve of Teflubenzuron.

Assessment of Precision

The intraday precision was estimated by assaying ten (10) solutions during the same day over a period of time, and injections were done in triplicate. The inter day precision was estimated by analyzing three replicates of standard solutions for five consecutive days. Precision describes the reproducibility of measurements. The main aim of intermediate precision validation is to find out whether the method will give same results in the same laboratory once the development phase is over. Reproducibility shows precision of analysis of the same sample by different analysts in different laboratories using operational and environmental conditions that may be different but are still within the specified parameters of the method (Jenke, 1998). Generally statistical terms like standard deviation, variance and coefficient of variance are terms widely used to describe the precision of a set of

replicate data. The results from the standard deviation reveals that the intra-day precision and inter-day precision was reproducible and repeatable (Table 30-31).

Table 30: Standard Deviation of Intraday Precision

Analytes	Intra-day standard deviation
Sarafloxacin	1.0327
Ethoxyquin	0.4216
Diflubenzuron	0.6749
Teflubenzuron	0.4714
Crystal Violet	0.4714
Malachite Green	0.9428

Source: Laboratory Analysis (2017- 2019)

Table 31: Standard Deviation of Inter-Day Precision

Analytes	Standard deviation (Day 1)	Standard deviation (Day 2)	Standard deviation (Day 3)	Standard deviation (Day 4)	Standard deviation (Day 5)
Sarafloxacin	0	0	0	0	0
Ethoxyquin	0	0.5773	0.5773	0.5773	0.5773
Diflubenzuron	0	0.5773	0.5773	0.5773	0.5773
Teflubenzuron	0	0.5773	0.5773	0.5773	0.5773
Crystal Violet	0.5773	0.5773	0.5773	0	0
Malachite green	0.5773	0.5773	0	0.5773	0.5773

Source: Laboratory Analysis (2017- 2019)

Recovery Studies Using SPE Extraction of Standards

Reversed-phase sorbents are commonly used in SPE when aqueous samples are involved. A reverse phase polymerically bonded sorbent was chosen for this research. This is because it can withstand high pH extremes, and also it is more appropriate for environmental applications for trapping organic compounds from acidified aqueous samples (Lucci *et al.*, 2012). The extracting solvent used for this work is different from that reported in literature. For instance, Cañada-Cañada *et al.* (2012) used m-phosphoric acid/acetonitrile, Li *et al.* (2009) used acetonitrile, Guidia *et al.* (2018) used trichloroacetic acid, Roudaut & Yorke, (2002) used acetonitrile basic solution as extracting solvent to extract antibiotics while Hormazabal, & Yndestad (1997), used acetone-tetrahydrofurane to extract pesticides etc. He & Ackman, (2000), Bohne *et al.* (2007), used acetonitrile and hexane respectively as extracting solvent for ethoxyquin. In this study, a single extracting solvent (ACN/ Mcllvaine buffer) was used to extract all the different groups of antiparasitic agents (antibiotic, dyes pesticides antioxidants). Different pH (2.5 and 3) of the extracting buffer was used in the extraction. This was done in order to find out at which pH will give more of the recovery of the analytes. The eluent used in the SPE extraction in this work was 80% acetonitrile and 20% EDTA. The use of EDTA has been reported to enhance quinolones extraction process (Arroyo-Manzanares *et al.* (2015); Bourdat-Deschamps *et al.* 2014), eventhough the mechanism has not been explained fully. In addition, the volumes of eluent (0.5 and 1 ml) used in elution were also checked for the best volume of eluent to elute the analyte with higher recoveries (Table 32-33).

Table 32: Recovery Studies of Analyte Extract Using 1.0 and 0.5 ml Eluents at Buffer pH 2.5

Standards	Standard Conc. (ppm)		Observed Conc. (ppm)		% Recovery	
	0.5 ml	1.0 ml	0.5 ml	1.0 ml	0.5 ml	1.0 ml
Sarafloxacin	15	7.50	13.50	9.00	90.00	120.00
Ethoxyquin	15	7.50	2.13	7.00	14.20	93.33
Diflubenzuron	15	7.50	6.37	7.15	42.47	95.33
Teflubenzuron	15	7.50	4.75	7.38	31.66	98.33
Malachite Green	15	7.50	5.45	7.35	36.33	98.00
Crystal violet	15	7.50	10.99	7.80	73.33	104.00

Source: Laboratory Analysis (2017- 2019)

In comparing the volume of eluent (0.5 ml and 1 ml) at buffer pH 2.5 with the corresponding standard, it can be seen that 1 ml of eluent at pH 2.5 extracted more of the analyte. At pH buffer of 3, concentration of all the analytes are lower as compared to the analyte extracted with pH buffer of 2.5. Finally, buffer of pH 2.5 and 1 ml eluting solvent was found to be more efficient for the SPE extraction.

Table 33: Recovery Studies of Analyte Using 0.5 ml and 1 ml Eluent with Buffer pH 3.0

Standards	Standard Conc. (ppm)		Observed Conc. (ppm)		% Recovery	
	0.5ml	1.0ml	0.5ml	1.0ml	0.5ml	1.0ml
Sarafloxacin	15	7.50	9.60	6.25	64.00	83.33
Ethoxyquin	15	7.50	4.99	4.68	33.33	62.33
Diflubenzuron	15	7.50	3.00	1.90	20.00	25.33
Teflubenzuron	15	7.50	4.35	3.23	29.00	43.00
Malachite Green	15	7.50	11.25	6.43	75.00	85.66
Crystal violet	15	7.50	9.09	5.78	60.66	77.00

Source: Laboratory Analysis (2017- 2019)

Extraction of Spiked Fish Samples

After obtaining the optimum condition of the buffer pH (2.5), the extraction of the spiked fish samples was performed. During the extraction of the spiked fish samples, fish was spiked with 0.08 ppm of all the standards. Different volumes of the acetonitrile Mcllvaine buffer was used to ascertain which volume of acetonitrile and Mcllvaine buffer would best extract the analytes in the spiked fish samples. Using extraction method 1 (Chapter 3 of this work) 50v/50v ACN/ Mcllvaine buffer, lower recoveries were recorded and also some of the analyte were lost (Table 34). The lower recoveries could be due to the fact that the extract which contained more acetonitrile and as a result made some of the analyte to be eluted during the concentration of the extract on the SPE cartridge.

Table 34: Extraction of Spiked Fish Sample Using Extraction Method 1

Standards	Standards (2 ppm)	Extract of Spiked fish (2 ppm)	% Recovery
Sarafloxacin	2	0.25	12.45
Ethoxyquin	2	0.07	3.28
Diflubenzuron	2	-	-
Teflubenzuron	2	0.29	14.30
Malachite Green	2	0.41	20.43
Crystal violet	2	0.38	17.88

Source: Laboratory Analysis (2017- 2019)

In view of the above results, the acetonitrile was further reduced to 2 ml with 23 ml Mcllvaine buffer (Extraction method 2, chapter 3). Results showed increased in percentage recoveries of the analytes as compared to extracting with

50v/50v ACN/ Mcllvaine buffer. Even though there was bit of improvement in the recoveries it was still very low (Table 35).

Table 35: Extraction of Spiked Fish Sample Using Extraction Method 2

Standards	Standards (2 ppm)	Extract of Spiked fish (2 ppm)	% Recovery
Sarafloxacin	2	0.21	10.79
Ethoxyquin	2	0.71	35.39
Diflubenzuron	2	0.10	7.13
Teflubenzuron	2	0.20	11.58
Malachite Green	2	0.71	35.33
Crystal violet	2	0.60	30.12

Source: Laboratory Analysis (2017- 2019)

The low recoveries were attributed to the sample matrix. The sample matrix was further reduced by using extraction method 3 as detailed in chapter 3 by spiking the fish with 5 times the concentration and taking 5 ml of the extract and finally diluting it with 20 ml of the Mcllvaine buffer in 25 ml volumetric flask. The results indicated very high recoveries (80.78-99.73 %) of all the analytes (Table 36).

Table 36: Extraction of Spiked Fish Sample Using Extraction Method 3

Standards	Standards (2 ppm)	Extract of Spiked fish (2 ppm)	% Recovery
Sarafloxacin	2	1.99	99.73
Ethoxyquin	2	1.87	93.82
Diflubenzuron	2	1.61	80.78
Teflubenzuron	2	1.93	96.61
Malachite Green	2	1.72	86.32
Crystal violet	2	1.64	82.49

Source: Laboratory Analysis (2017- 2019)

Percentage recoveries, obtained in this work for all the standards in the spiked fish were 99.73, 93.80.78, 96.61, 86.3, 82.49% for sarafloxacin, ethoxyquin, diflubenzuron, teflubenzuron, crystal violet and malachite green respectively and their recoveries were higher compared to other research works like Roudaut &, Yorke (2002), (sarafloxacin) 56.9-71%, Tyrpenou *et al.* (2002) 82.14%, Li *et al.* (2009), 79-94%, (ethoxyquin), He & Ackman (2000), 90-100%, Ying-Jiang *et al.* (2011), crystal violet > %75, Ying-Jiang *et al.* (2011), malachite green >75%.

Castillo-Garcia *et al.* (2015) method was used in the extraction and identification of Sarafloxacin. The method was used on the same fish samples to compare with the new develop method (Table 37).

Table 37: Mean Recovery Results

Sarafloxacin	Standard (ppm)	Extract of spiked fish (ppm)	% Recovery
Extract 1	0.0800	0.0685	85.60
Extract 2	0.0800	0.0682	85.25
Extract 3	0.0800	0.0681	85.12

Source: Laboratory Analysis (2017- 2019)

The results of the nanomaterial and the dispersive extraction step for Quinolones (Sarafloxacin) can be seen at the appendix Z of this work. In comparing the results of Castillo-Garcia *et al.* (2015) method to this new developed method, it could be seen that the average recovery obtained for Sarafloxacin in the Terbium coated nanomaterial was 85.50% (Table 37) but the average recovery for this new method was 99.73% (Table 36) indicating that the new developed method possess higher recoveries as compared to the Terbium coated nanomaterial method.

Method of Validation

The validation of the proposed method was done in terms of linearity, sensitivity, selectivity and accuracy (trueness and precision), according to the European Directive 2002/657. Spiked fish samples were used to establish all quality parameters.

Linearity

Linearity of the calibration graphs was determined. The value obtained for R^2 was 99.9% for each of the analytes with a linear graph (Fig. 32). The rest of the regression graphs can be seen at the appendix X. Where Col 1 is the concentration and col 2 is the peak area.

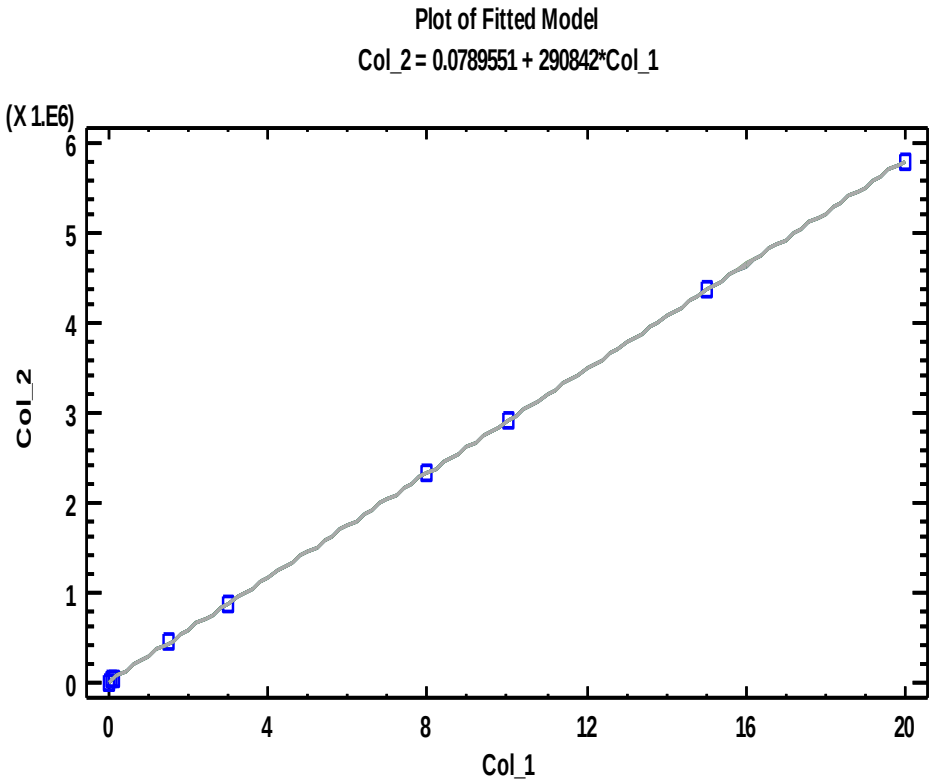


Figure 32: Linearity of Sarafloxacin.

Sensitivity

The sensitivity was determined in terms of the LoDs obtained. The estimation of the limits of detection (LoDs) was done following IUPAC recommendations, which involve the use of a signal-to noise ratio of 3 (Long & Winefordner, 1983). The LoDs are estimated as the minimum concentration of analyte that the method can detect with a signal-to-noise ratio of 3 (Table 38).

Comparing the LoDs obtained in this new method to the Maximum Residual Limits (MRL) set by the European commission (EC, 2009), the LoDs obtained in this work were much lower than their respective MRL for Sarafloxacin, Ethoxyquin, Diflubenzuron, Teflubenzuron, Malachite green and Crystal violet to be: 30, 3000, 1000, 500, 2 and 2 µ/Kg respectively.

Table 38: Quality Control Analysis Results

Standards	Limit of Detection (LoD) µ/kg	Limit of Quantitation (LoQ) µ/kg	P-value	Correlation Coefficient	% R-Squared
Sarafloxacin	0.0560	0.1890	0.0000	0.9999	99.9999
Ethoxyquin	0.0122	0.0407	0.0000	0.9999	99.9999
Diflubenzuron	5.0800	16.9000	0.0000	0.9999	99.9999
Teflubenzuron	1.5750	5.2500	0.0000	0.9999	99.9999
Malachite Green	0.3870	1.2900	0.0000	0.9999	99.9999
Crystal violet	0.2654	0.8847	0.0000	0.9999	99.9999

Source: Laboratory Analysis (2017- 2019)

Research carried out by Roudaut & Yorke (2002), recorded LoDs for sarafloxacin at 2 µg/Kg. Cañada- Cañada *et al*. (2012), also had LoDs for sarafloxacin between 0.2-9.5 µg/Kg. Guidia *et al*. (2018) and Tyrpenou *et al.* (2002), also had LoDs for sarafloxacin at 12.5 µg/Kg and 1 µg/Kg. Comparing the LoDs obtained in this work to the above referenced research, the LoD obtained in this work (0.056 µg/Kg) is far lower signifying the sensitive nature of the developed method. In the case of Ethoxyquin, LoD obtained in this work is 0.0122 µg/Kg, which is also lower than literature research works by Rodríguez-Gómez *et al.* (2018) and Berdikova Bohne *et al.* (2007), with LoDs 0.05 µg/Kg and 0.02 µg/Kg) respectively. LoDs of Diflubenzuron and Teflubenzuron obtained in this work were 5.080 µg/Kg and 1.575 µg/Kg respectively (Table 38), and were lower than 32 µg/Kg and 0.40 g/Kg reported by Luvizotto-Santos *et al.* (2009) and Hormazhal &Yndestad (2006) respectively. For Crystal Violet and Malachite Green, LoDs in

this work were 0.265 µg/Kg and 0.387 µg/Kg respectively lower than 1.4 µg/Kg and 2.5 µg/Kg reported by Sadeghi & Nasehi (2018) and Halme *et al.* (2016) respectively which confirms the efficiency and sensitivity of the developed method.

Selectivity

In comparison between a chromatogram of a blank sample and the one corresponding to a spiked fish sample, the selectivity of the method was determined. At the retention time of the analytes, no interferences from endogenous substances were seen. The chromatograms showed the blank fish sample and spiked fish sample with Sarafloxacin (Fig. 33-34).

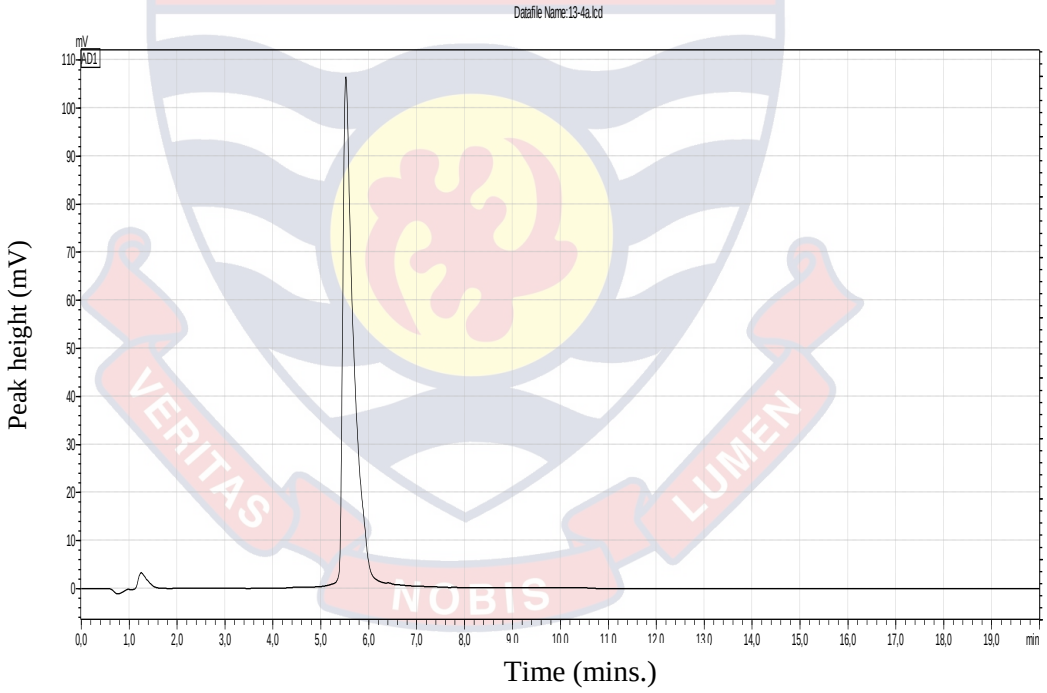


Figure 33: Chromatogram of Spiked Fish with Sarafloxacin (Analyte).

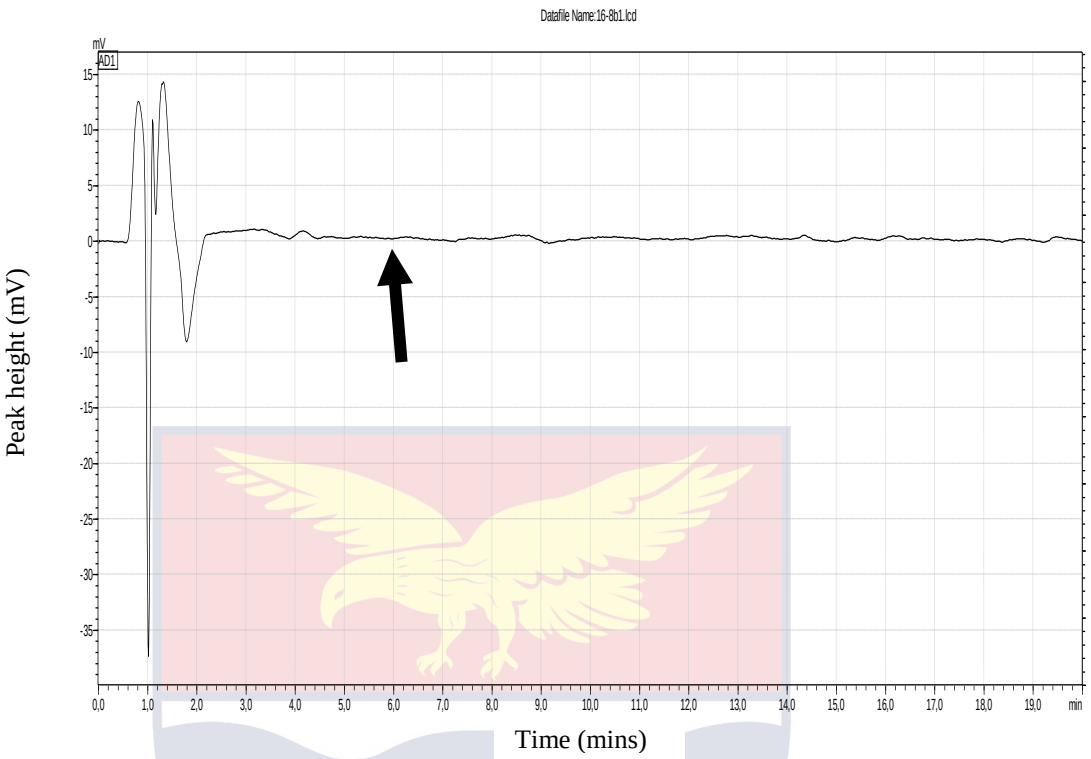


Figure 34: Chromatogram of Blank Fish Sample.

Accuracy (Precision and Trueness)

Since there was no certified material and to assess the trueness and the precision of the method, a recovery study with spiked fish samples using three concentrations levels for each compound (0.5×MRL, 1.0×MRL and 2×MRL), was done for five consecutive days. MRL for sarafloxacin, ethoxyquin, diflubenzuron and teflubenzuron, crystal violet and malachite green are 30 µg/Kg, 3000 µg/Kg, 1000 µg/Kg, 500 µg/Kg, 2 µg/Kg and 2 µg/Kg respectively. The precision was expressed as relative standard deviation (% RSD) and the trueness was estimated with the percentage of recovery (% recovery). In Table 40, the precision and the trueness of the proposed analytical method is shown. The trueness was estimated by determining the recovery of known amounts in spiked fish samples. In all cases, the recoveries were between 80% and 99.73% (Table 39). Precision was lower than

15% for all compounds (Table 39) and this was within the acceptable limits for the validation guide of European Directive (2002). The data (Table 39) revealed that the proposed method was repeatable. Hence, precision and trueness data has indicated that the methodology was highly accurate.

Table 39: RSD and % Recoveries of Standards at their Individual MRL

Analyte	MRL (µg/kg)	MRL Level	Recovery %	Relative Standard deviation (RSD %)
Sarafloxacin	30	0.5	98.76	0.0029
		1.0	98.76	0.0007
		2.0	98.76	0
Ethoxyquin	3000	0.5	93.09	0.0000
		1.0	92.49	0.0000
		2.0	93.08	0
Diflubenzuron	1000	0.5	80.95	0.0576
		1.0	80.83	0.0038
		2.0	80.83	0
Terflubenzuron	500	0.5	93.96	0.1417
		1.0	93.25	0.0032
		2.0	93.25	0
Crystal Violet	2	0.5	83.09	0
		1.0	83.22	0.0000
		2.0	83.22	0
Malachite Green	2	0.5	85.72	0
		1.0	85.50	0
		2.0	85.50	0

Source: Laboratory Analysis (2017- 2019)

Chapter Summary

This chapter focused on all the results of the processes that led to the development of the new analytical method. The gradient elution separation method was used in this method development. The best mobile phase combination for identifying and quantifying all the anti-parasitic agents studied in this work was

acetonitrile, acetic acid and magnesium nitrate (gradient method 14). Method was validated and all the standards gave distinct peaks with recoveries between 93.33 - 120.00% and lower LoDs (0.0122 - 5.080 µ/Kg).

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary

The new analytical method that has been developed showed that employing a single mobile phase composition of 0.01 M acetic acid 0.04 M magnesium nitrate with acetonitrile was the best mobile phase composition for identifying, separating quantifying the various group of antiparasitic agents. Gradient method 14 was the LC gradient method, pH of 3.5 of the aqueous phase, flow rate of 0.6mil/min, 1 ml of eluent used in SPE extraction was the best conditions. Interference of fat and sample matrix was reduced. The analytical method was optimized and validated.

Conclusion

For the first time, a new analytical method has been developed for the identification and quantification of different groups of antiparasitic agents (antibiotics, dyes, pesticides and antioxidants) in a single run. The newly developed method was able to detect lower concentrations of the antiparasitic agents (0.056, 0.0122, 5.080, 1.575, 0.2654, 0.3870 µ/Kg) for Sarafloxacin, Ethoxyquin, diflubenzuron, Teflubenzuron, Crystal violet and Malachite green respectively. The limit of detection obtained were lower than the Maximum Residual Limit set by the European Union. Recoveries obtained for all the various types of antiparasitic agents in fish were between 80% and 99.73%. The new developed method used less solvent, and fewer chemicals, less generated waste, and less analysis time, to detect the antiparasitic agents. For instance, only 1 ml of eluent was used in the elution, there was no need for the use of nitrogen gas to concentrate the extract. In

addition, it took only 10 minutes to run the analysis. The pH of the buffer used in the SPE was optimized (pH 2.5). In the optimization of the method, pH 3.5 was used as the pH of the aqueous phase and the concentration of the acetic acid was also optimised. The concentrations of mobile phase used were 0.01M Acetic, 0.04 M magnesium Nitrate, Acetonitrile and the gradient time range was also optimised (gradient method 14). Separation of the analytical method was carried out by reverse phase chromatography at an optimised flow rate of 0.6 ml/min with optimised injection volume of 50 µl under optimised gradient elution, combining an organic phase A (Acetonitrile) and an aqueous phase (B) as follows: (5% A) in the range of 0.01-2.5 minutes, then the % A was increased up to 60 % in the range of 2.5-6 minutes, the % A of was kept at 60% again in the range of 6-7minutes, the % A was increased up to 90% in the range of 7-8 minutes, , the % A of was kept again at 90% in the range of 8-9 minutes and the % A was decreased to 5% in the range of 9-10 minutes finally this percentage was maintained at 20 minutes for column re-equilibration. Temperature was maintained at 35°C in the column oven. The chromatograms were recorded at $\lambda_{exc.}$ 350nm and λ_{em} 451nm for Sarafloxacin and Ethoxyquin, Diflubenzuron and Teflubenzuron at λ_{abs} 251nm and finally, crystal violet and malachite green at λ_{abs} 596 nm.

Recommendations

The outcome of this research work have led to the identification of some areas that require further research because there were questions that either remains unanswered or asked. These recommendations include:

1. There are other antibiotics used in the treatment of fish in aquaculture. It is recommended that antibiotics such as tetracyclines should be added in the identification and separation of different groups of antiparasitic agents.

2. There are other pesticides used in the treatment of fish in aquaculture. It is recommended that pesticides such as pyrethroids should be added in the identification and separation of different groups of antiparasitic agents.

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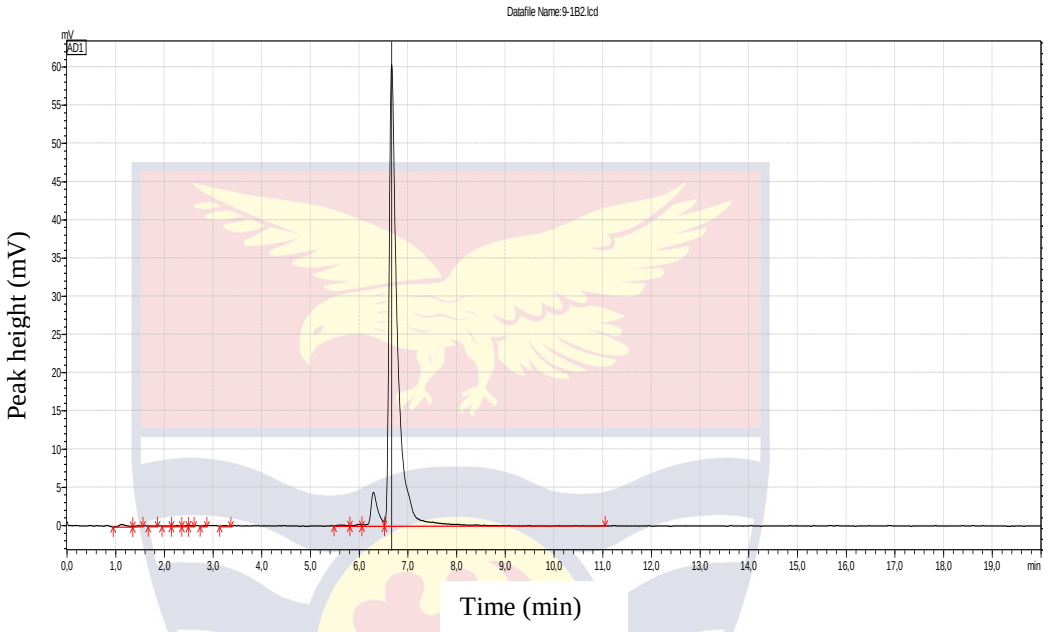
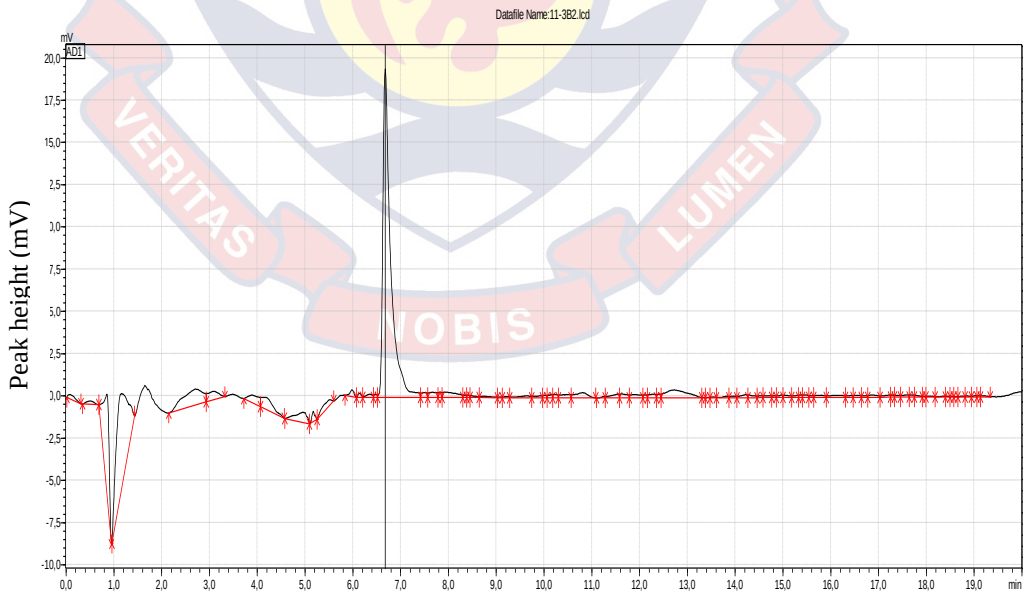
APPENDICES

APPENDIX A

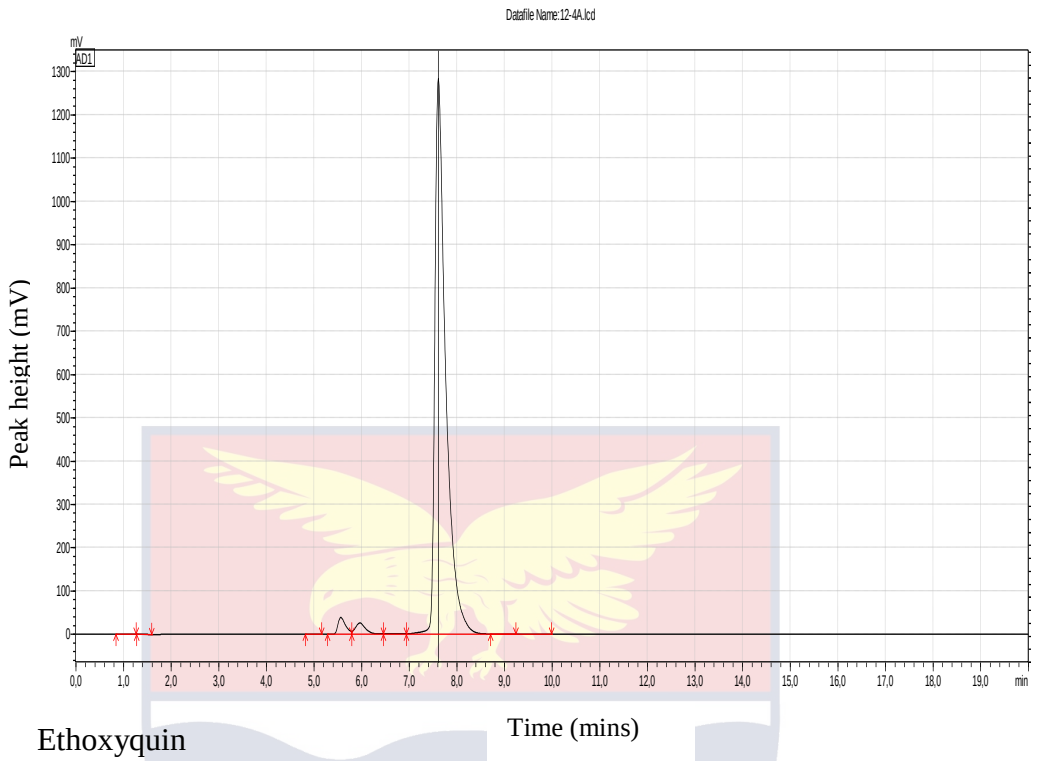
CHROMATOGRAPHS REPRESENT MOBILE PHASE ACETONITRILE, 0.05M SODIUM ACETATE AND 0.04 M MAGNESIUM NITRATE USING GRADIENT METHOD 11



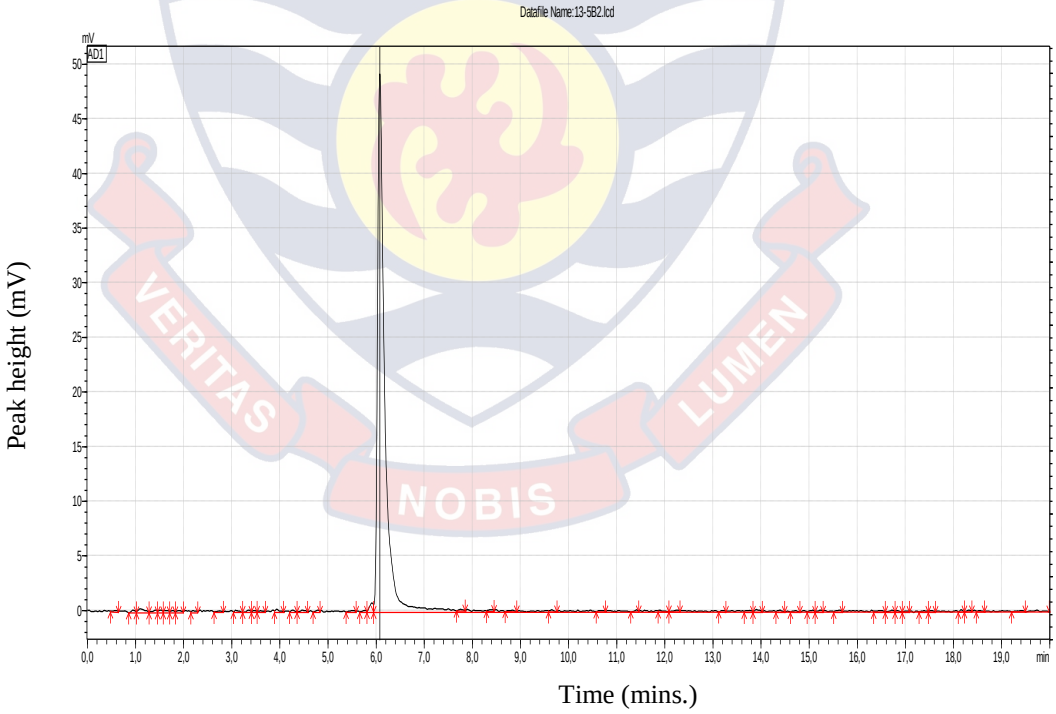
Crystal Violet



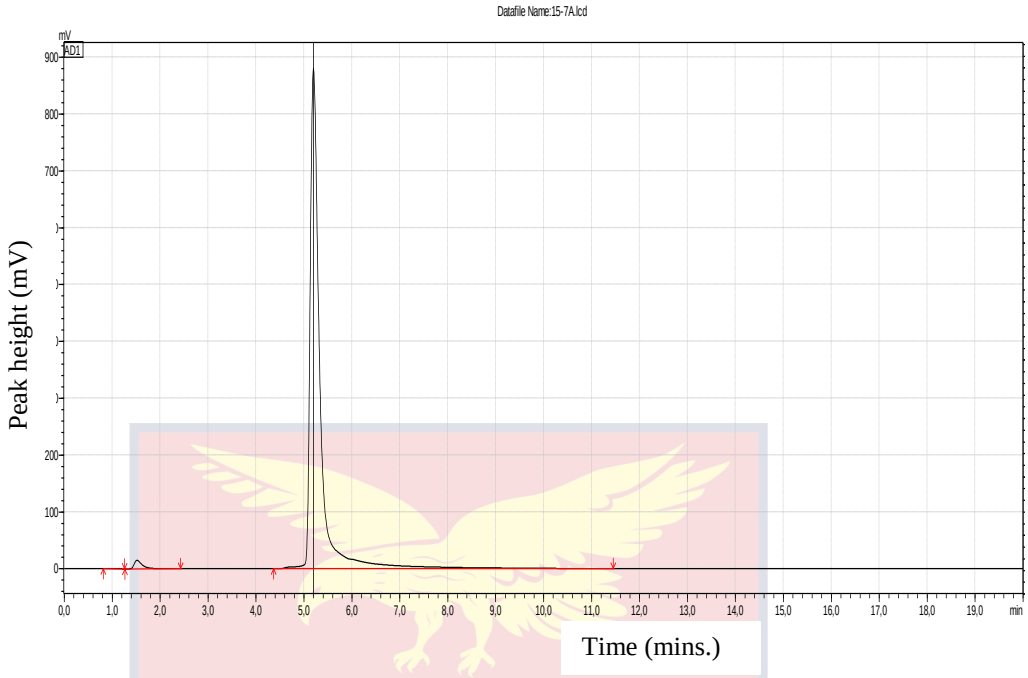
Diflubenzuron



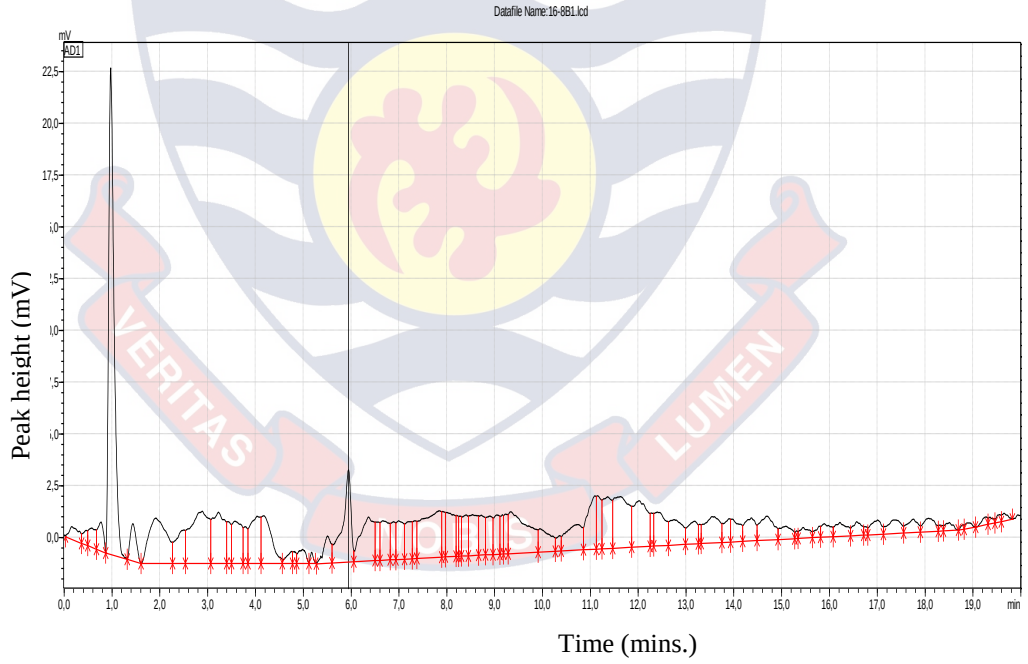
Ethoxyquin



Malachite Green



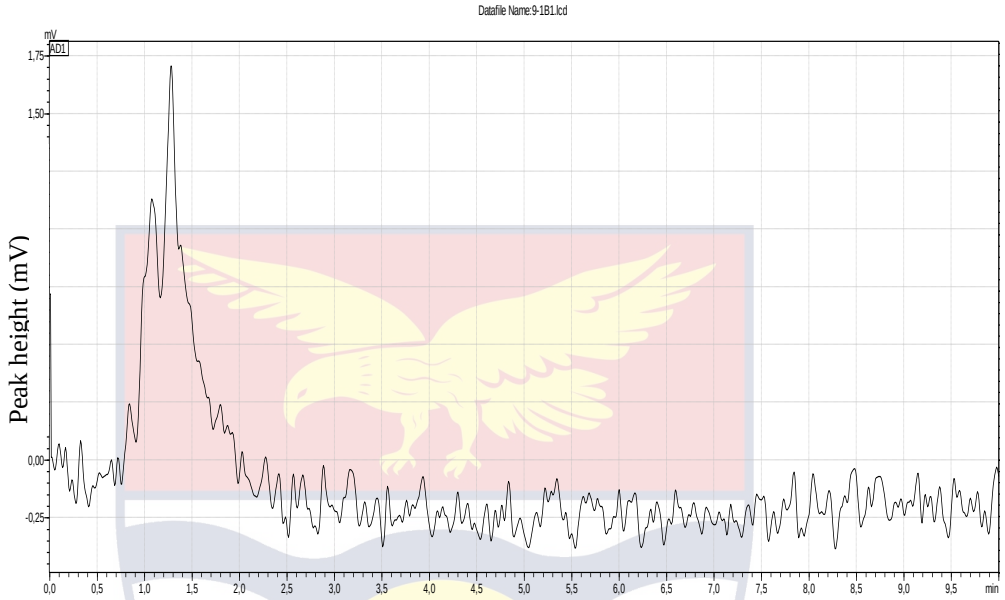
Sarafloxacin



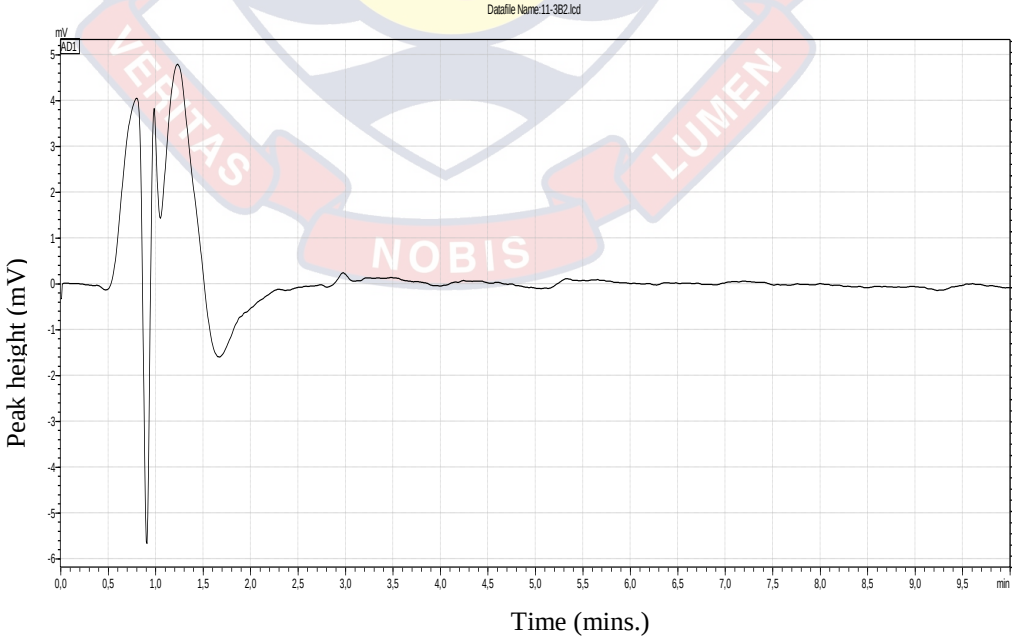
Teflubenzuron

APPENDIX B

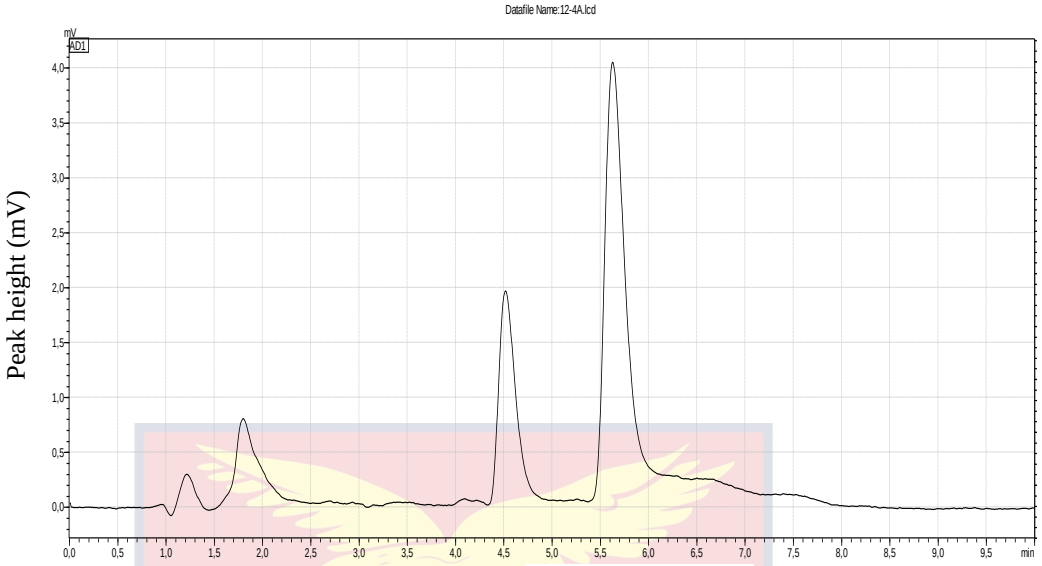
CHROMATOGRAPHS REPRESENT MOBILE PHASE 50% METHANOL, 0.05 M SODIUM ACETATE AND 0.04 M MAGNESIUM NITRATE USING GRADIENT METHOD 11



Crystal Violet



Diflubenzuron



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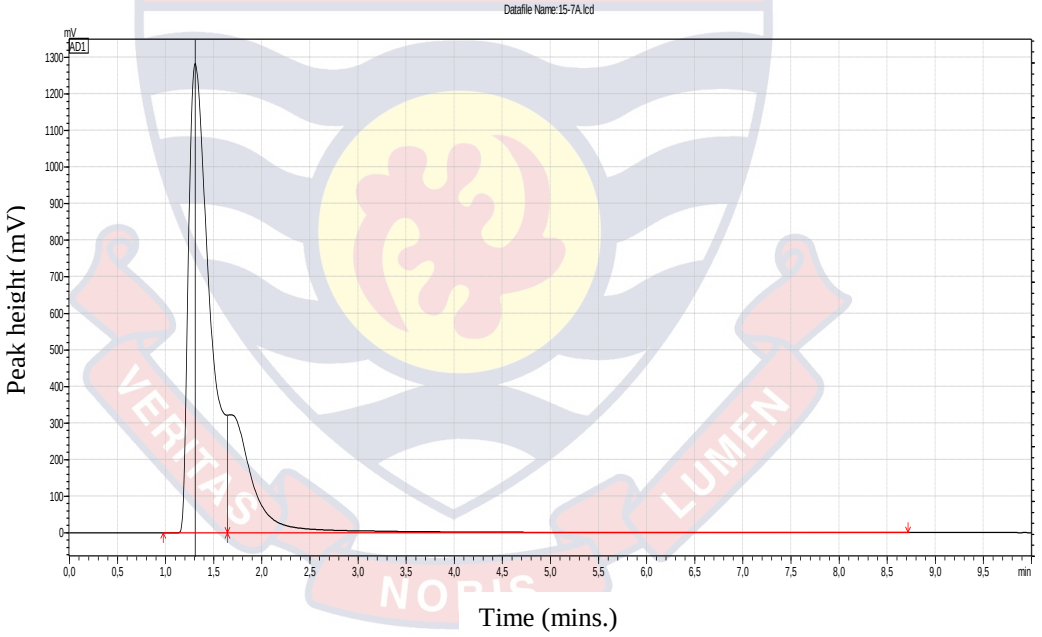
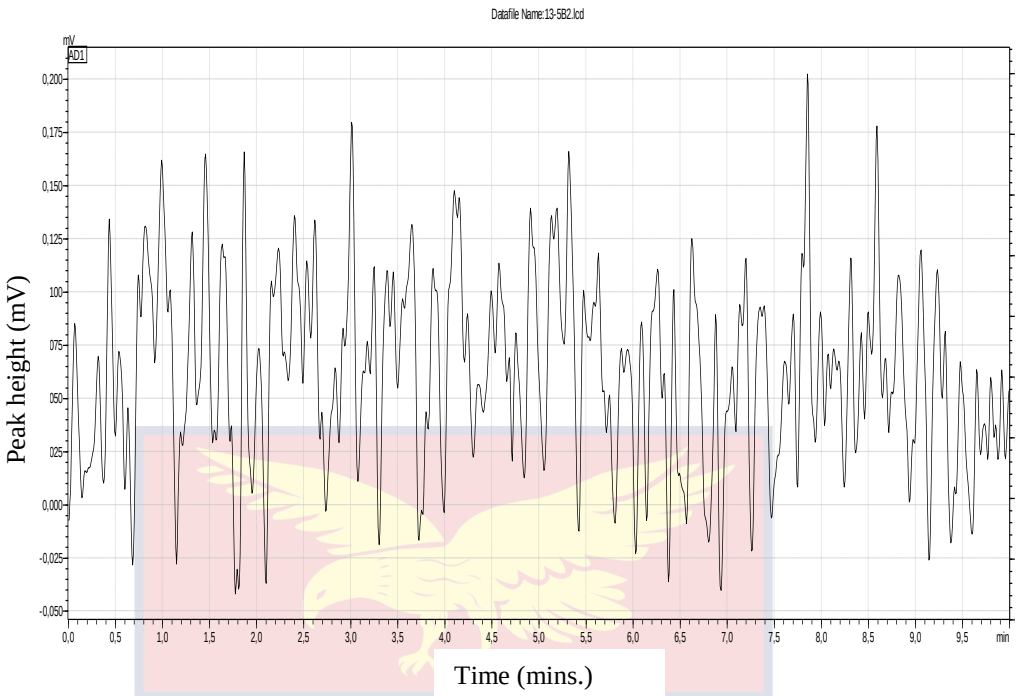


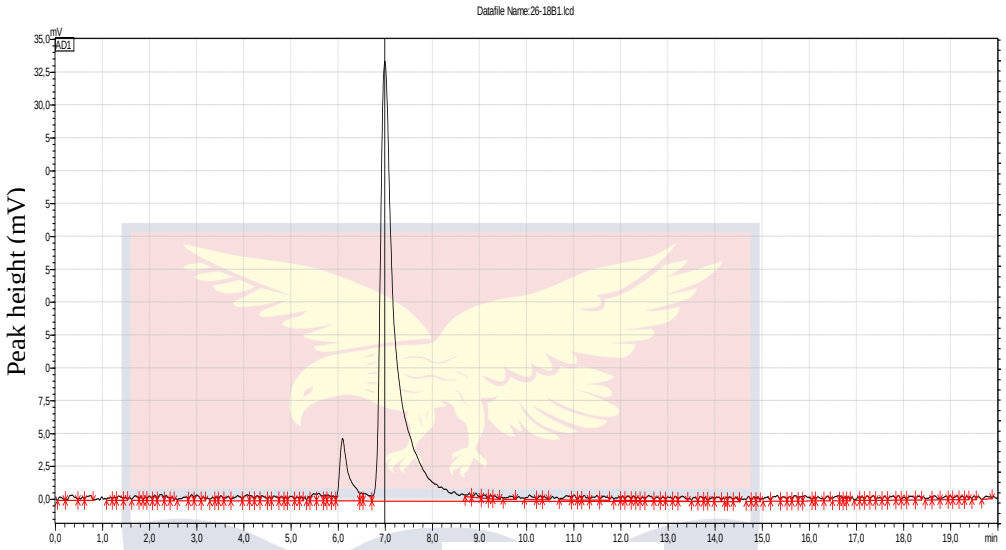
Sarafloxacin.



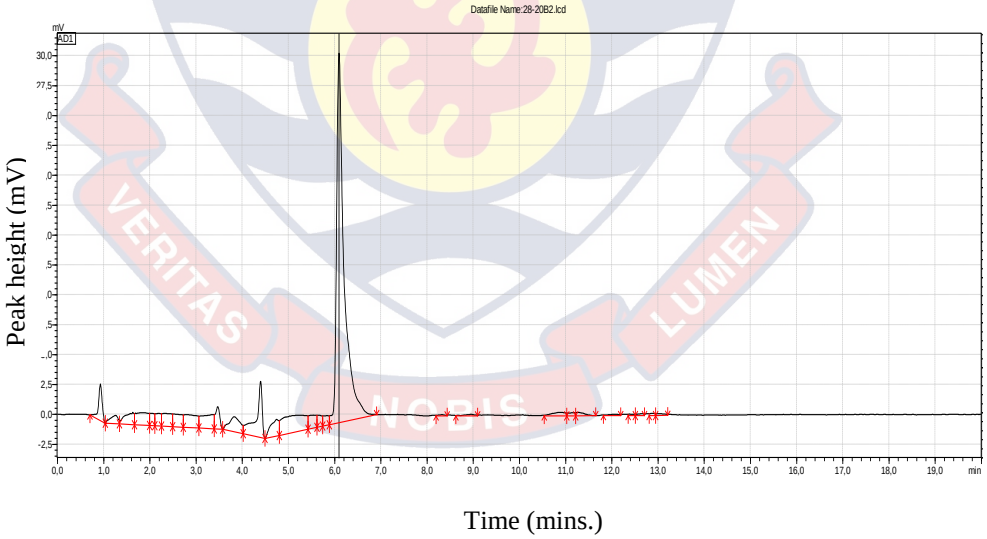
Malachite Green

APPENDIX C

CHROMATOGRAPHS REPRESENT MOBILE PHASE ACETONITRILE, 0.05 M SODIUM ACETATE AND 0.04 M MAGNESIUM NITRATE USING GRADIENT METHOD 8



Crystal Violet



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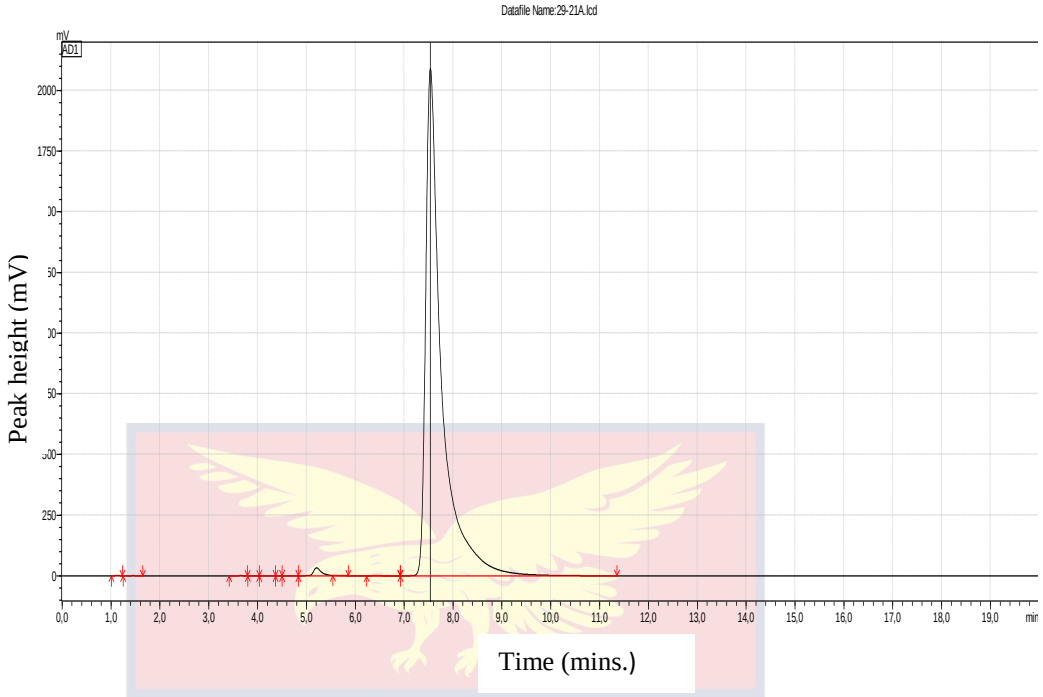

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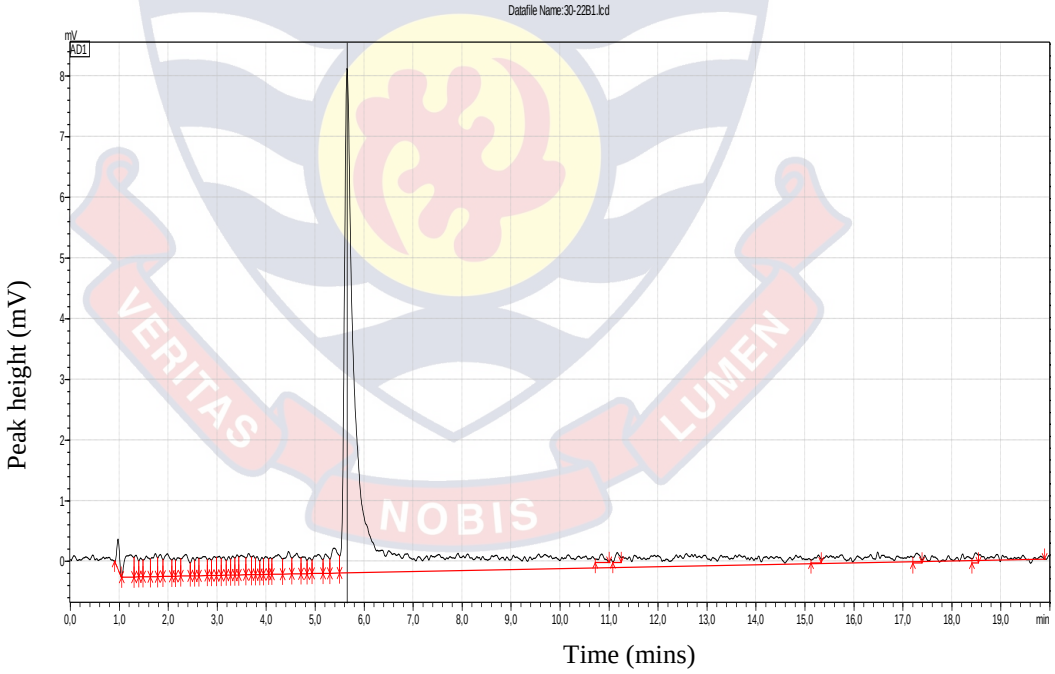
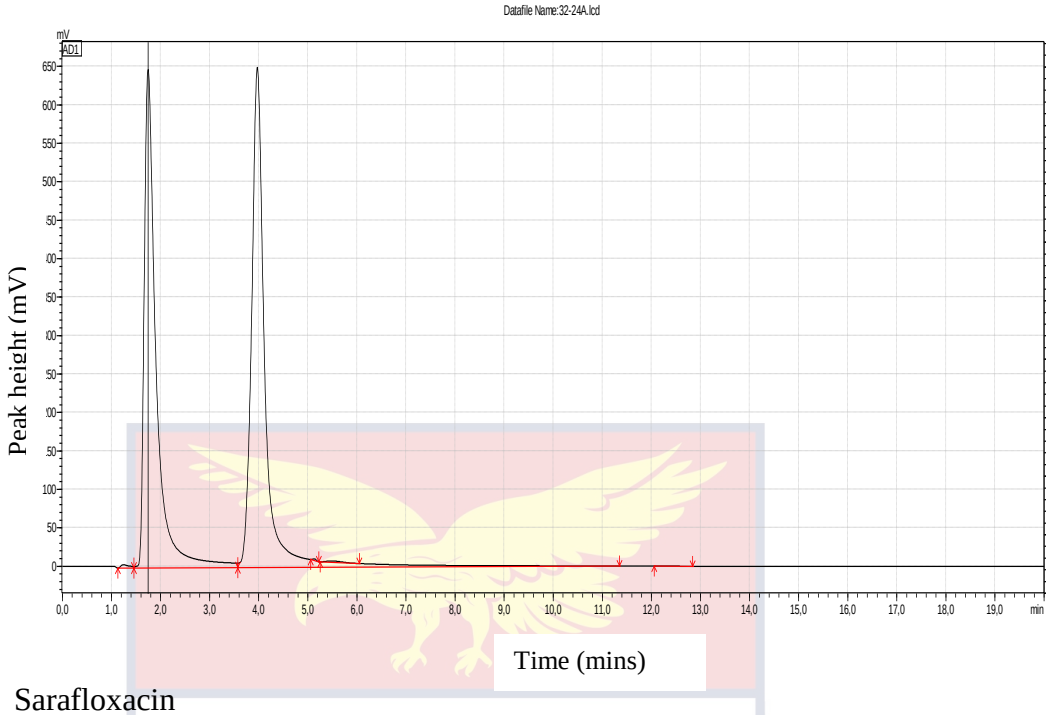
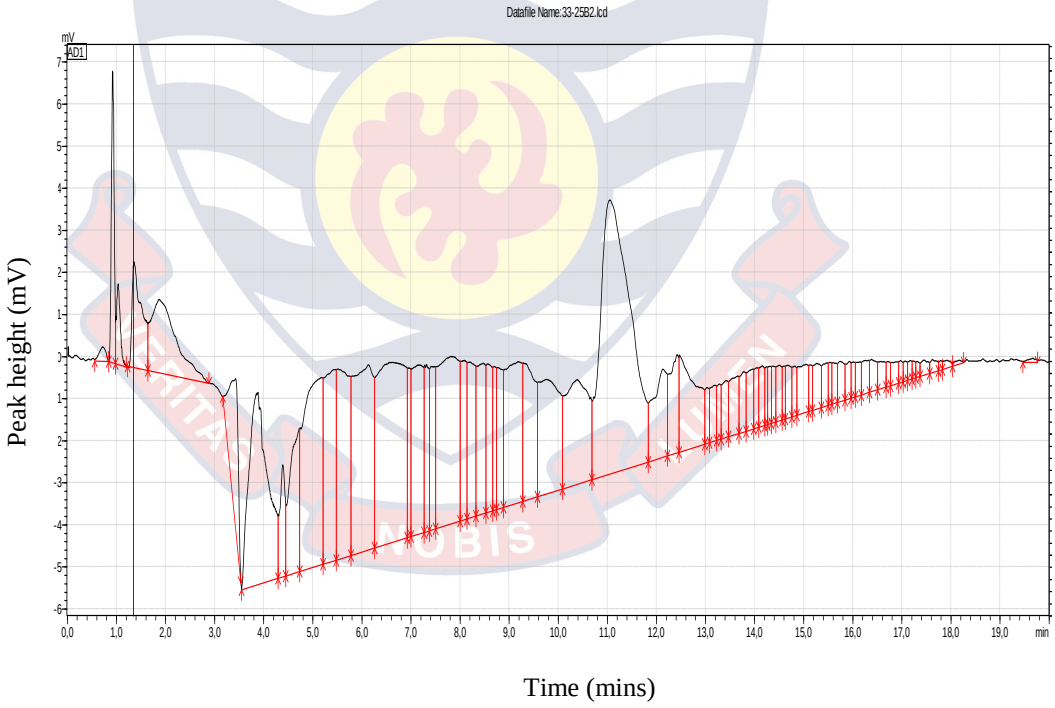


Malachite Green.



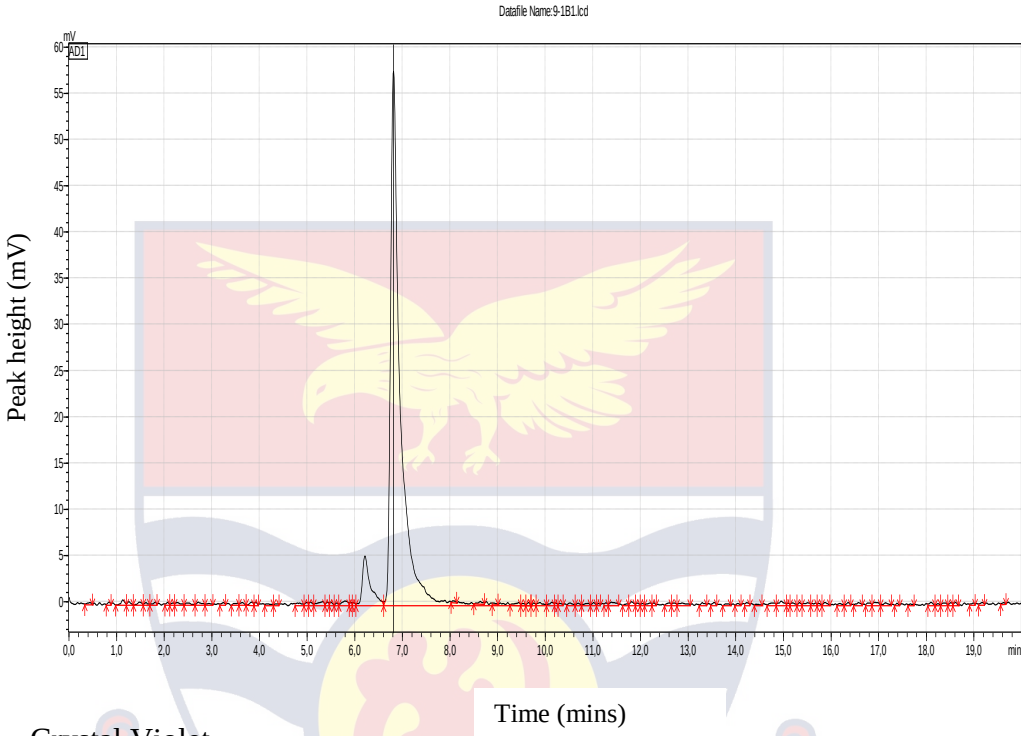
Sarafloxacin



Teflubenzuron

APPENDIX D

CHROMATOGRAPHS REPRESENT MOBILE PHASE ACETONITRILE, 0.05 M SODIUM ACETATE AND 0.04 M MAGNESIUM NITRATE USING GRADIENT METHOD 15



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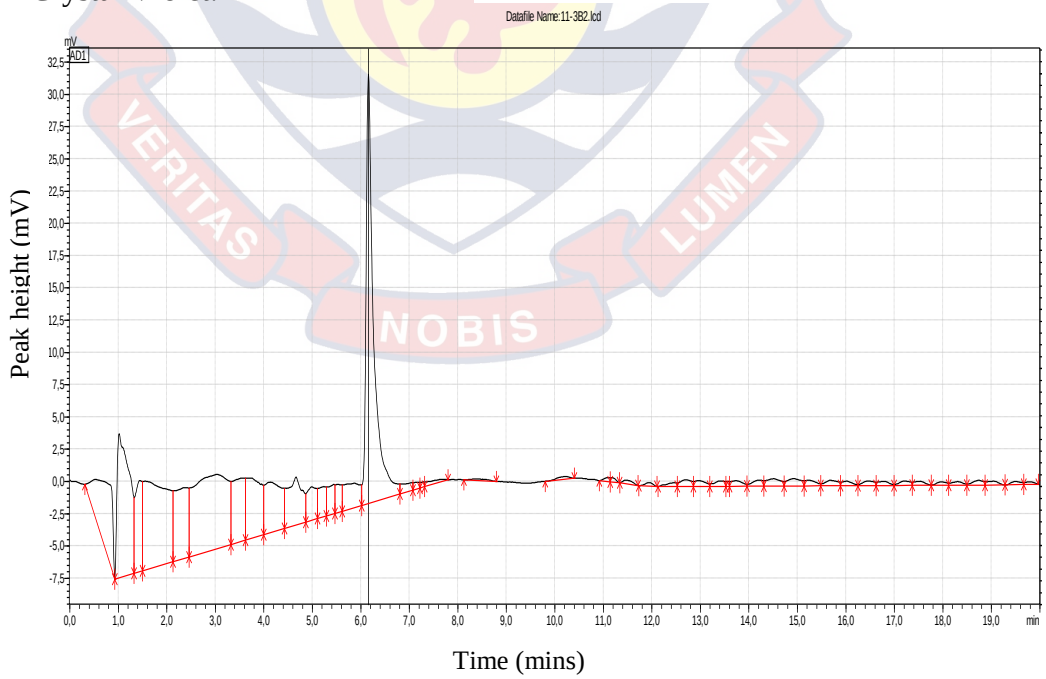

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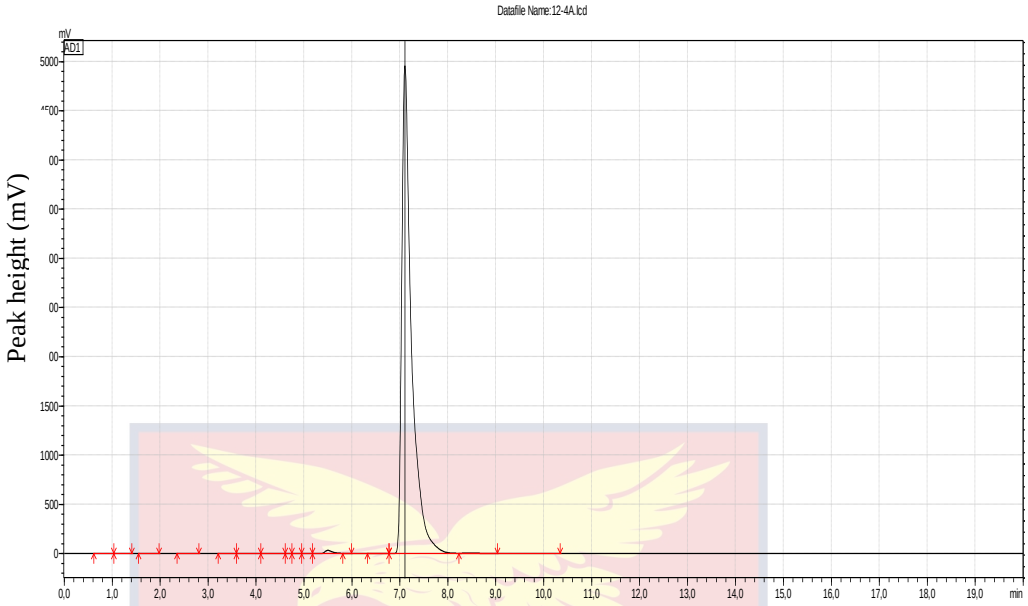
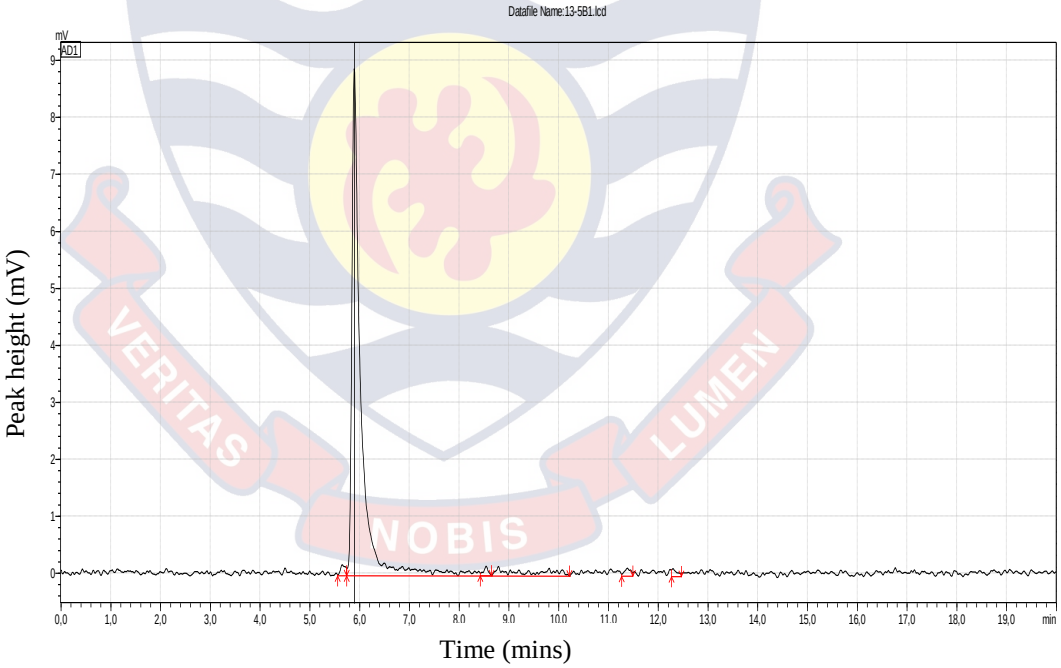
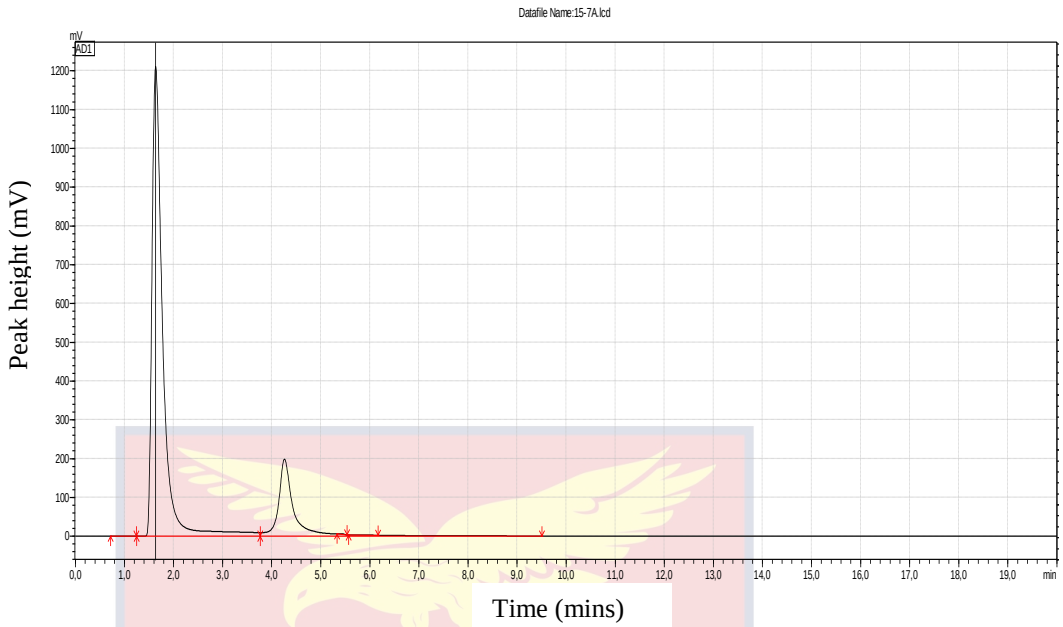


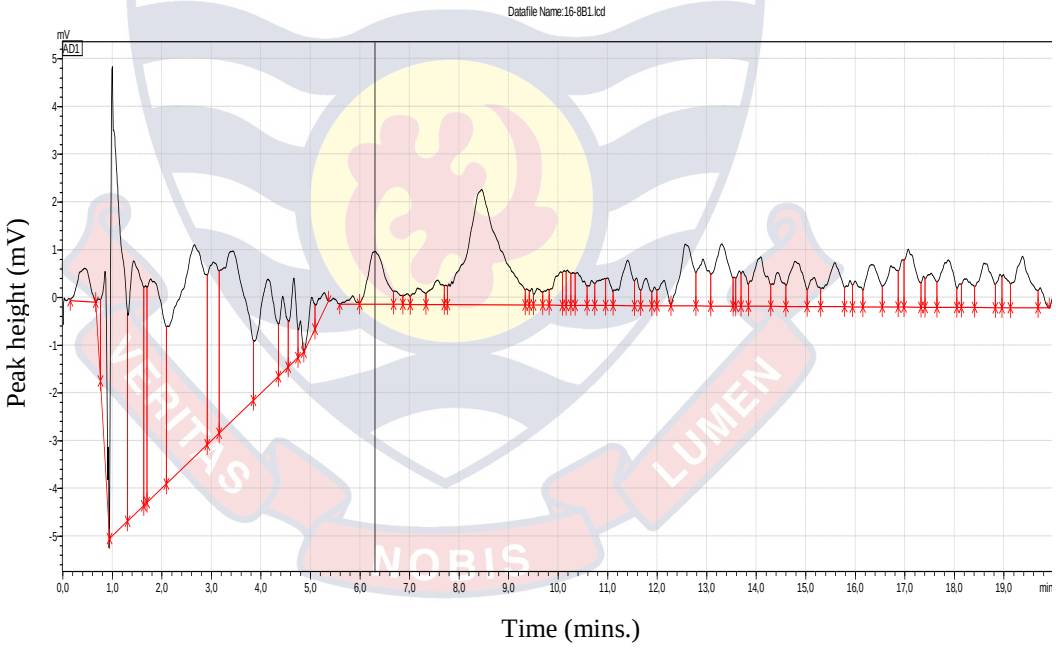
Ethoxyquin



Malachite Green.



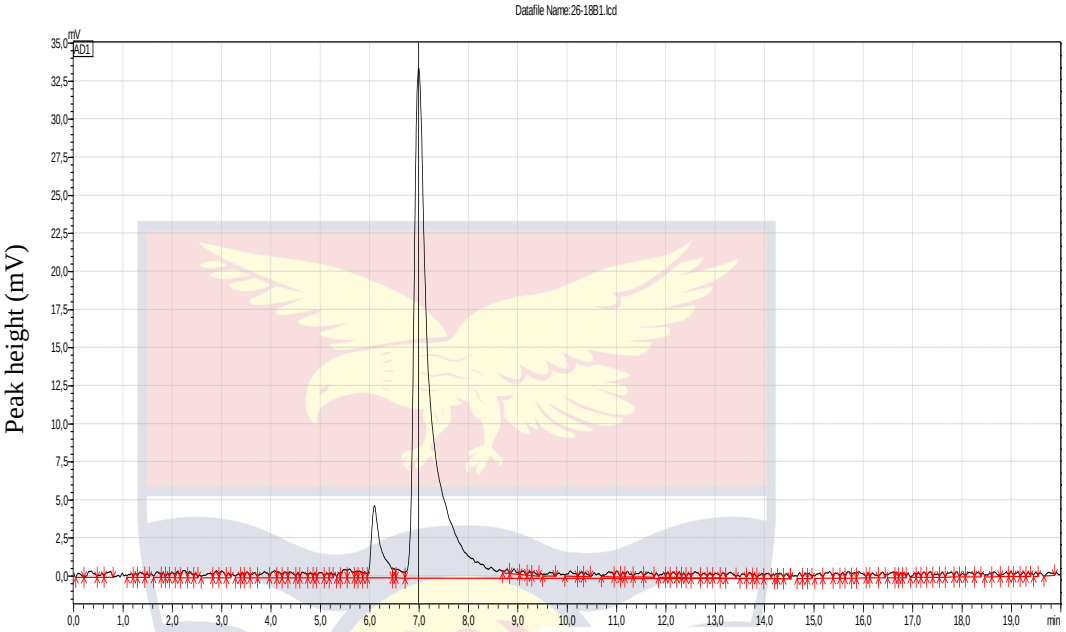
Sarafloxacin



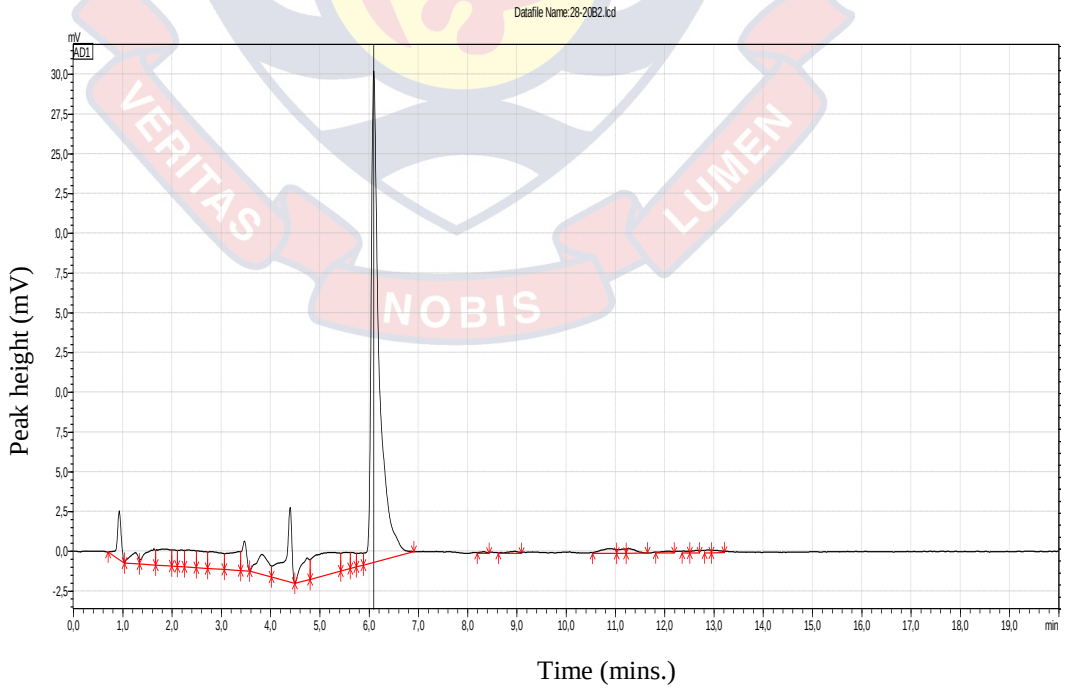
Teflubenzuron

APPENDIX E

CHROMATOGRAPHS REPRESENT MOBILE PHASE ACETONITRILE, 0.05 M AMMONIUM ACETATE AND 0.04 M MAGNESIUM NITRATE USING GRADIENT METHOD 15



Crystal Violet



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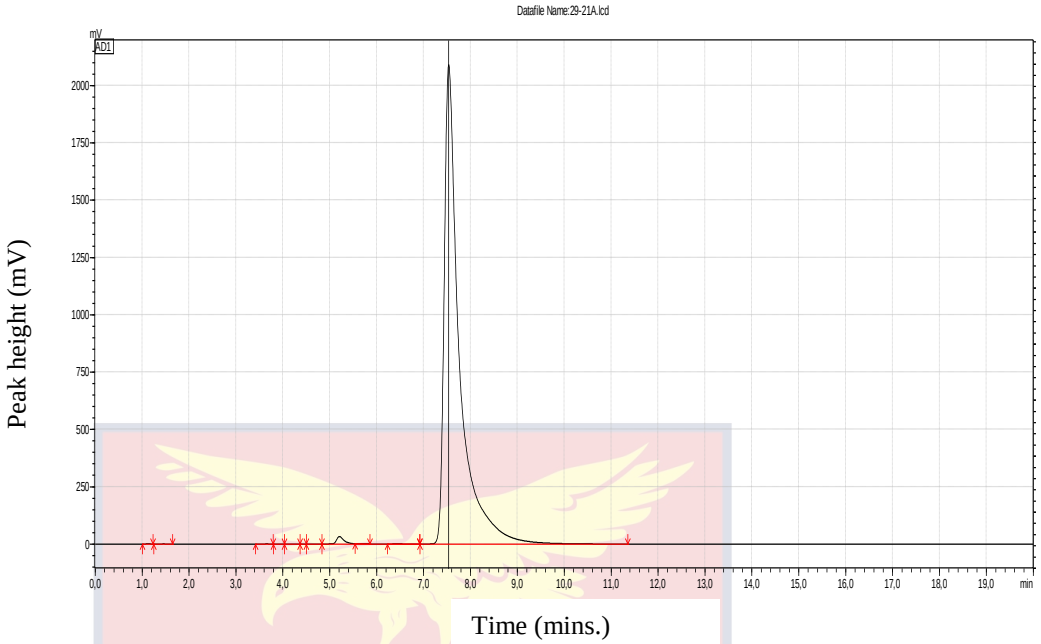
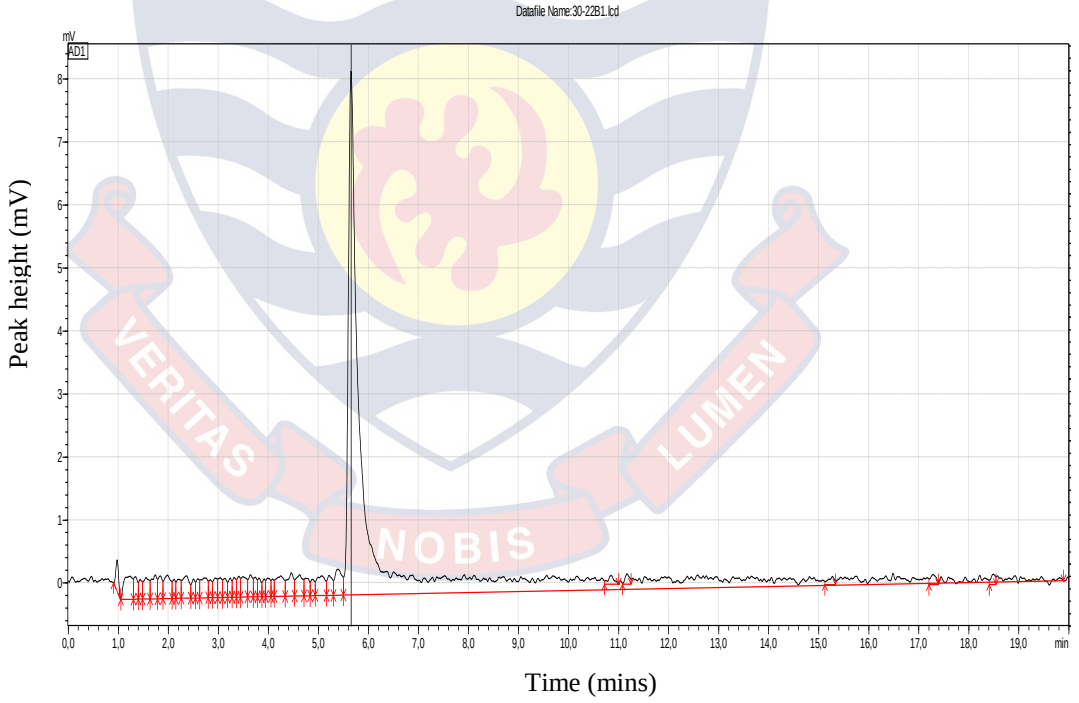
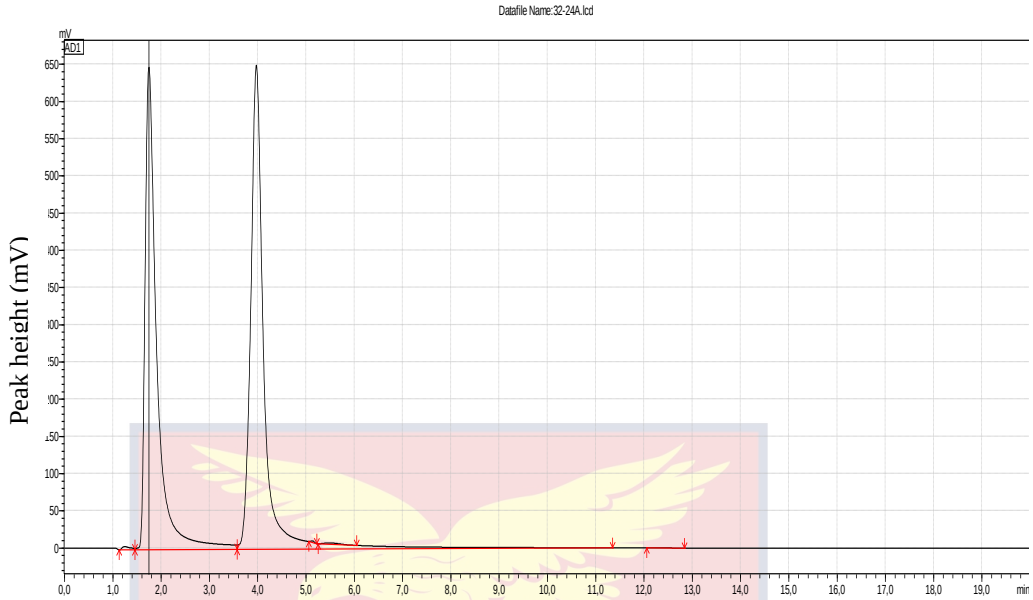


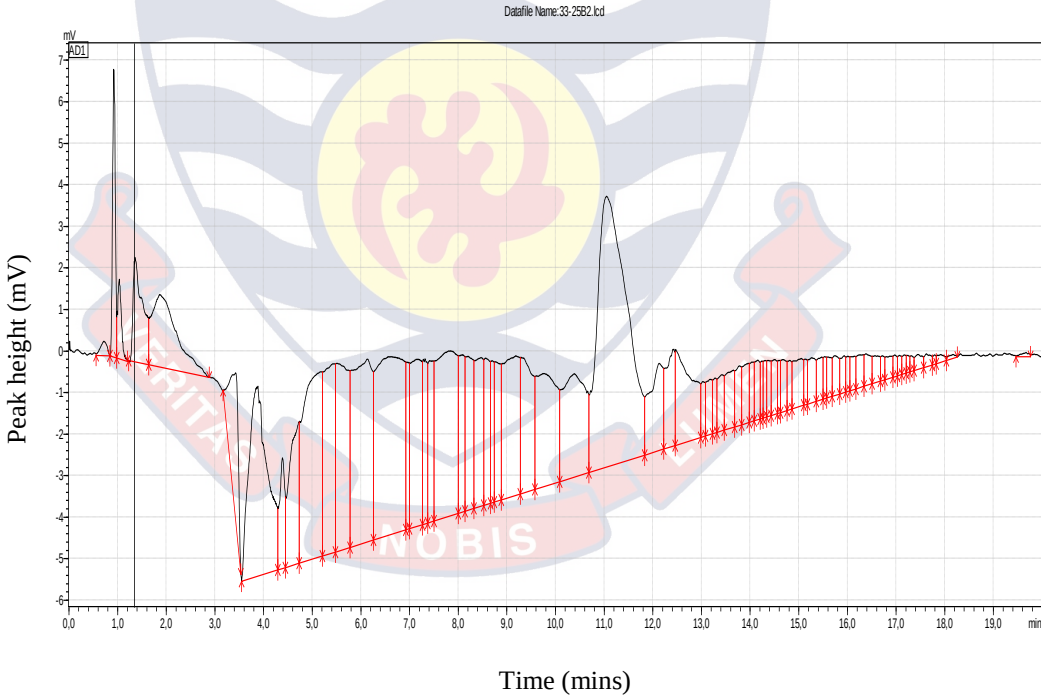
Ethoxyquin



Malachite Green



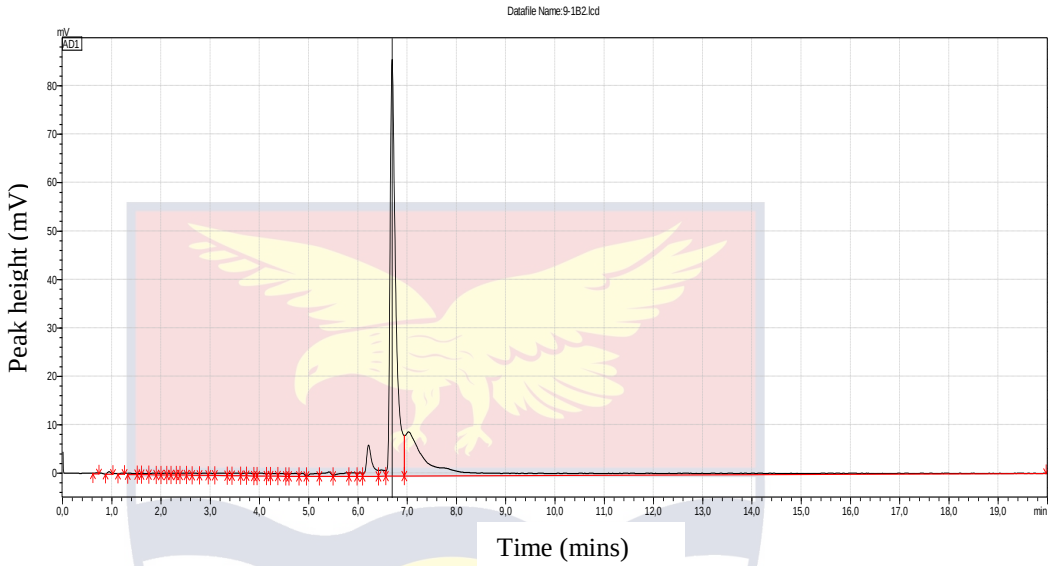
Sarafloxacin



Teflubenzuron

APPENDIX F

CHROMATOGRAPHS REPRESENT MOBILE PHASE ACETONITRILE, 0.05 M AMMONIUM ACETATE AND 0.04 M MAGNESIUM NITRATE USING GRADIENT METHOD 11



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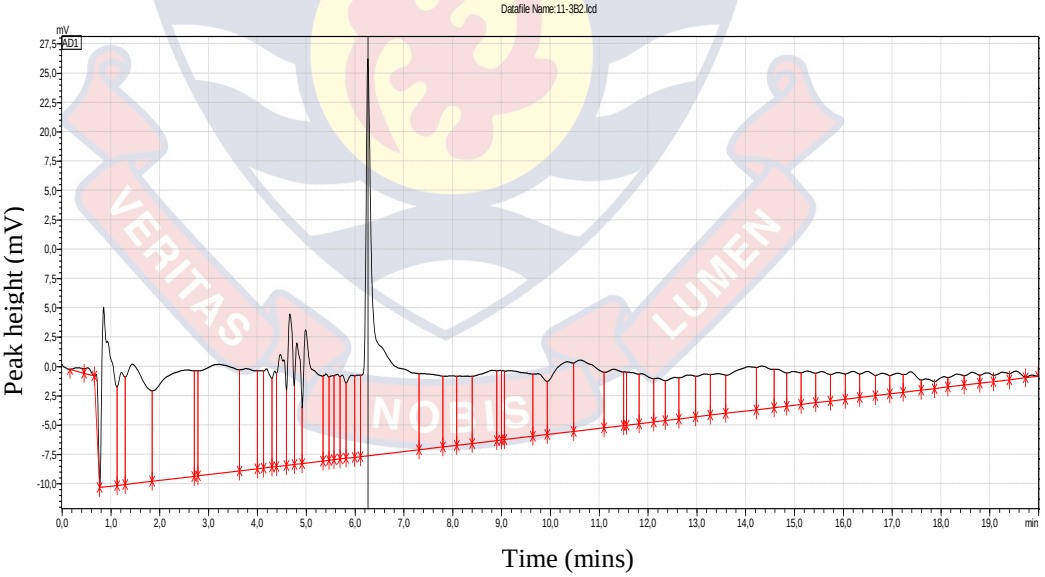

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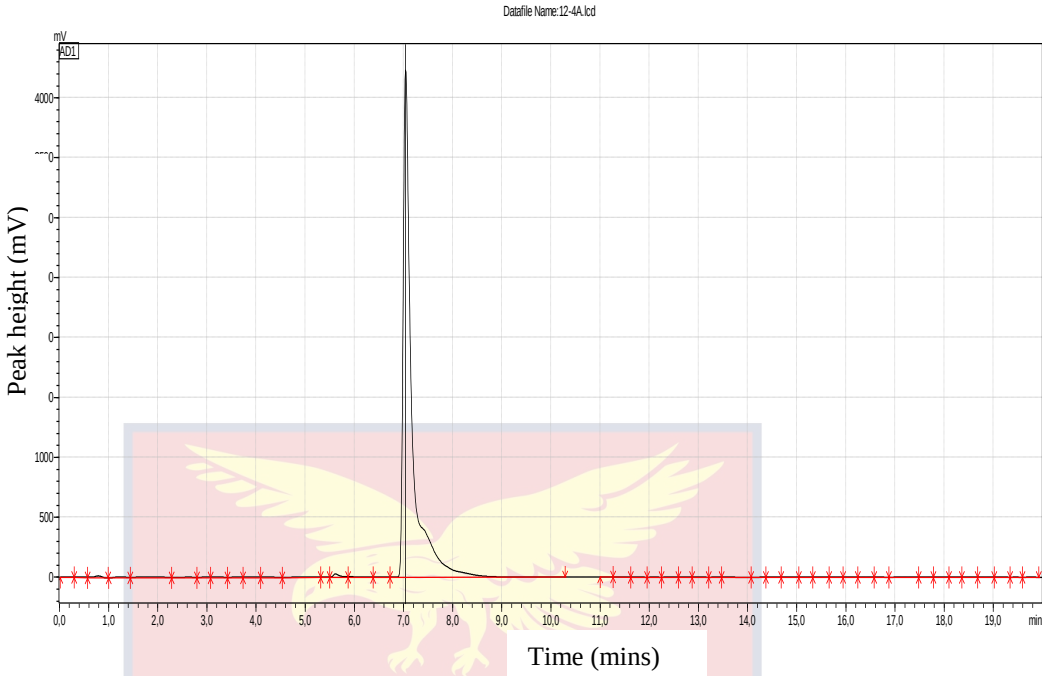
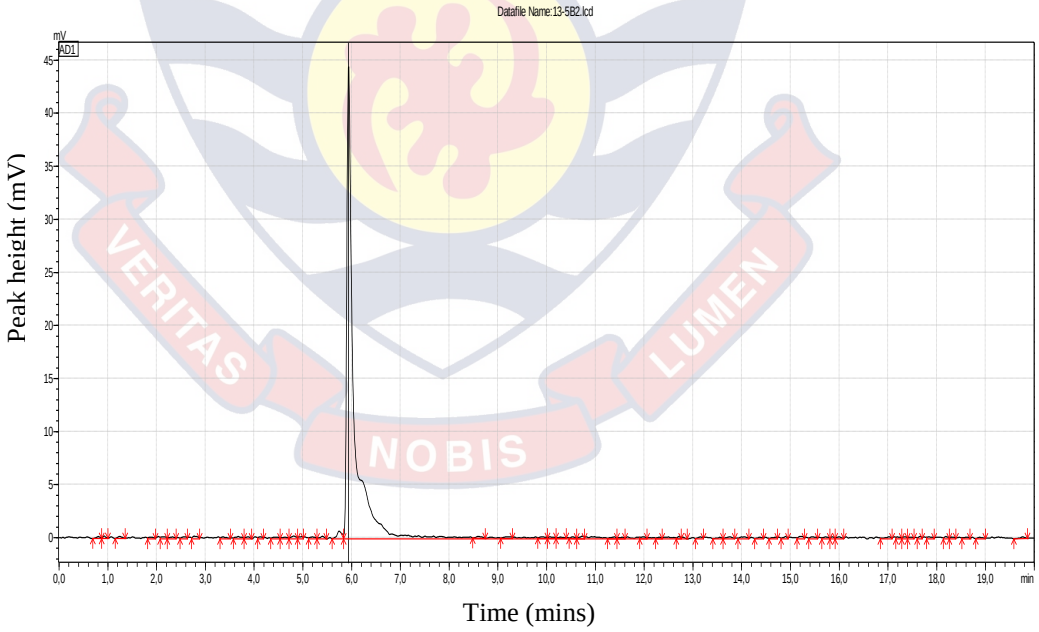
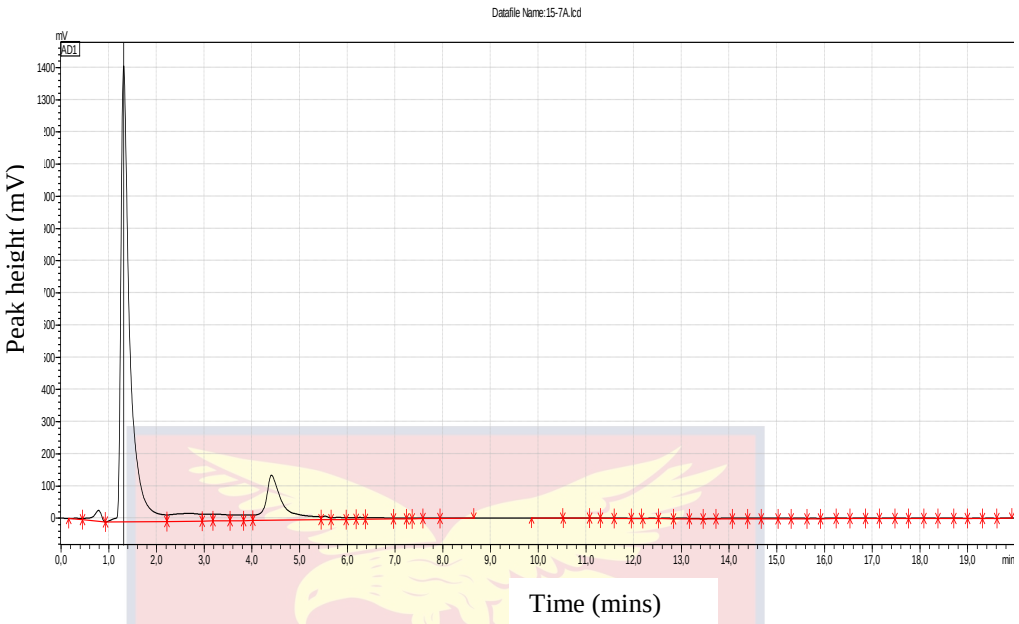


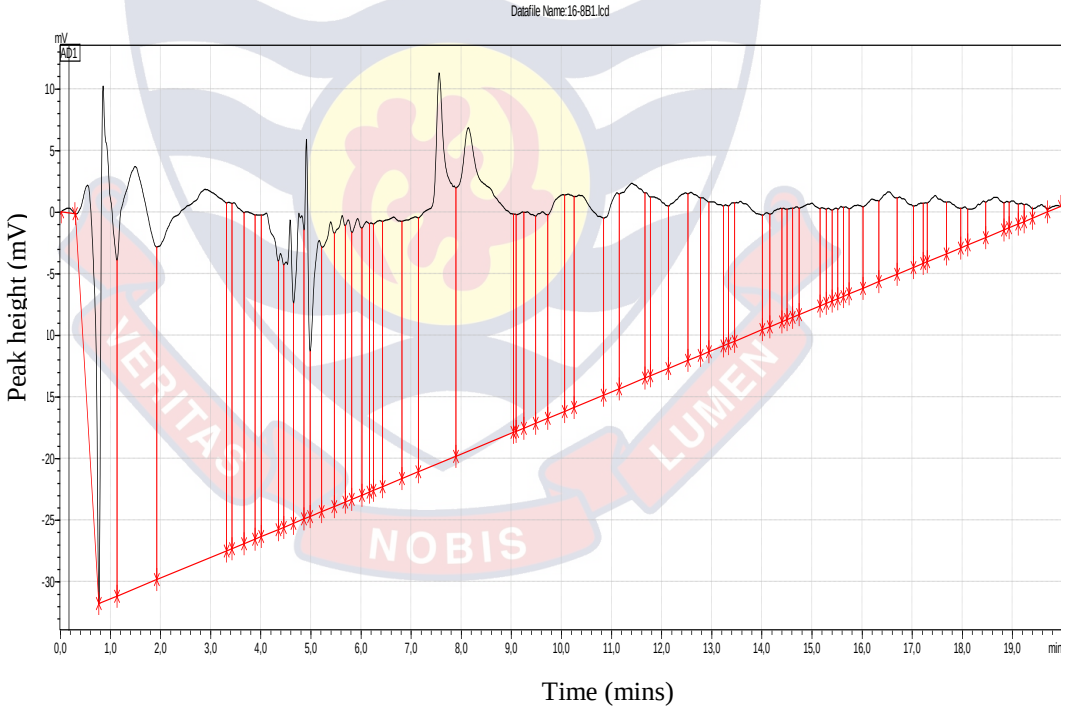
Ethoxyquin.



Malachite Green.



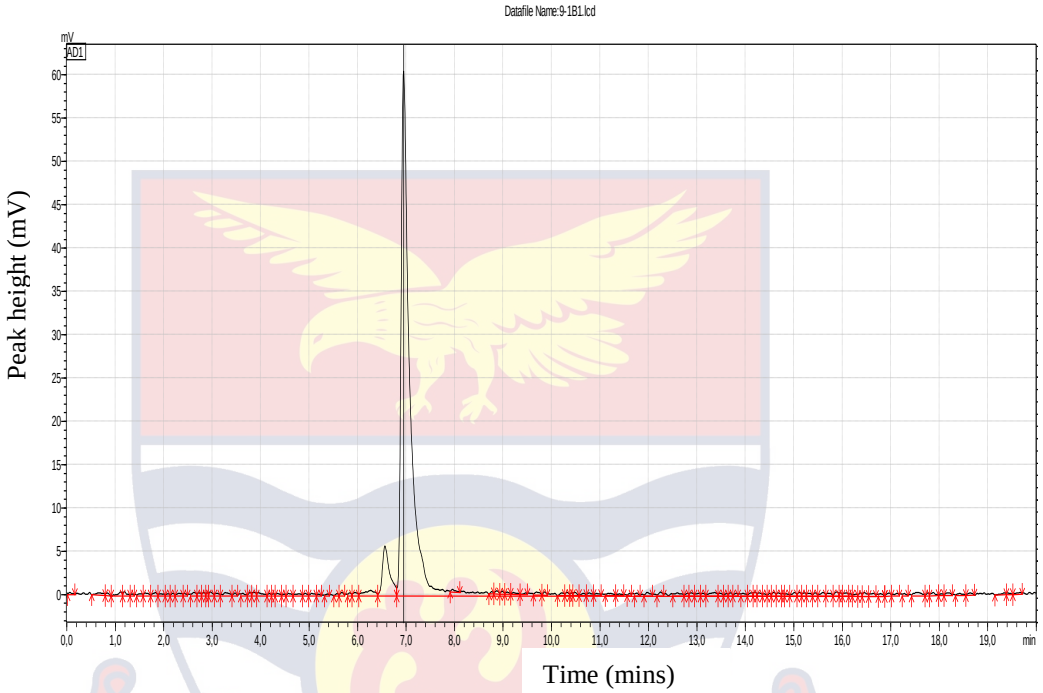
Sarafloxacin.



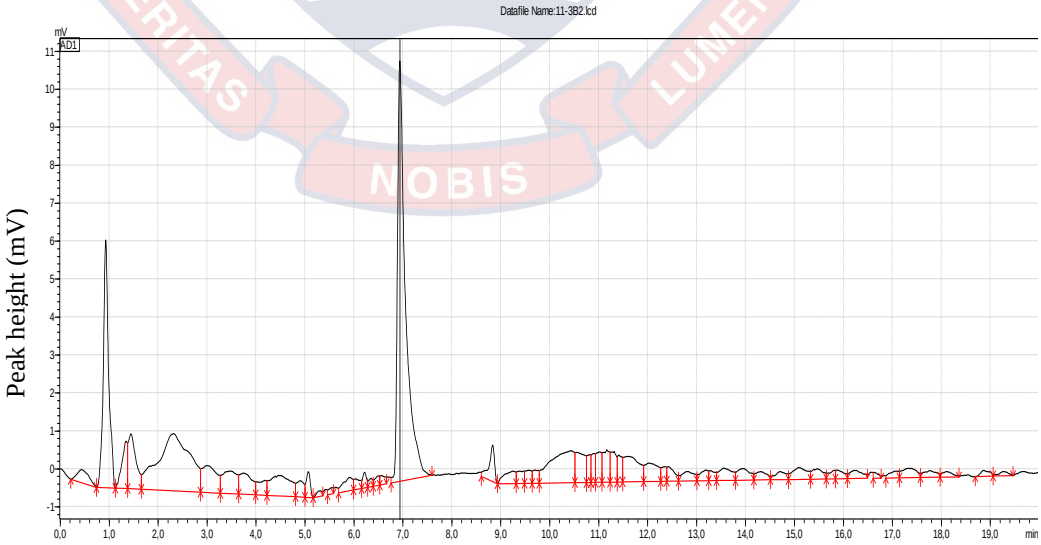
Teflubenzuron

APPENDIX G

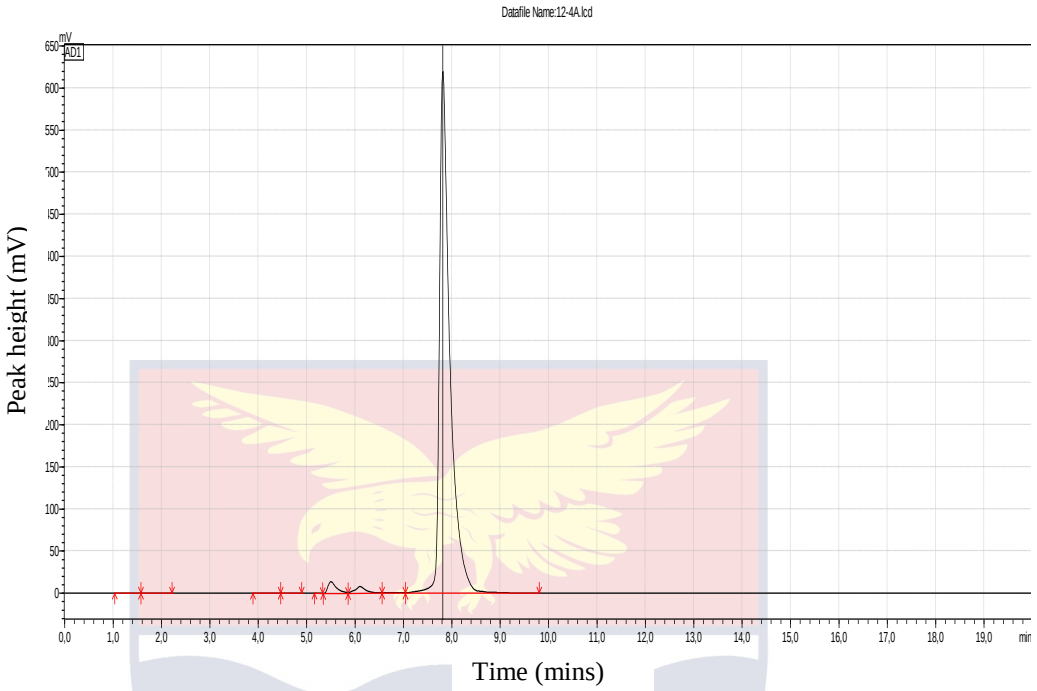
CHROMATOGRAPHS OF MOBILE PHASE 0.05 M ACETONITRILE, SODIUMACETATE AND 0.04 M MAGNESIUM NITRATE USING GRADIENT METHOD 9 AT pH 4



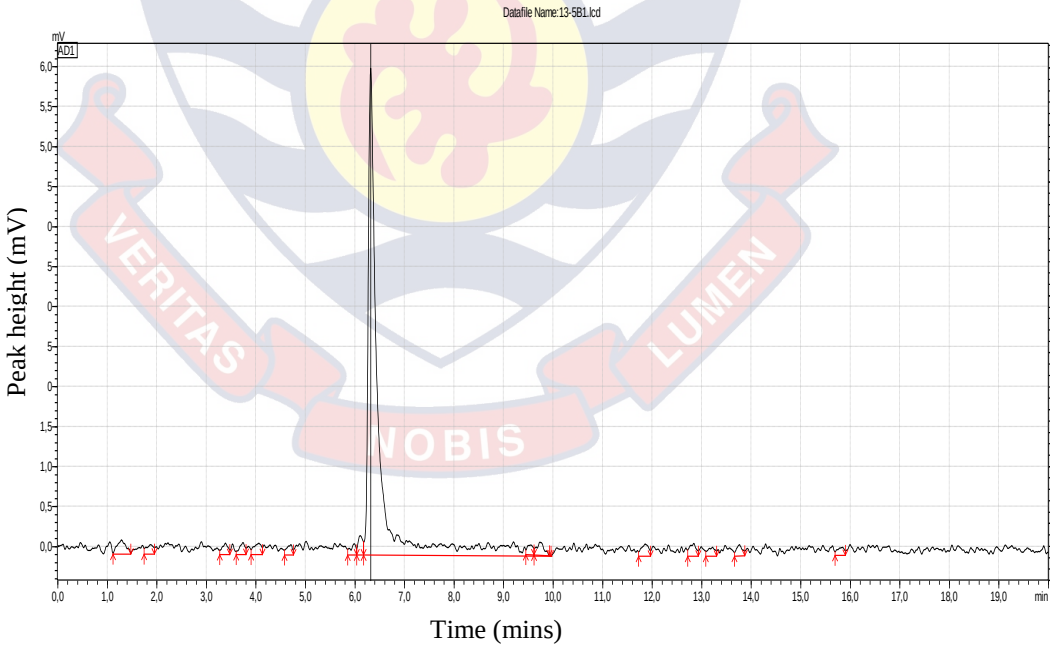
Crystal Violet



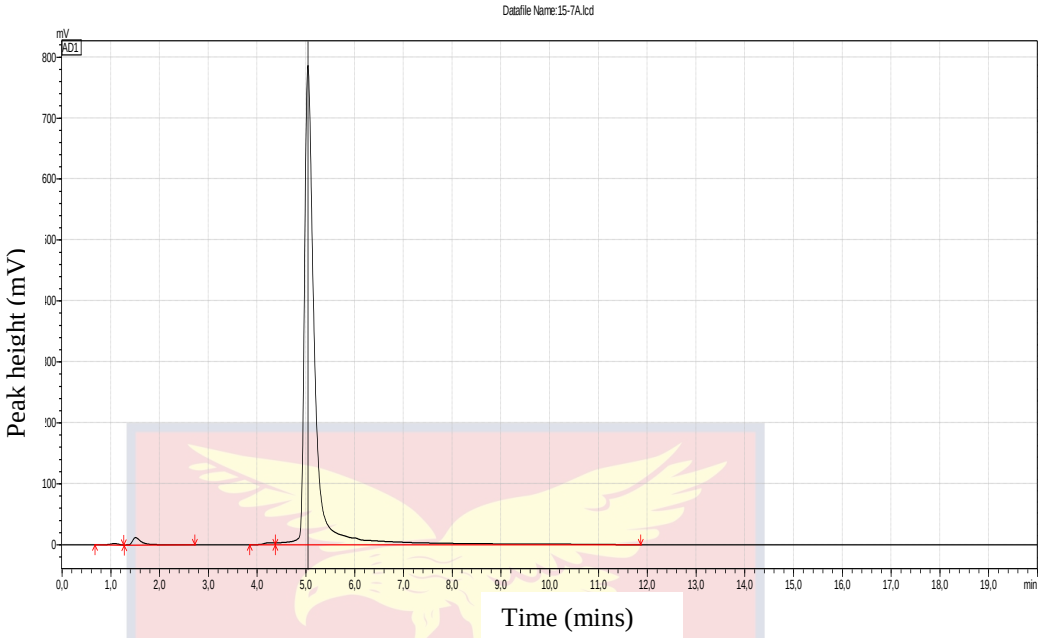
Diflubenzuron



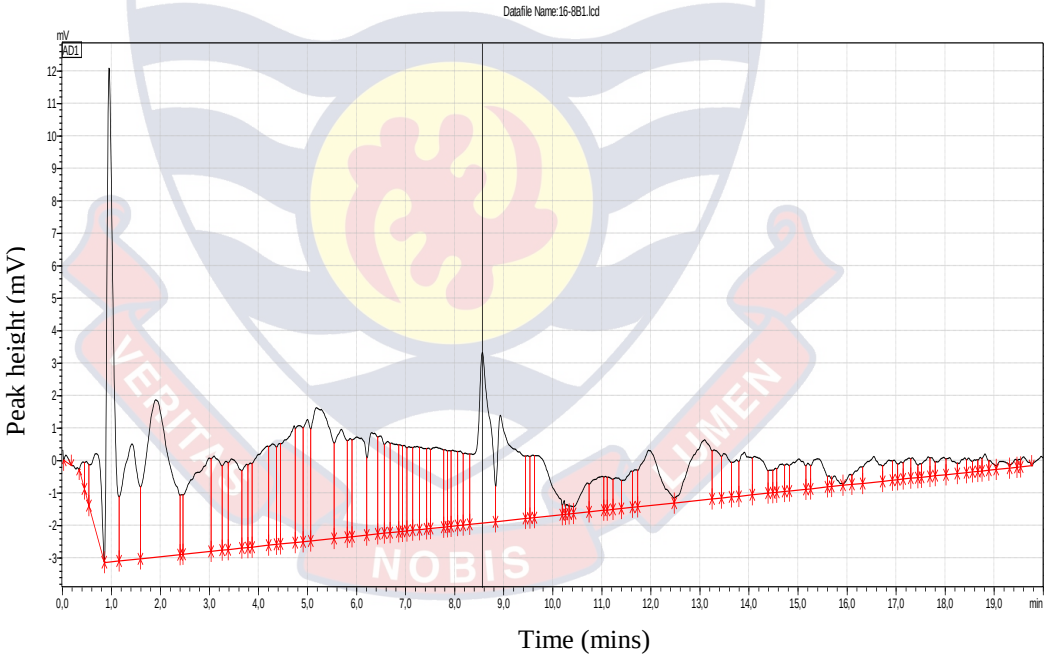
Ethoxyquin.



Malachite Green



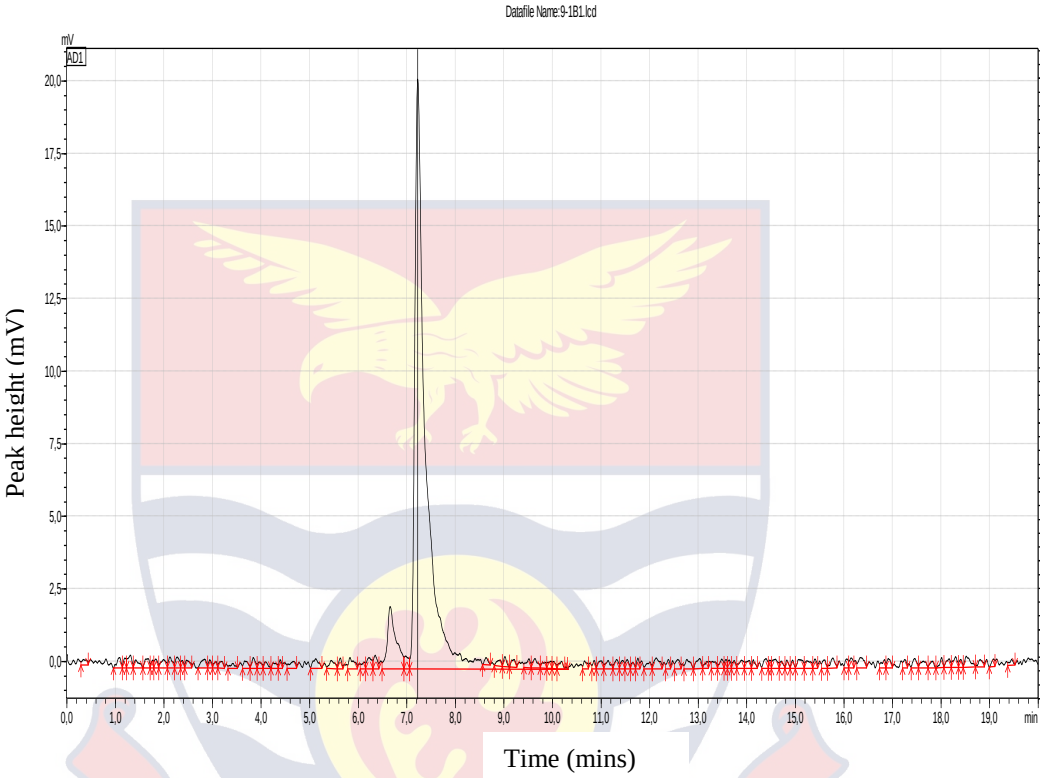
Sarafloxacin.



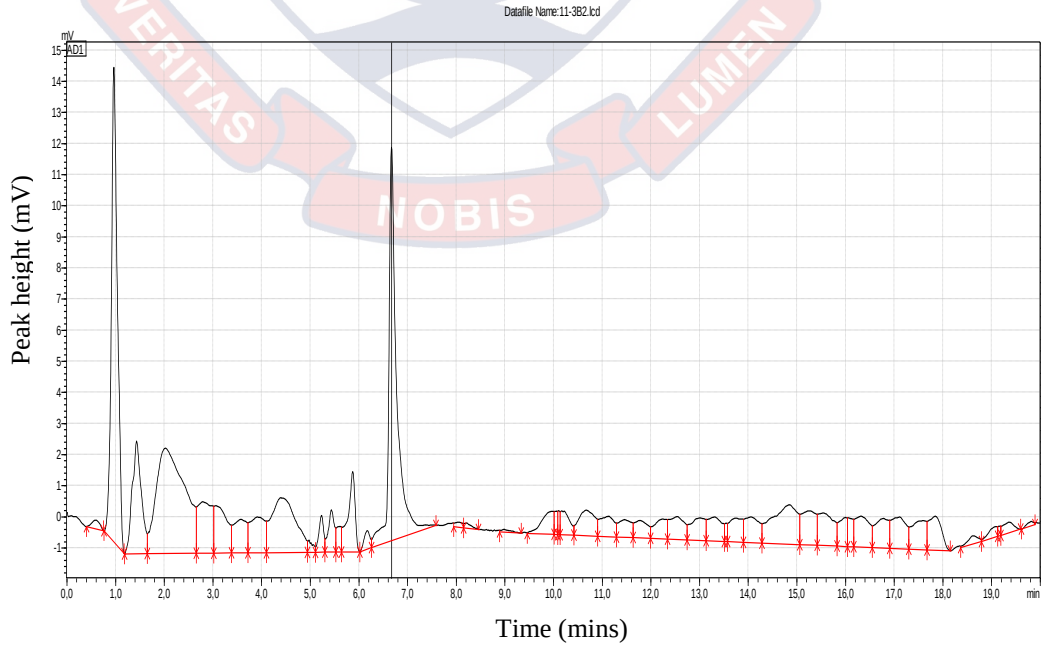
Teflubenzuron.

APPENDIX H

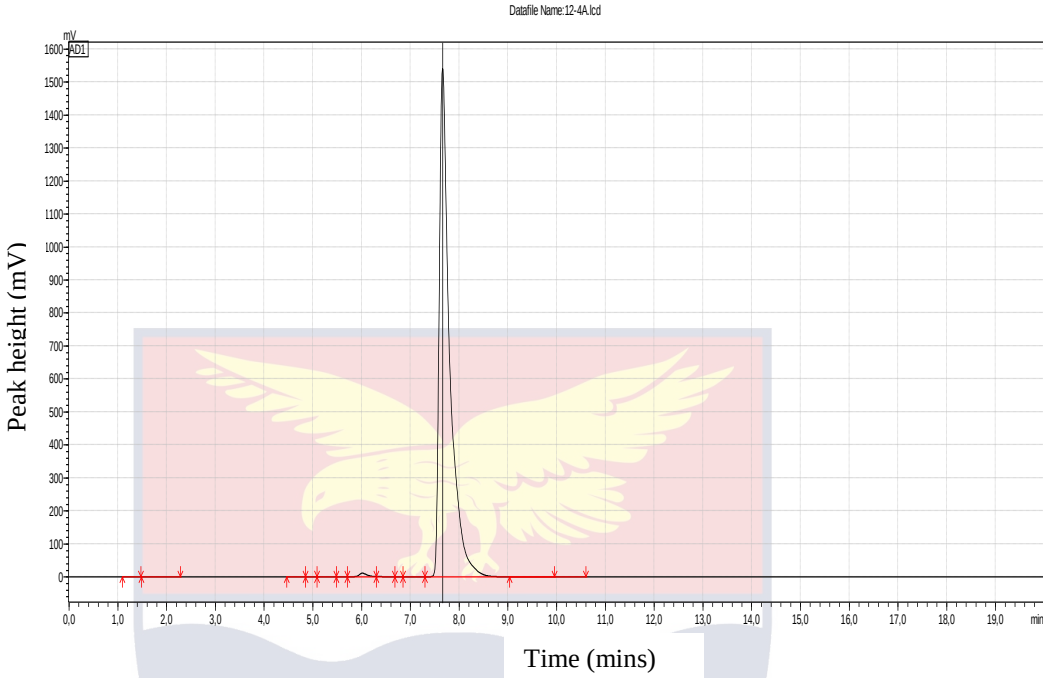
CHROMATOGRAPHS OF MOBILE PHASE 0.05 M ACETONITRILE, SODIUM ACETATE AND 0.04 M MAGNESIUM NITRATE USING GRADIENT METHOD 9 AT pH 6



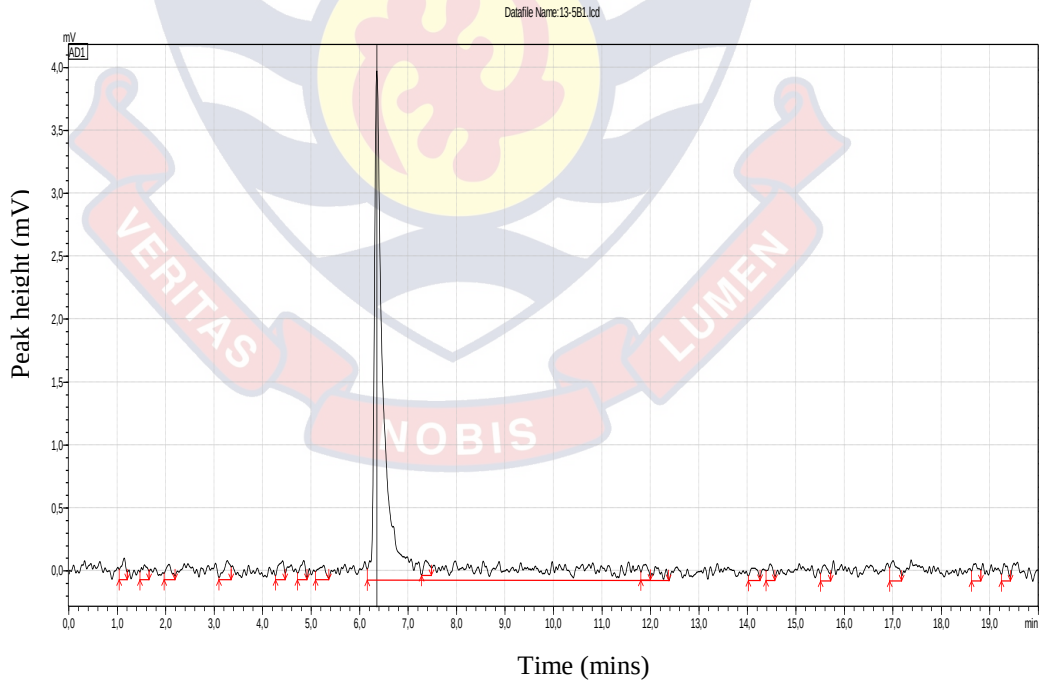
Crystal Violet



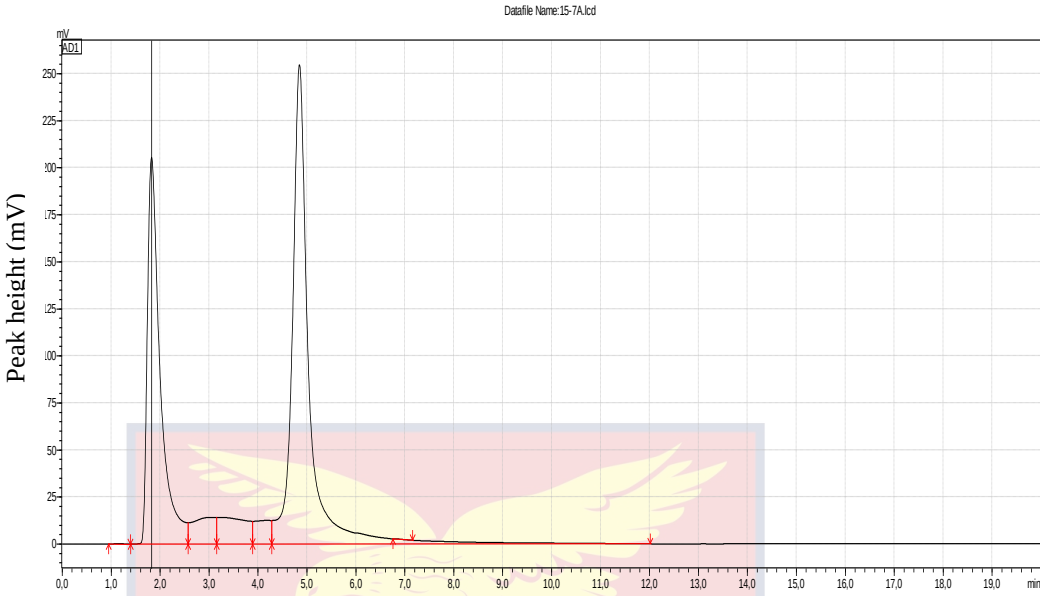
Diflubenzuron



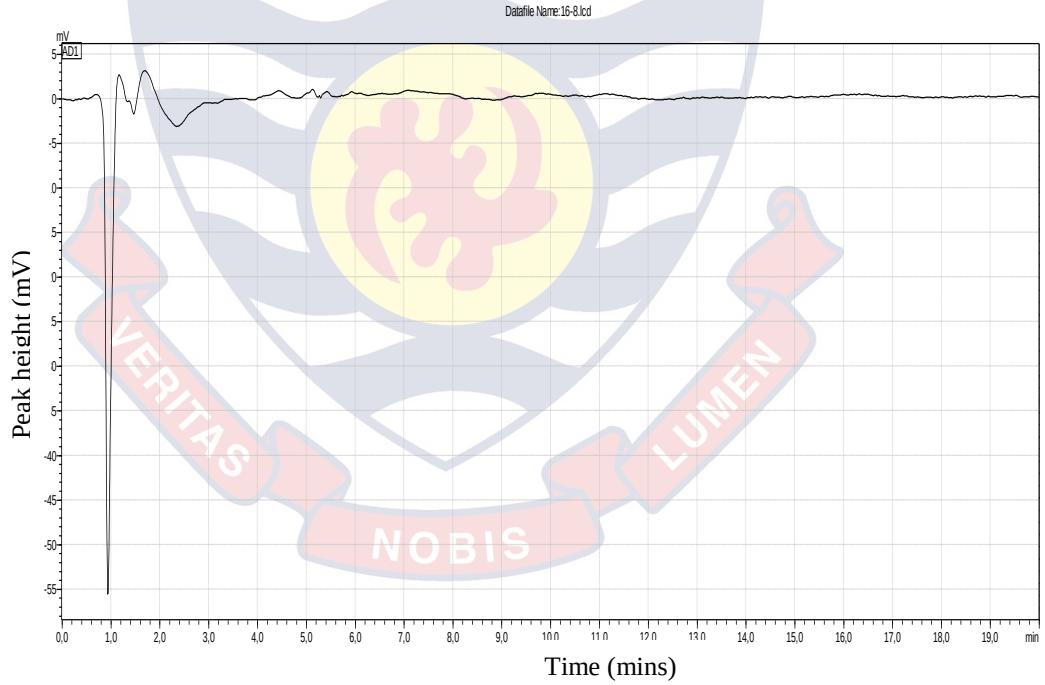
Ethoxyquin



Malachite Green



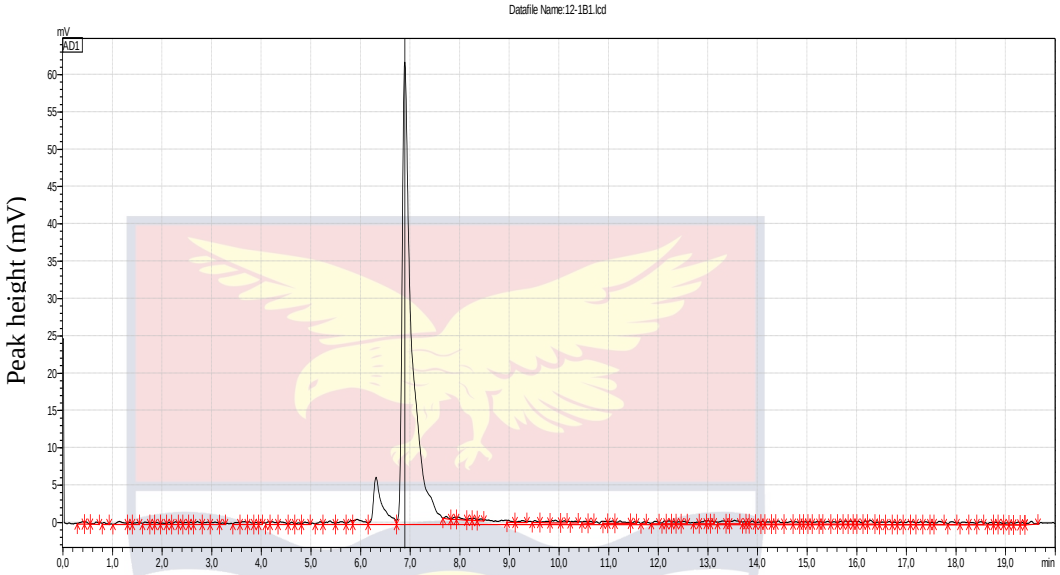
Sarafloxacin



Teflubenzuron.

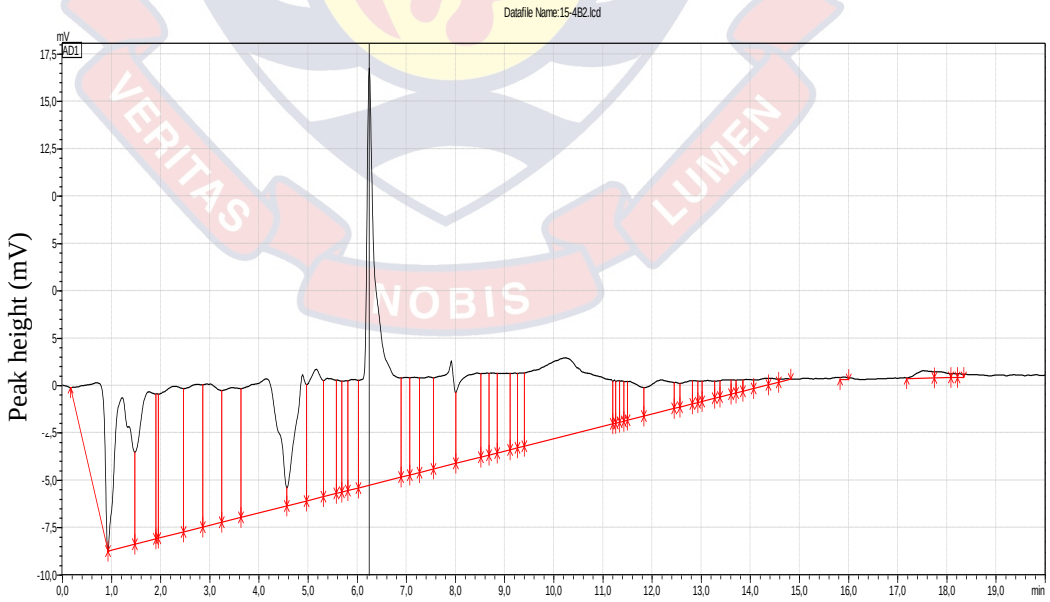
APPENDIX I

CHROMATOGRAPHS OF MOBILE PHASE 0.05 M ACETONITRILE, AMMONIUM ACETATE AND 0.04 M MAGNESIUM NITRATE USING GRADIENT METHOD 9 AT pH 8.50



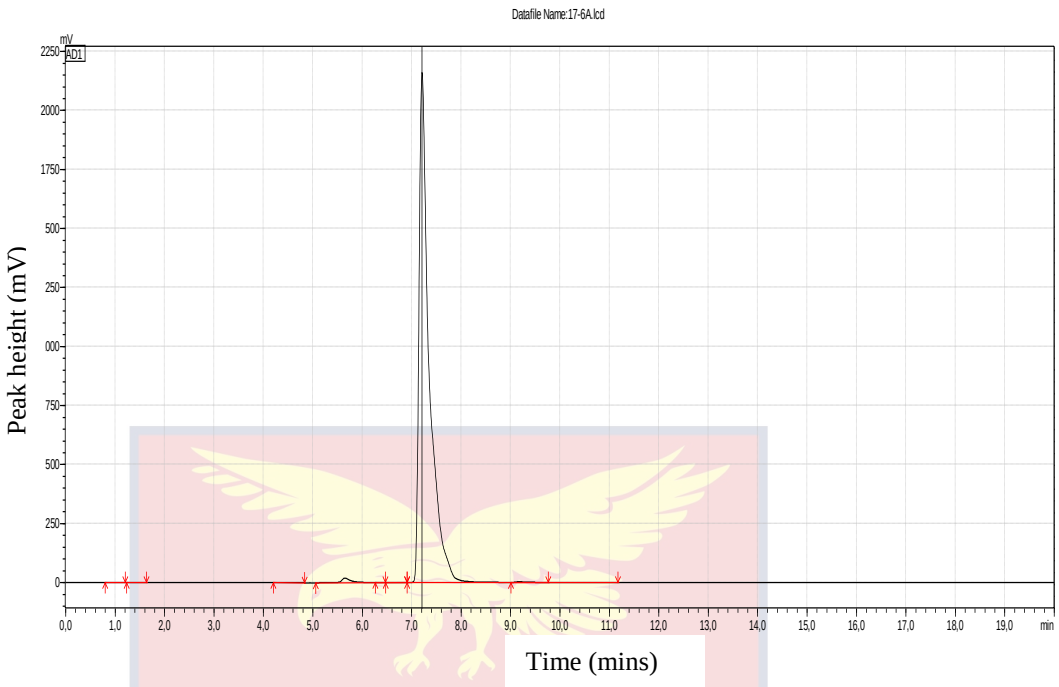
Crystal Violet

Time (mins)

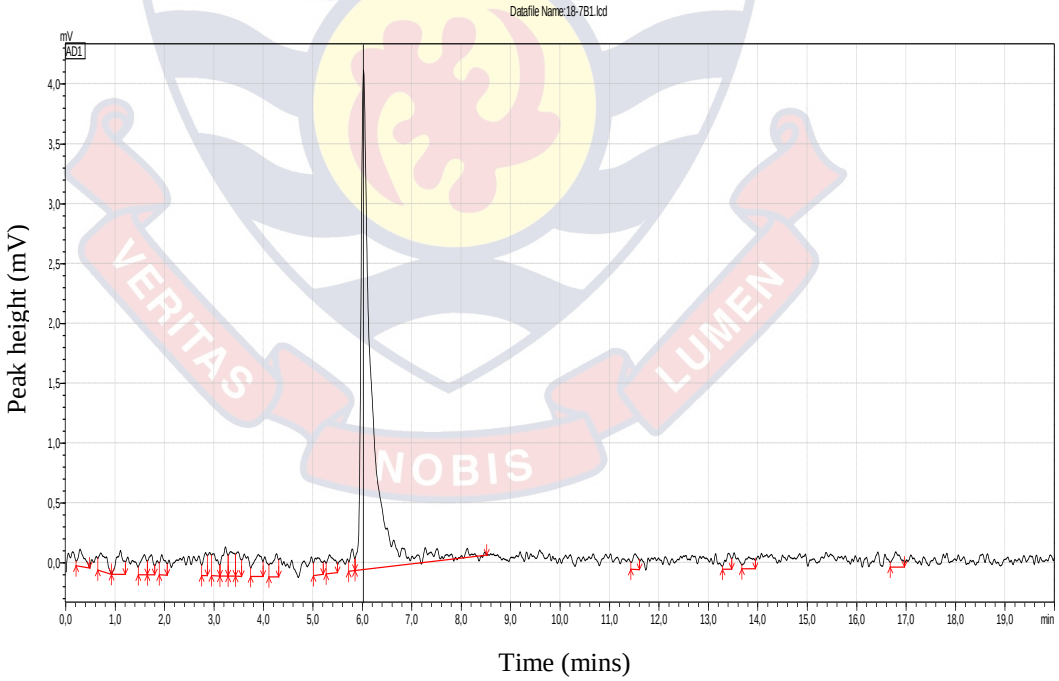


Time (mins)

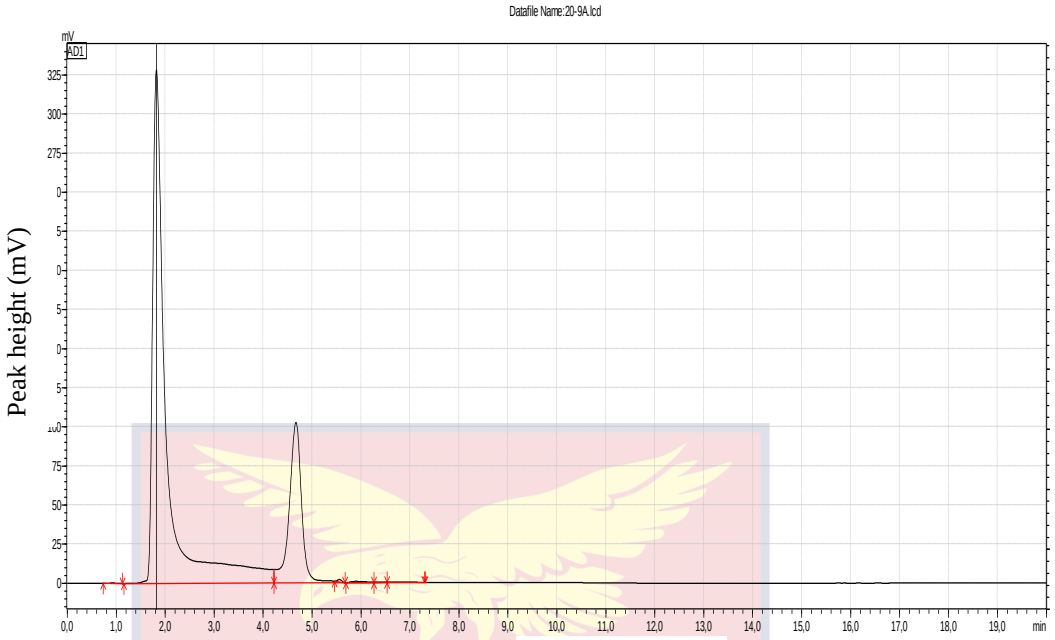
Diflubenzuron



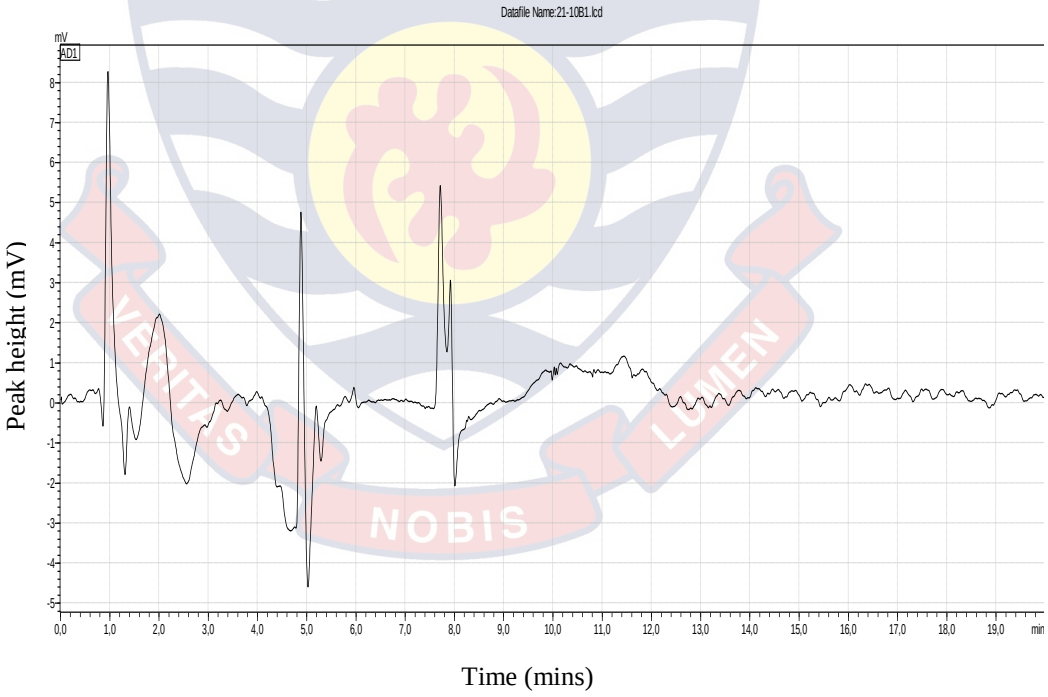
Ethoxyquin.



Malachite Green



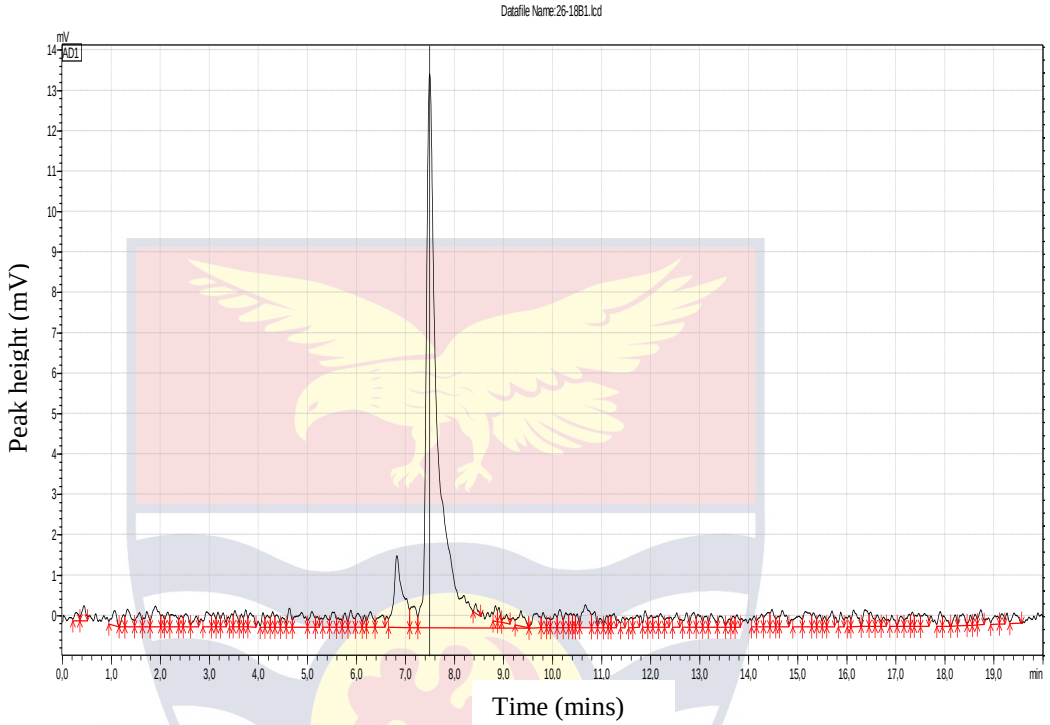
Sarafloxacin.



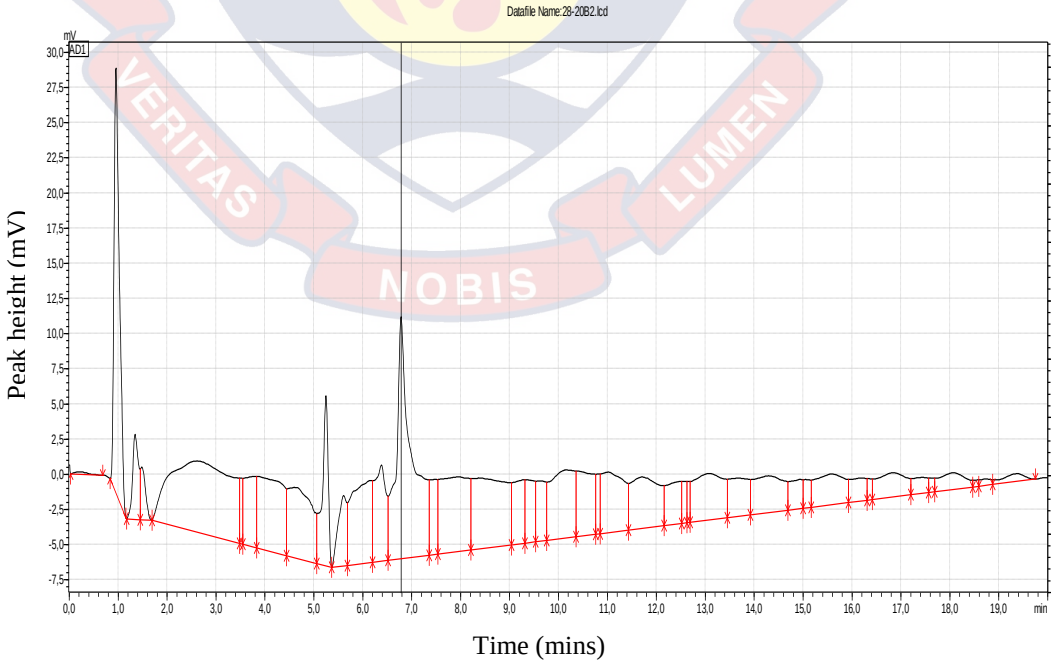
Teflubenzuron.

APPENDIX J

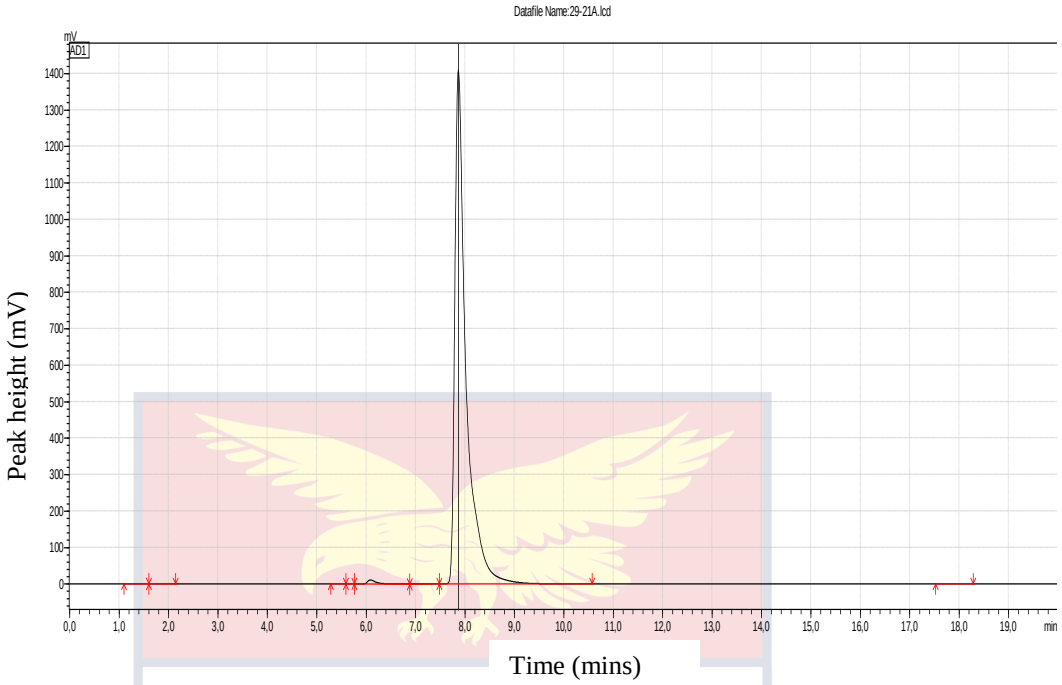
CHROMATOGRAPHS OF GRADIENT METHOD 7 WITH MOBILE PHASE ACETONITRILE, 0.05 M AMMONIUM ACETATE AND 0.04 M MAGNESIUM NITRATE AT pH 8.50



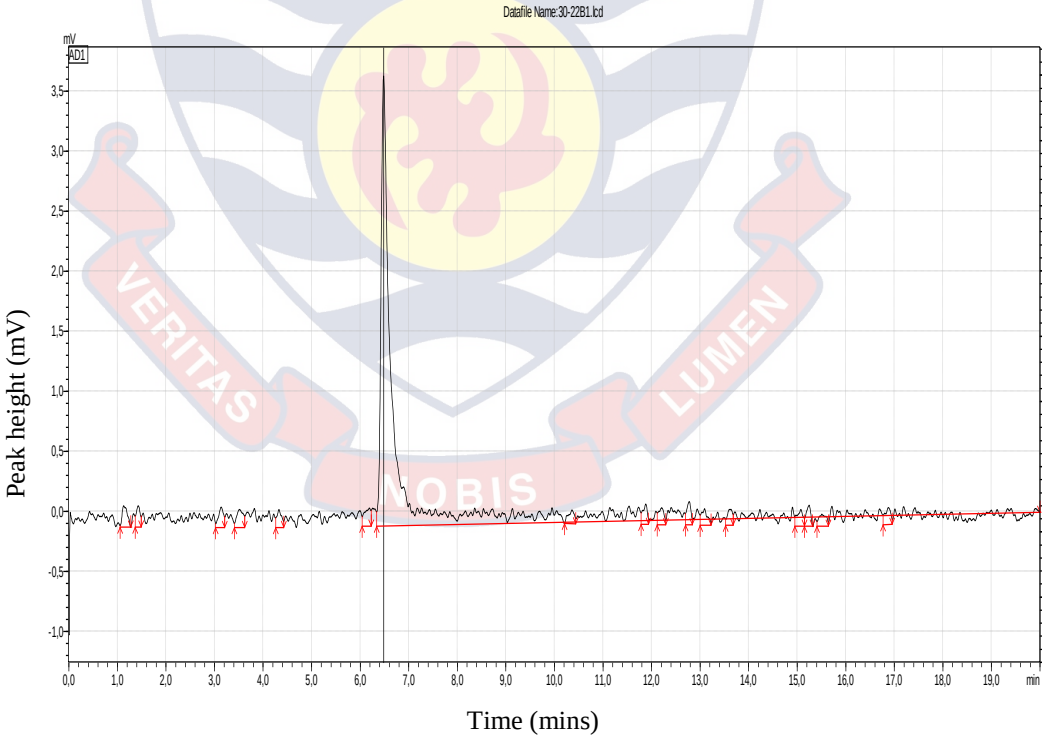
Crystal Violet.



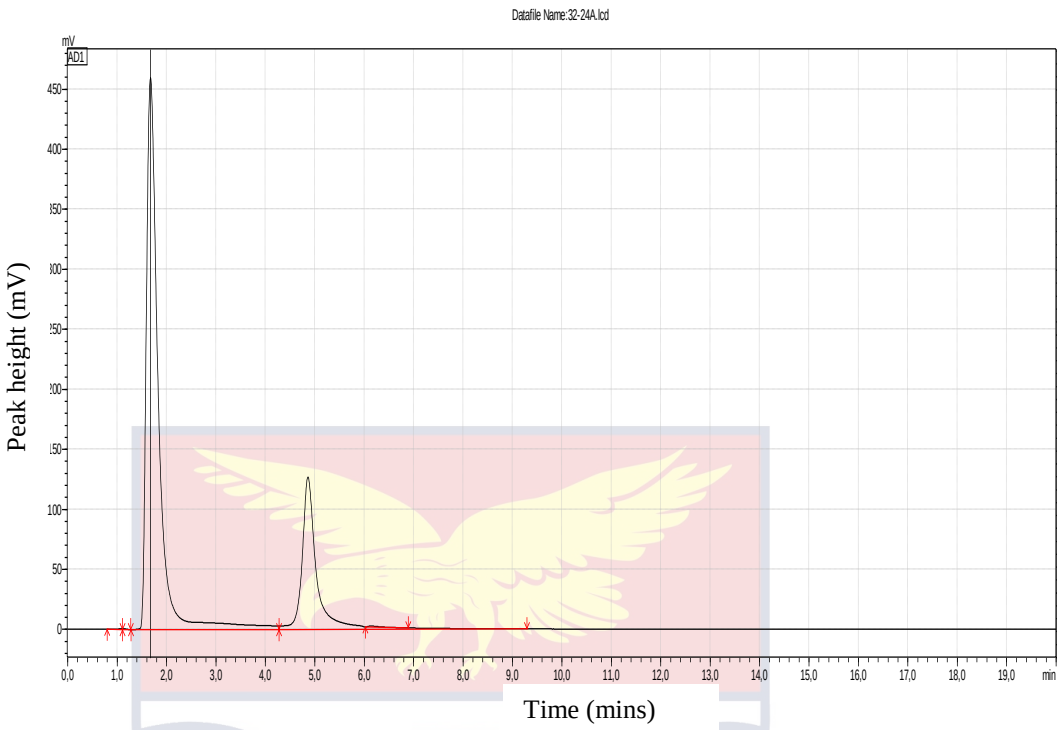
Diflubenzuron.



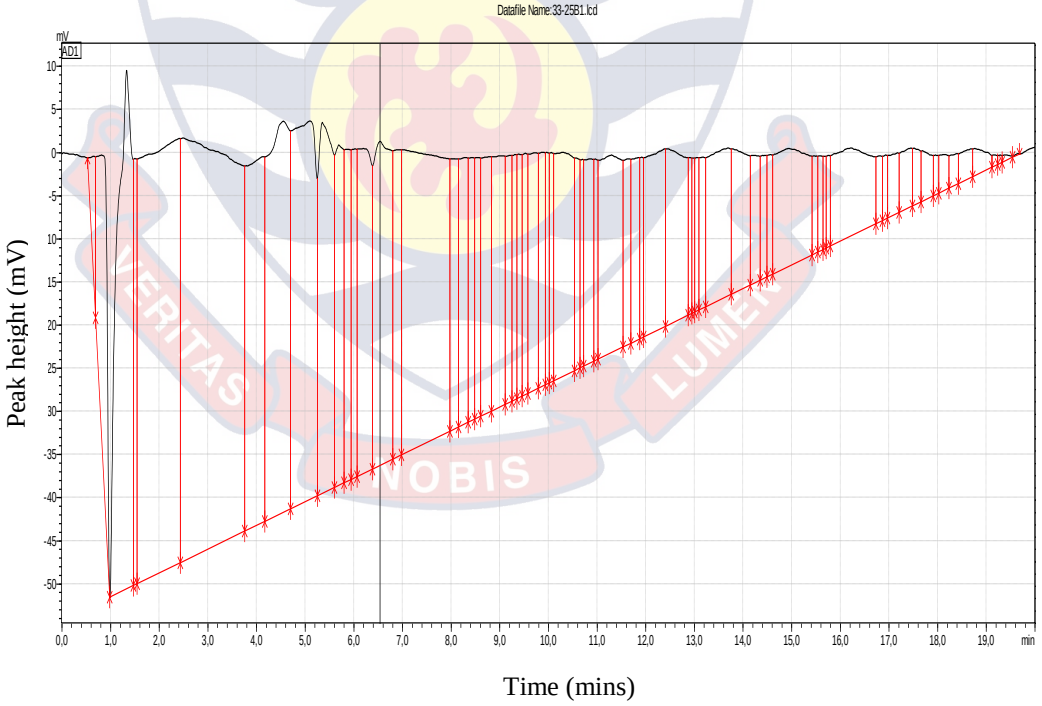
Ethoxyquin.



Malachite Green.



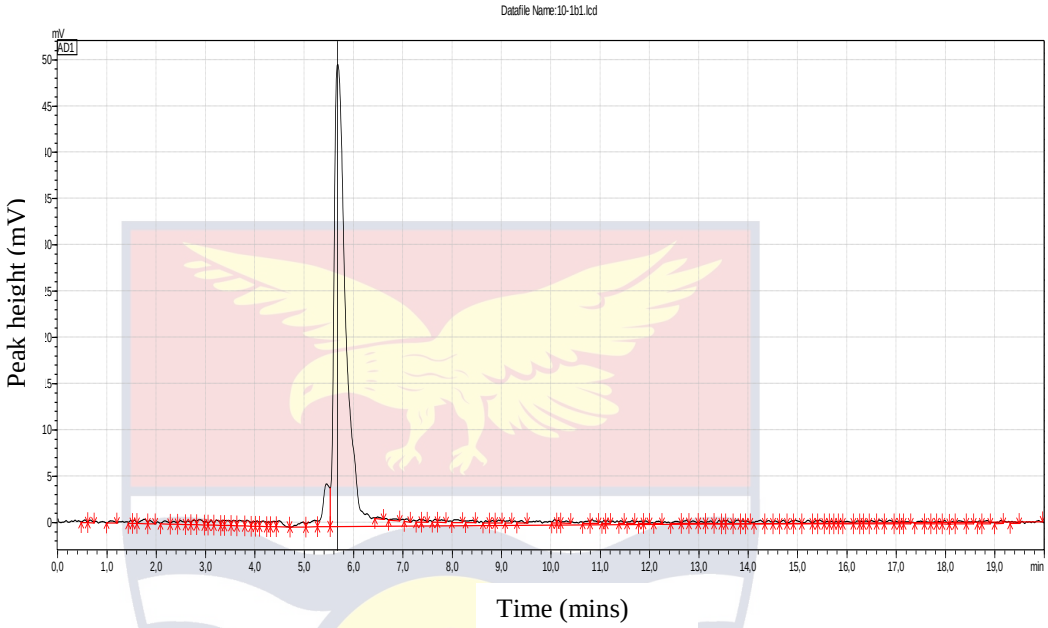
Sarafloxacin.



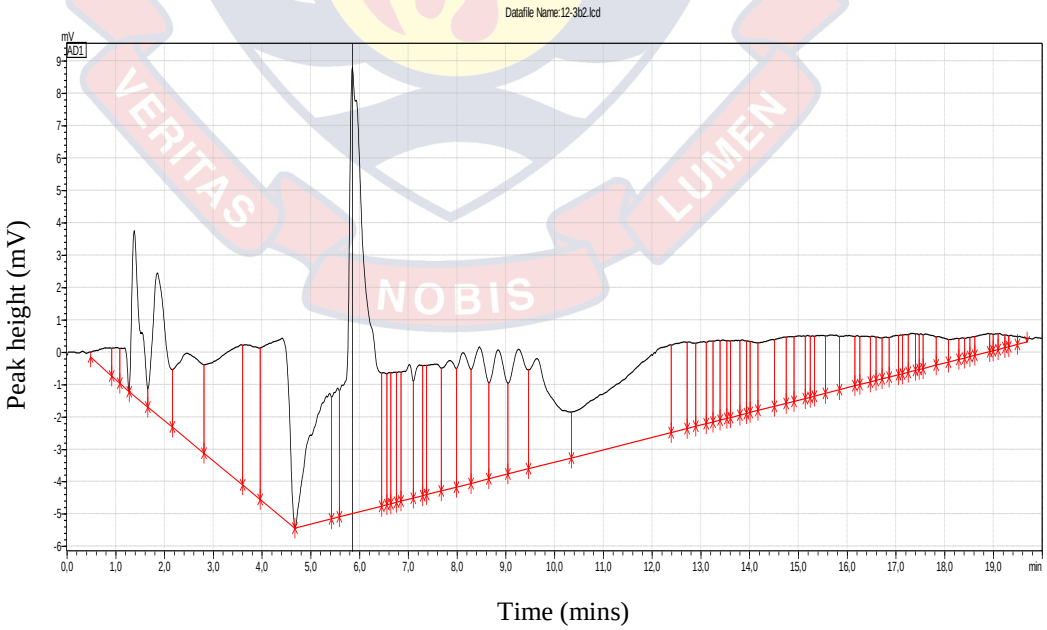
Teflubenzuron

APPENDIX K

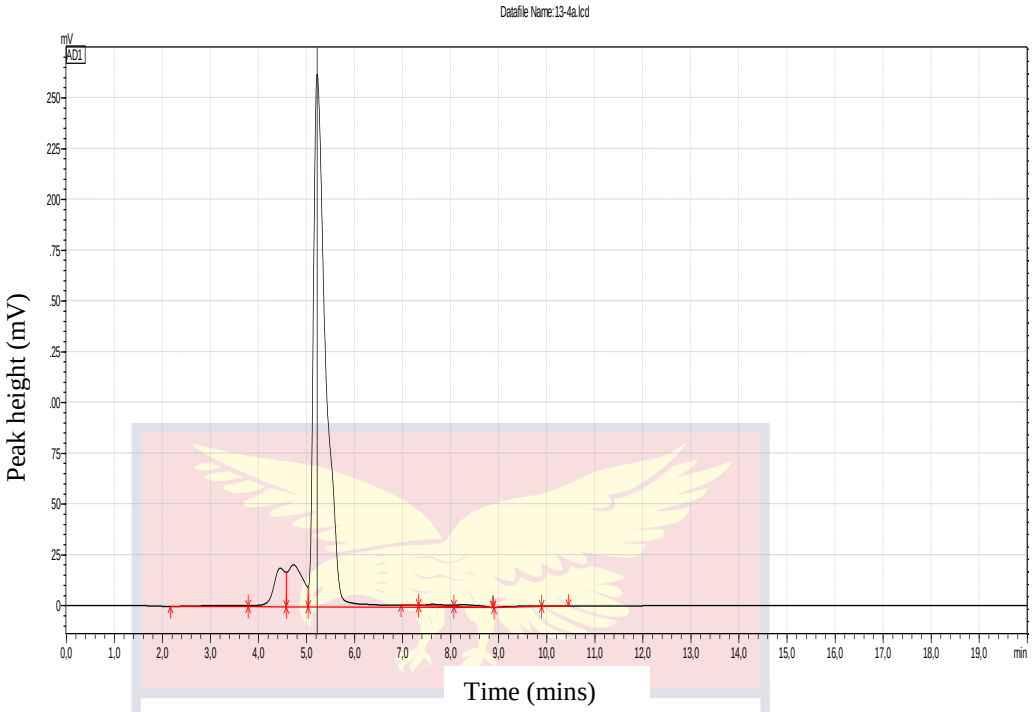
CHROMATOGRAPHS OF GRADIENT METHOD 13 WITH MOBILE PHASE ACETONITRILE, 0.05 M AMMONIUM ACETATE AND 0.04 M MAGNESIUM NITRATE AT pH 8.5



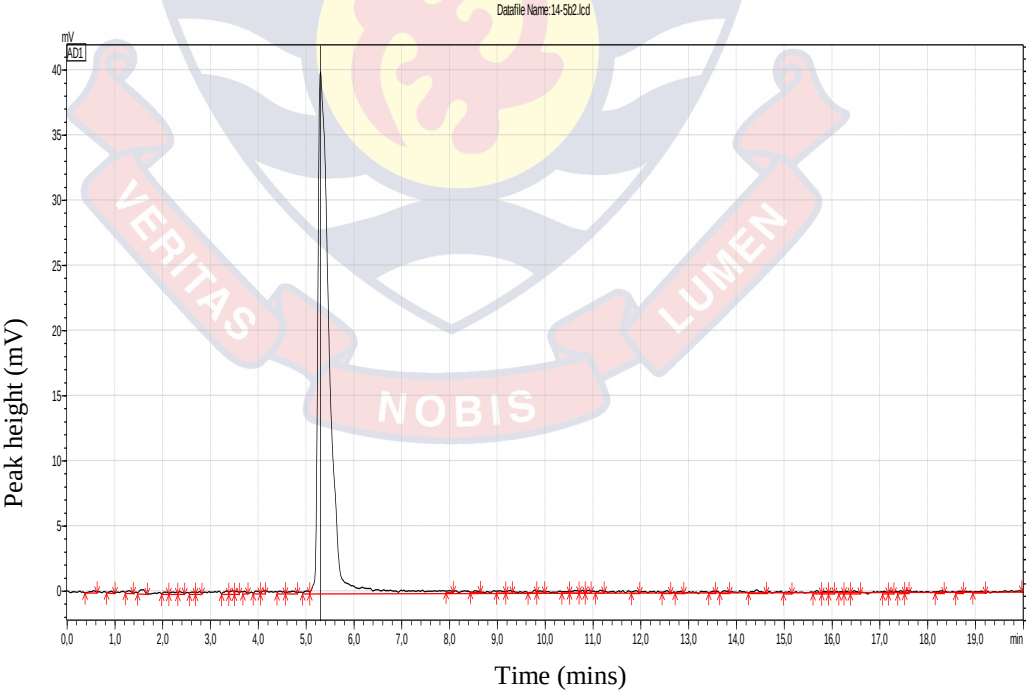
Crystal violet.



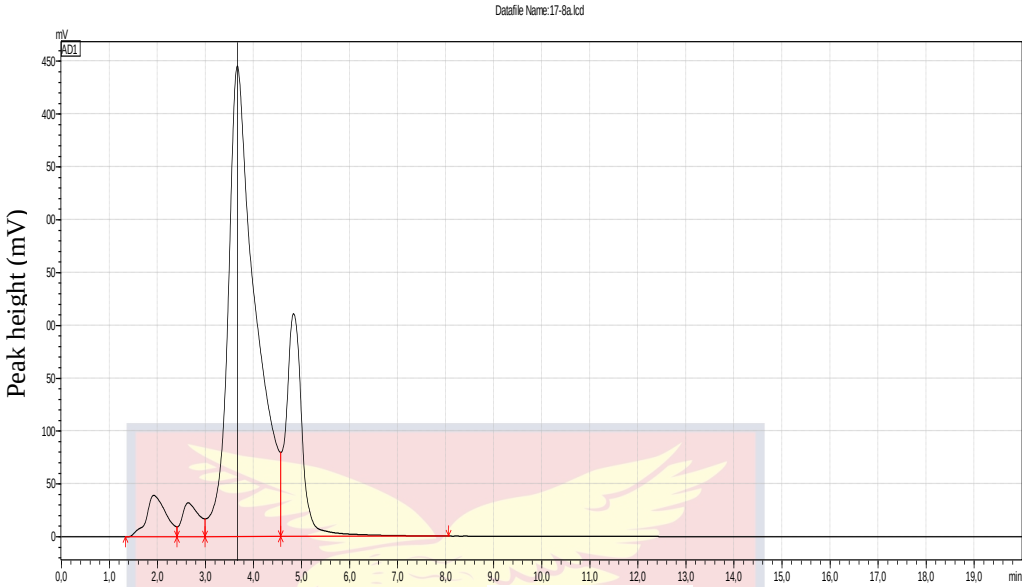
Diflubenzuron



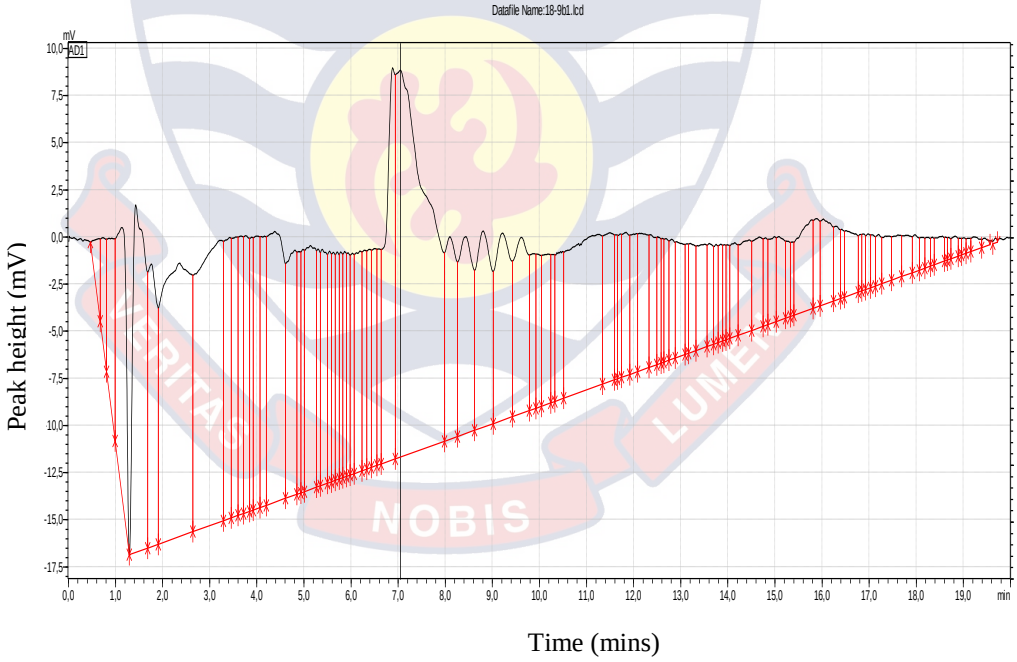
Ethoxyquin.



Malachite green.



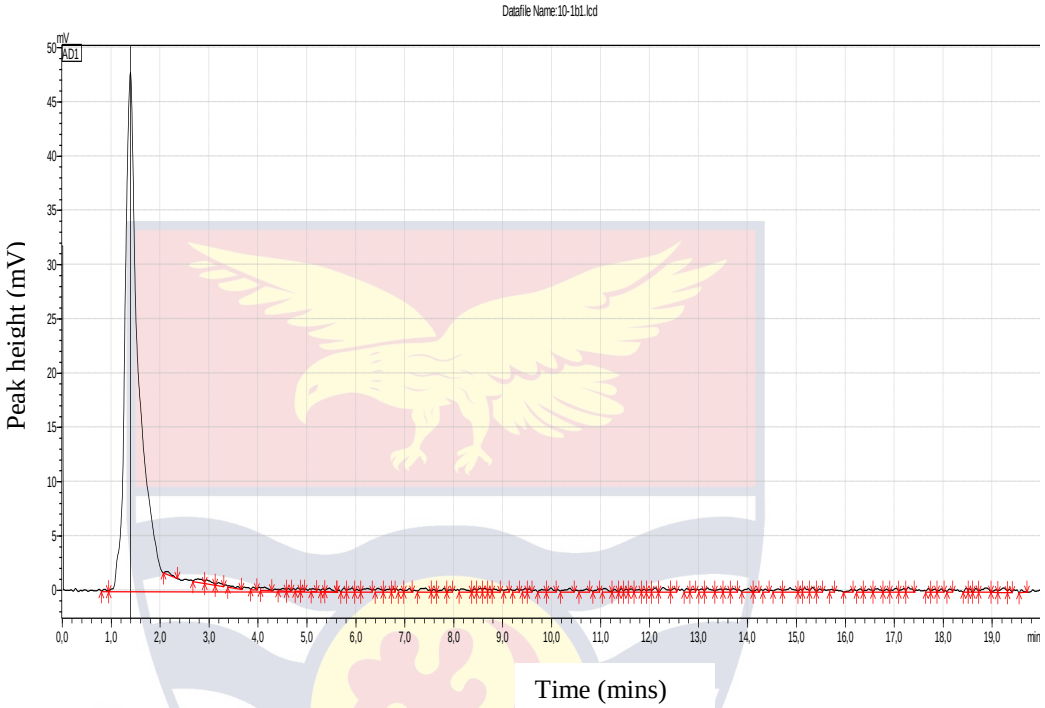
Sarafloxacin.



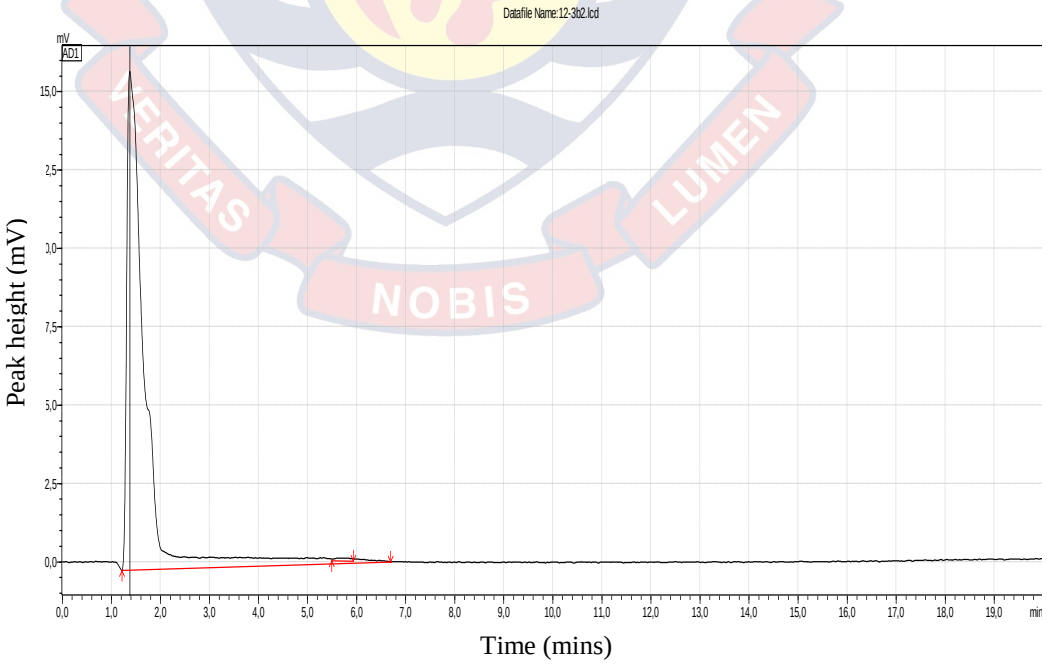
Teflubenzuron

APPENDIX L

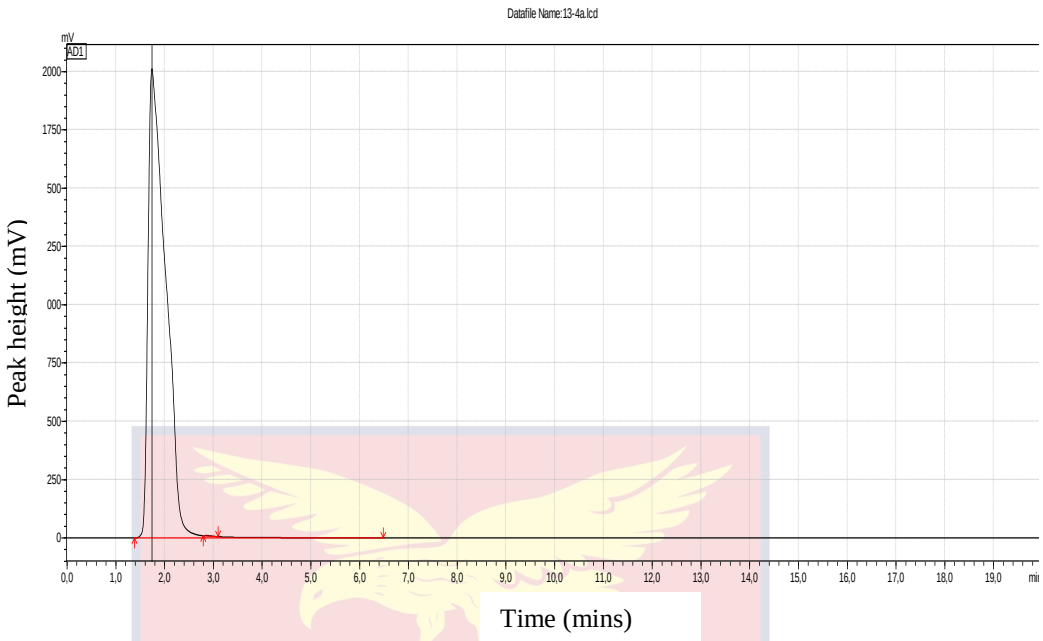
CHROMATOGRAPHS OF GRADIENT METHOD 12 WITH MOBILE PHASE ACETONITRILE, 0.05 M ACETIC ACID AND 0.04 M MAGNESIUM NITRATE AT pH 8.5



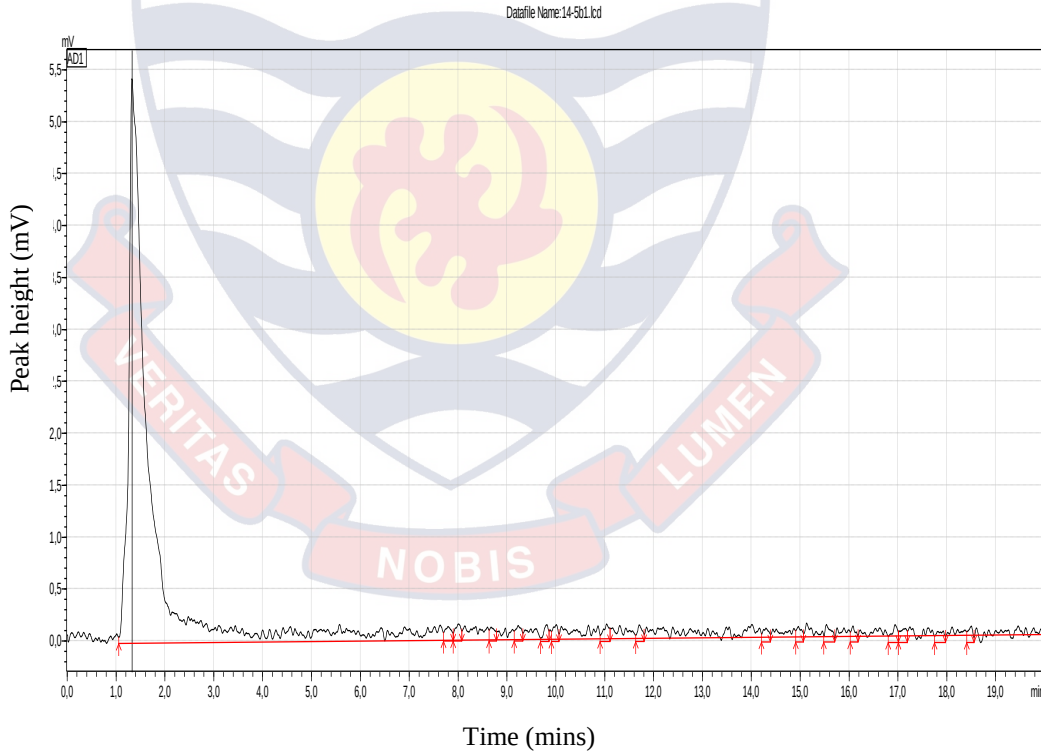
Crystal Violet



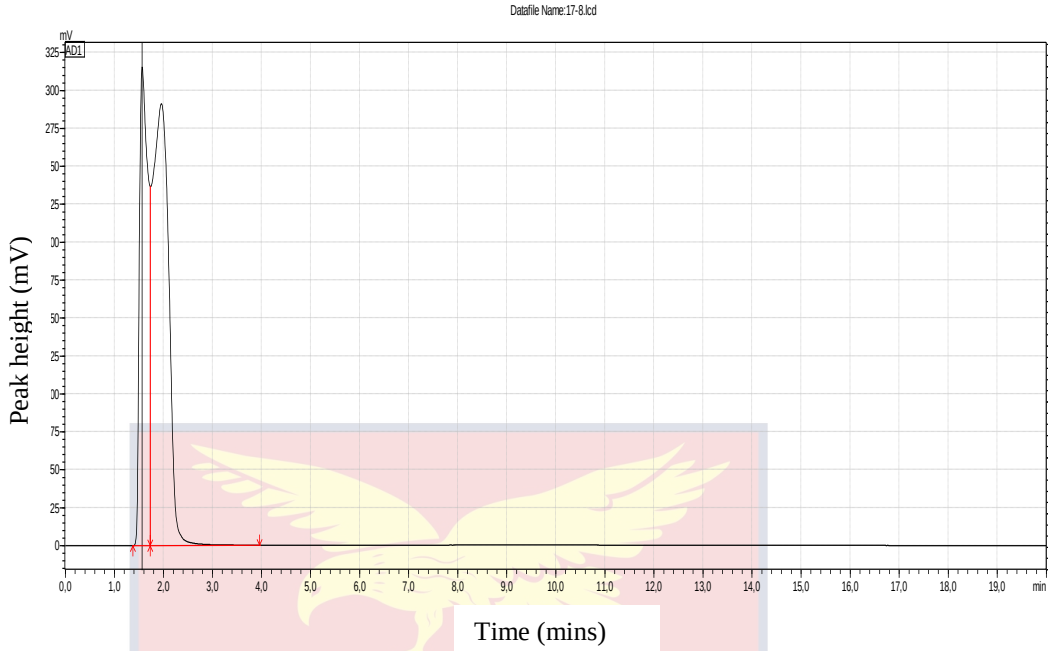
Diflubenzuron.



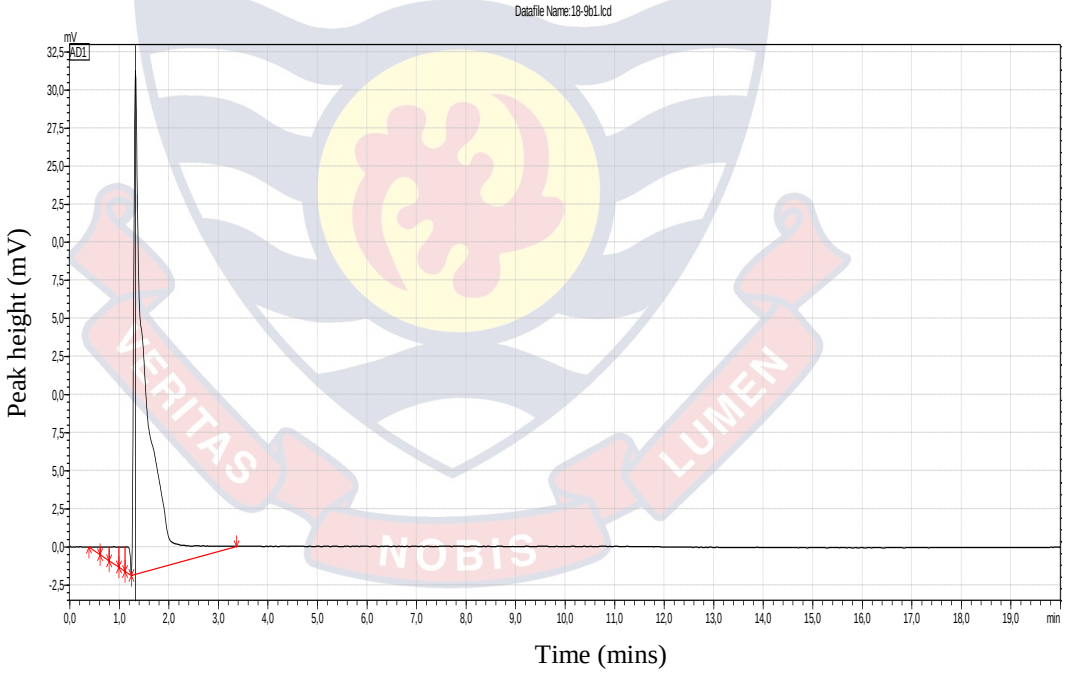
Malachite Green



Sarafloxacin



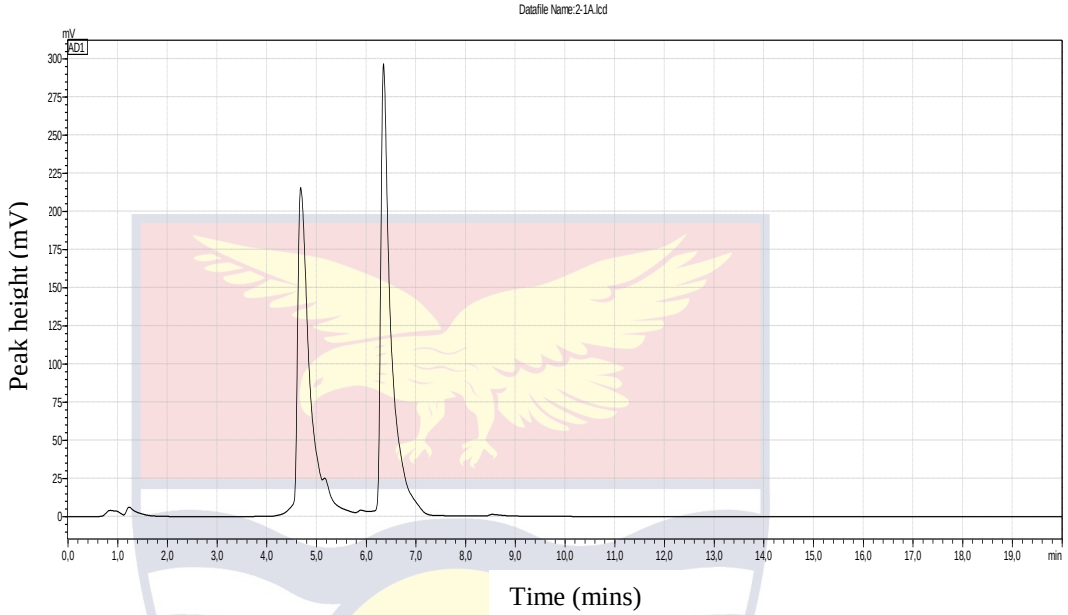
Teflubenzuron.



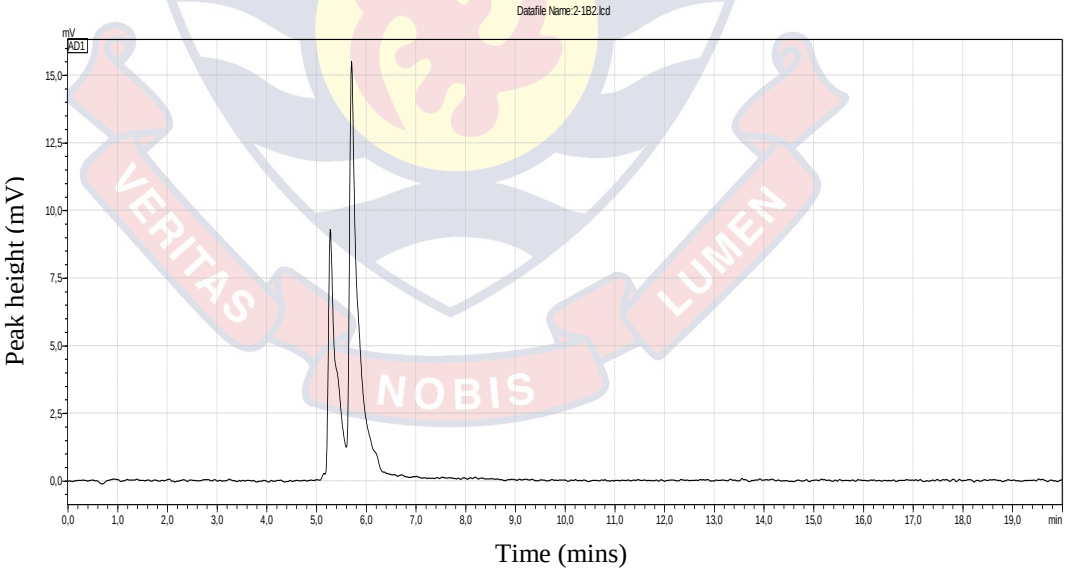
Ethoxyquin.

APPENDIX M

CHROMATOGRAMS OF MIXTURE USING GRADIENT METHOD 9 WITH MOBILE PHASE ACETONITRILE, 0.05 M ACETIC ACID AND 0.04 M MAGNESIUM NITRATE AT pH 3.3



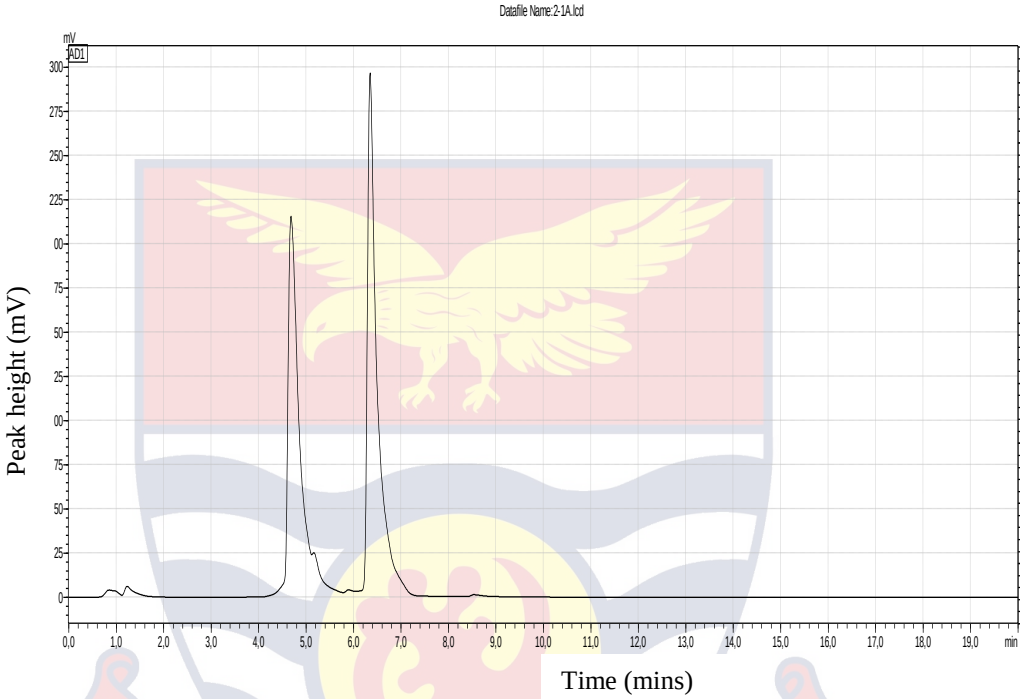
Detector A.



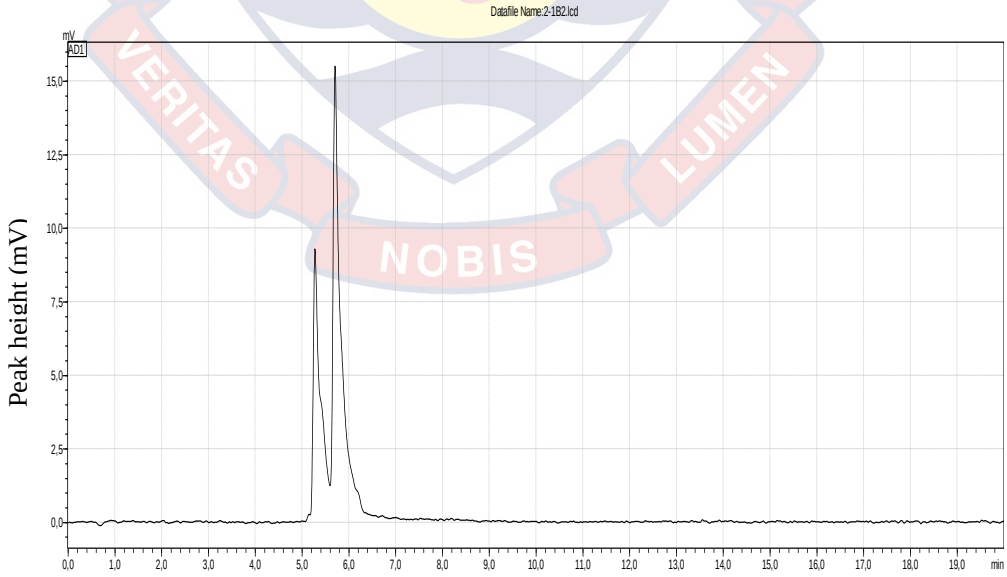
Detector B2.

APPENDIX N

CHROMATOGRAMS OF MIXTURE USING GRADIENT METHOD 9 WITH MOBILE PHASE ACETONITRILE, 0.05 M ACETIC ACID AND 0.04 M MAGNESIUM NITRATE AT pH 3.8



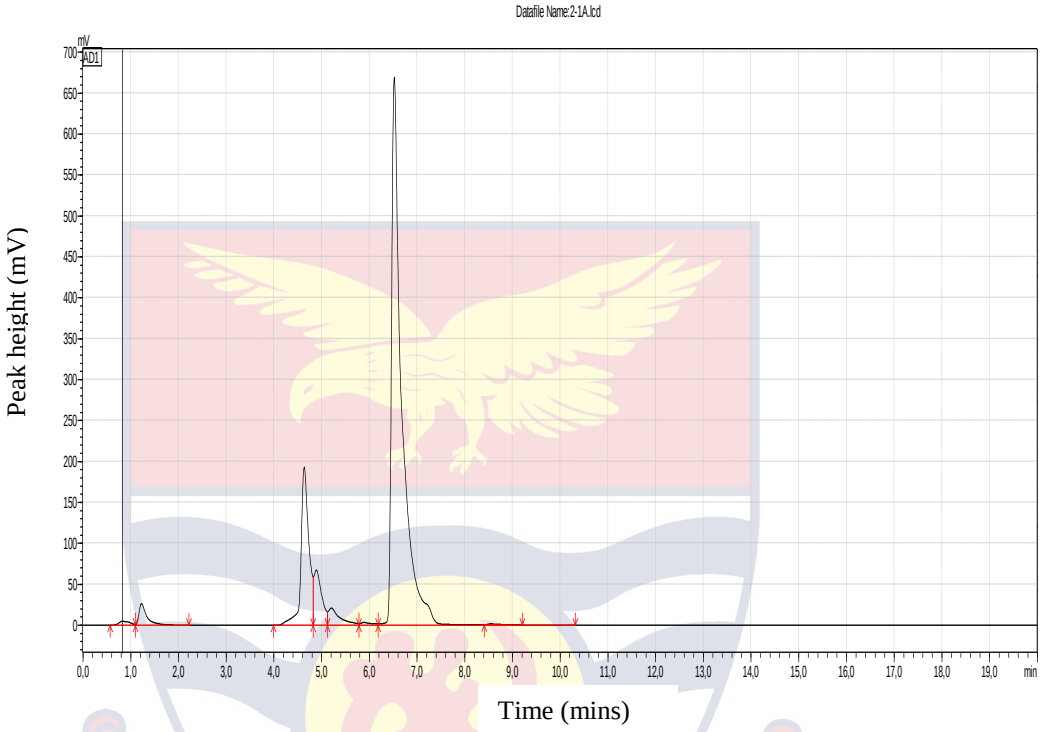
Detector A.



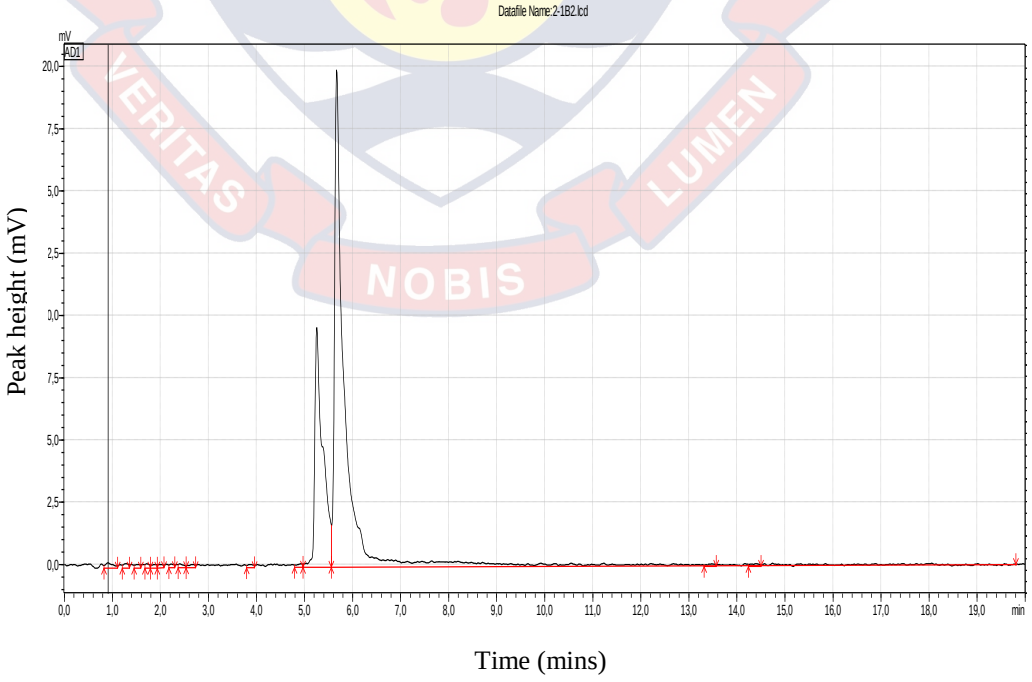
Detector B Time (mins)

APPENDIX O

CHROMATOGRAMS OF MIXTURE USING GRADIENT METHOD 9 WITH MOBILE PHASE ACETONITRILE, 0.05 M ACETIC ACID AND 0.04 M MAGNESIUM NITRATE AT pH 4



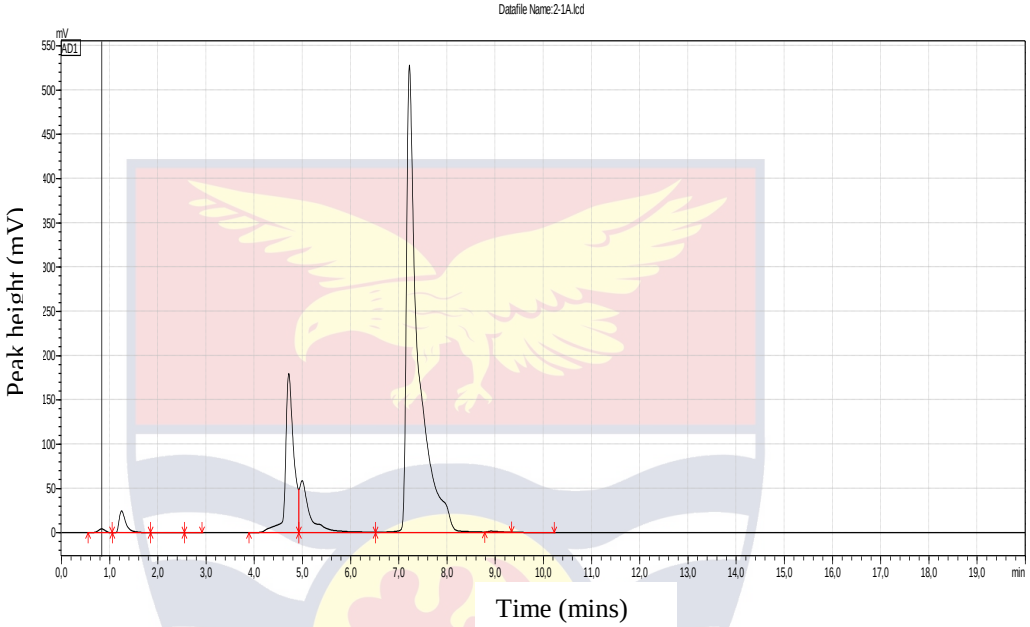
Detector A.



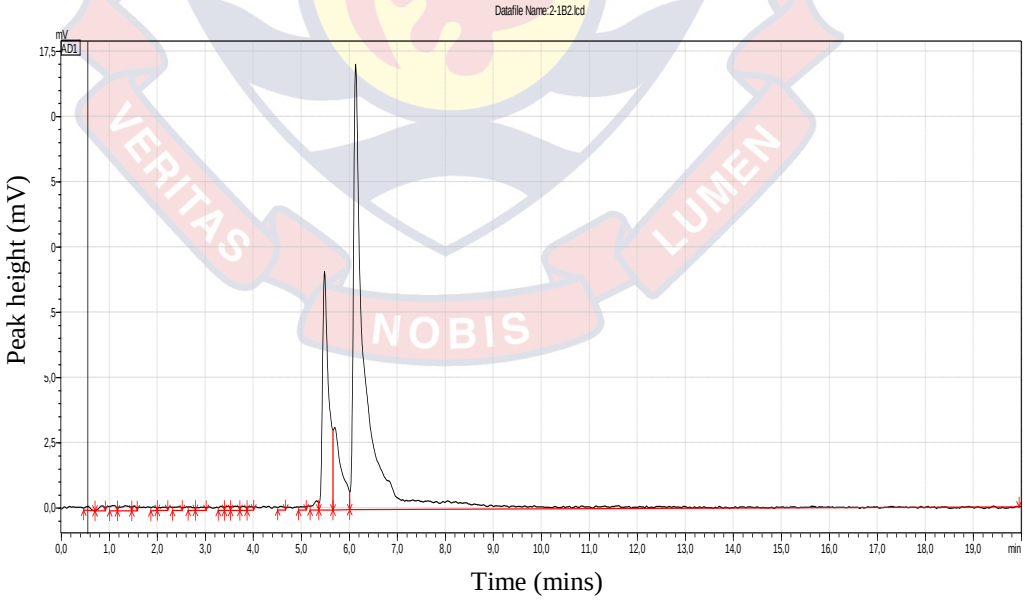
Detector B

APPENDIX P

CHROMATOGRAMS OF MIXTURE USING GRADIENT METHOD 13 WITH MOBILE PHASE ACETONITRILE, 0.05 M ACETIC ACID AND 0.04 M MAGNESIUM NITRATE AT pH 4



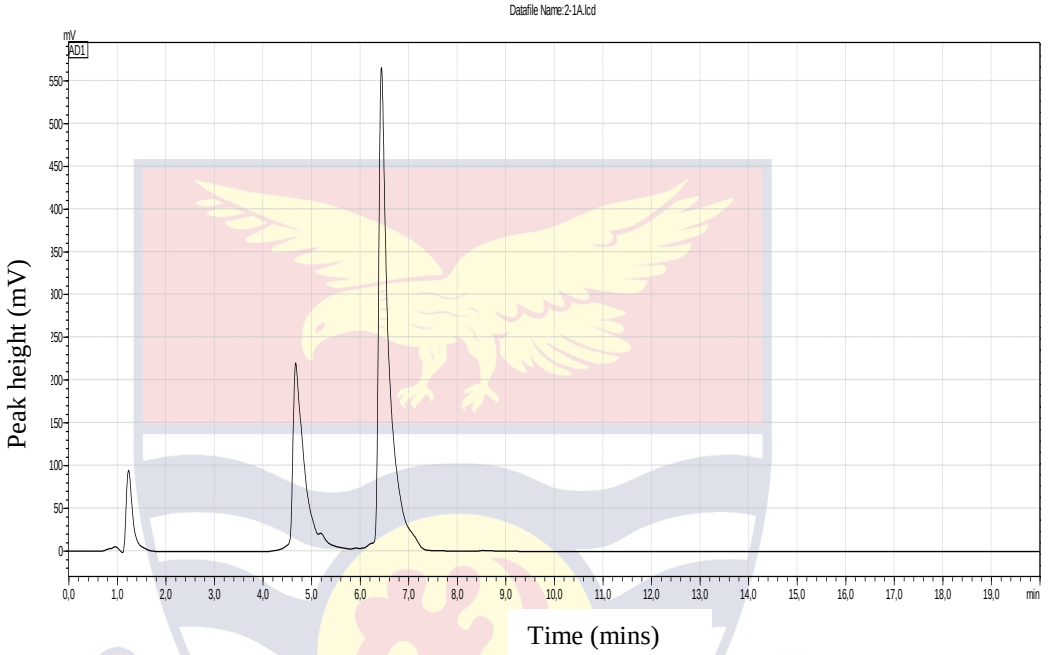
Detector A.



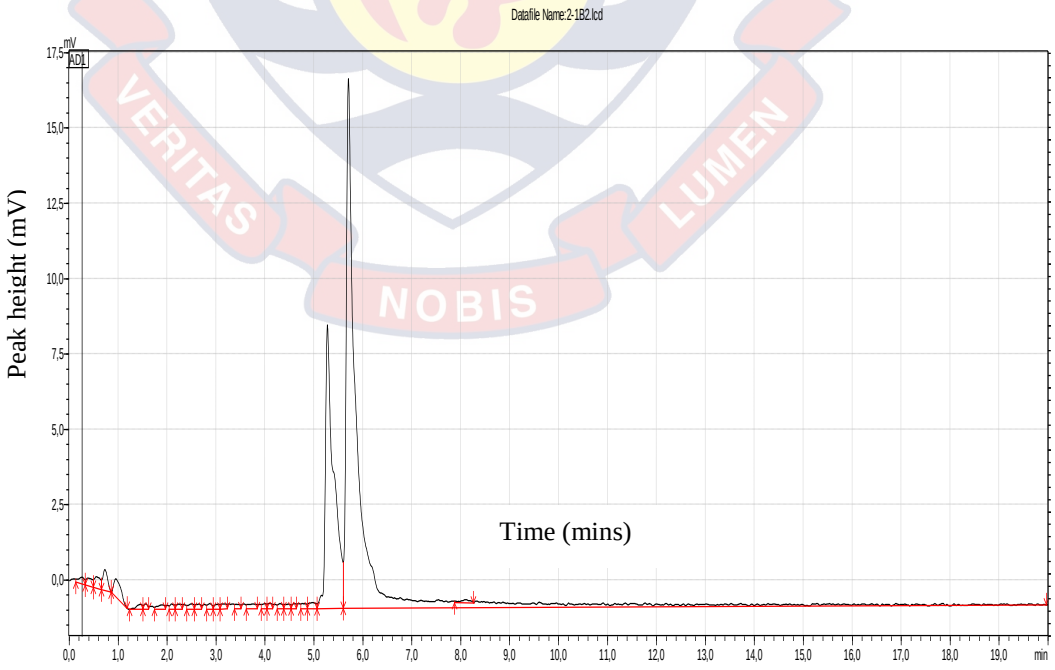
Detector B.

APPENDIX Q

CHROMATOGRAMS OF MIXTURE USING GRADIENT METHOD 9 WITH MOBILE PHASE ACETONITRILE, 0.005 M ACETIC ACID AND MAGNESIUM NITRATE AT pH 3.5



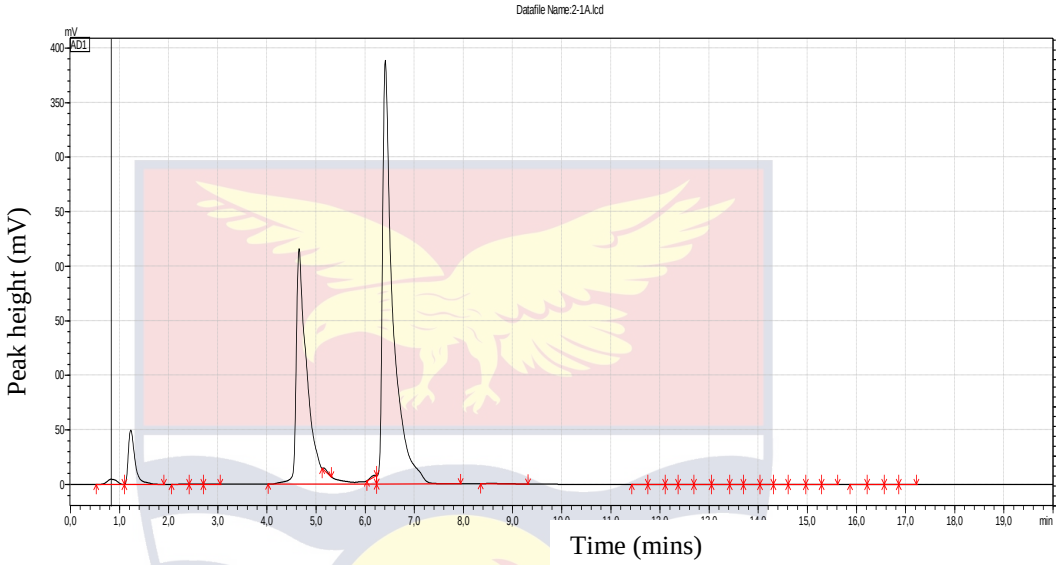
Detector A.



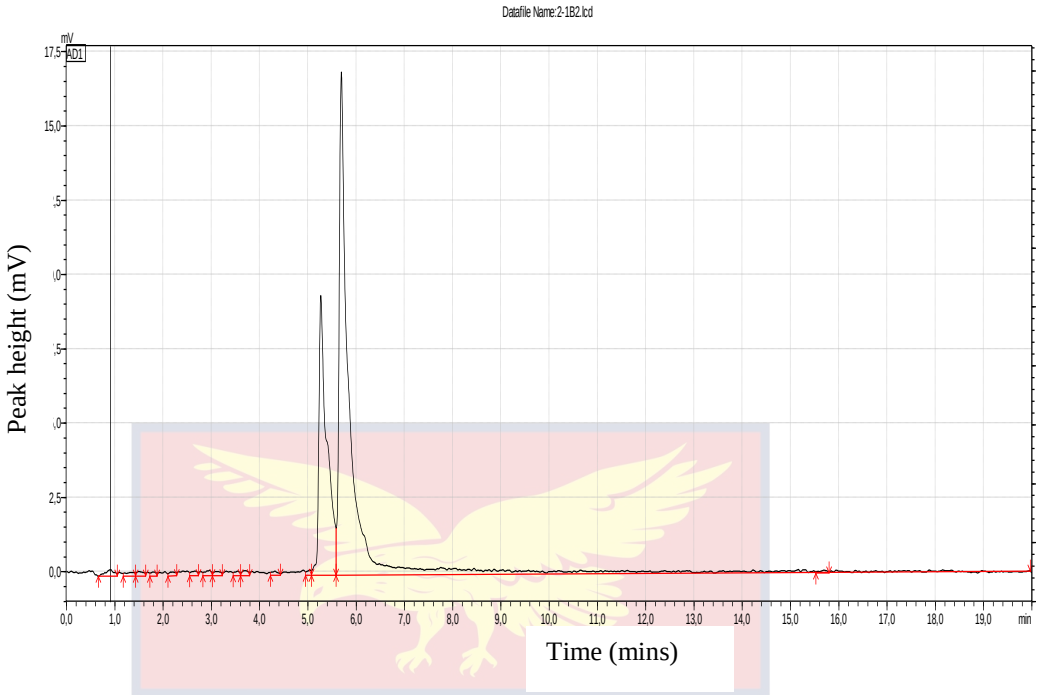
Detector B.

APPENDIX R

CHROMATOGRAMS OF MIXTURE USING GRADIENT METHOD 9 WITH MOBILE PHASE 0.025 M ACETIC ACID AND MAGNESIUM NITRATE AT pH 3.5



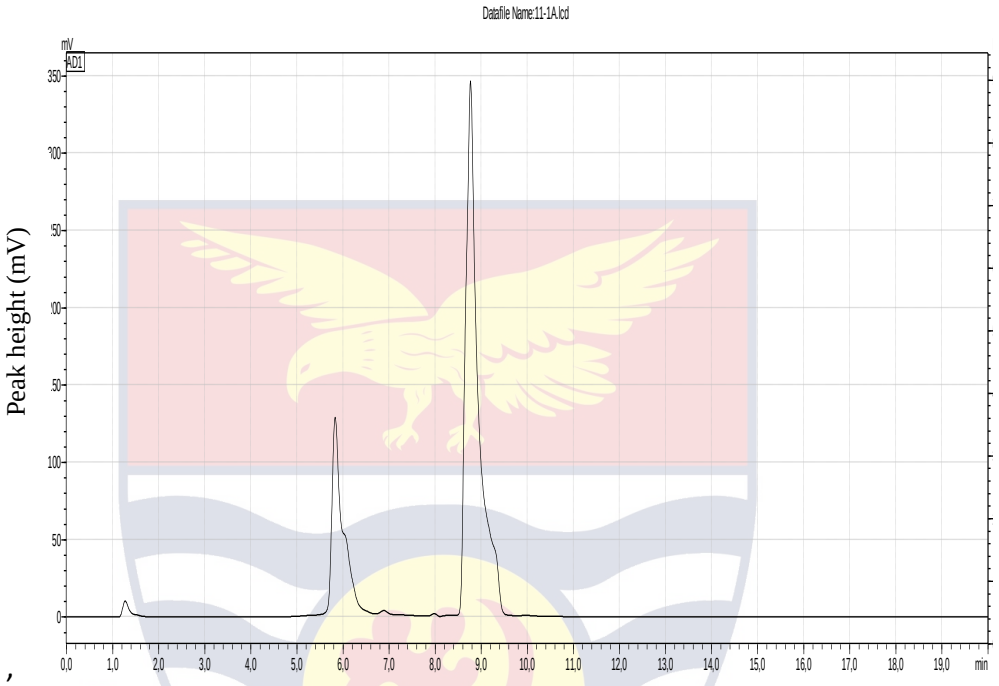
Detector A.



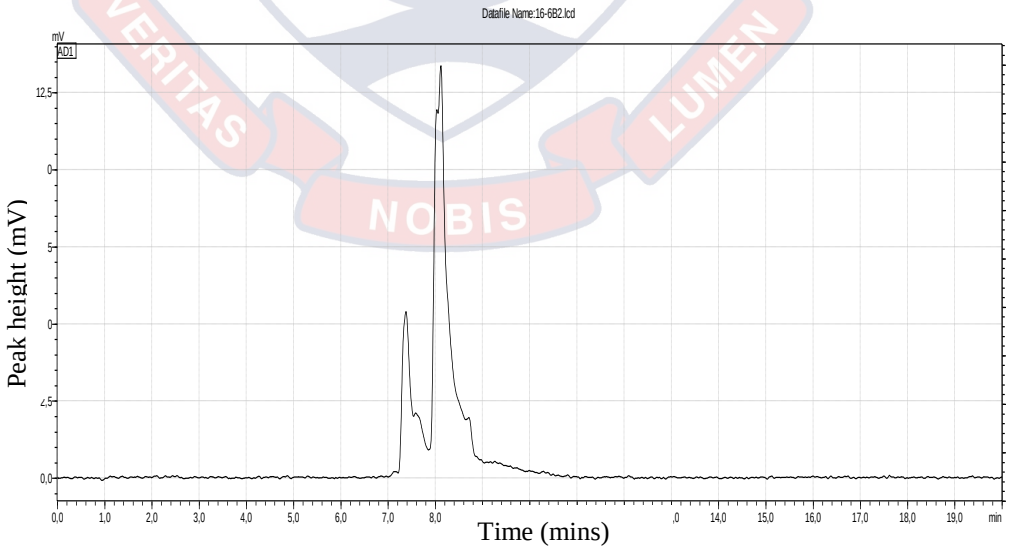
Detector B.

APPENDIX S

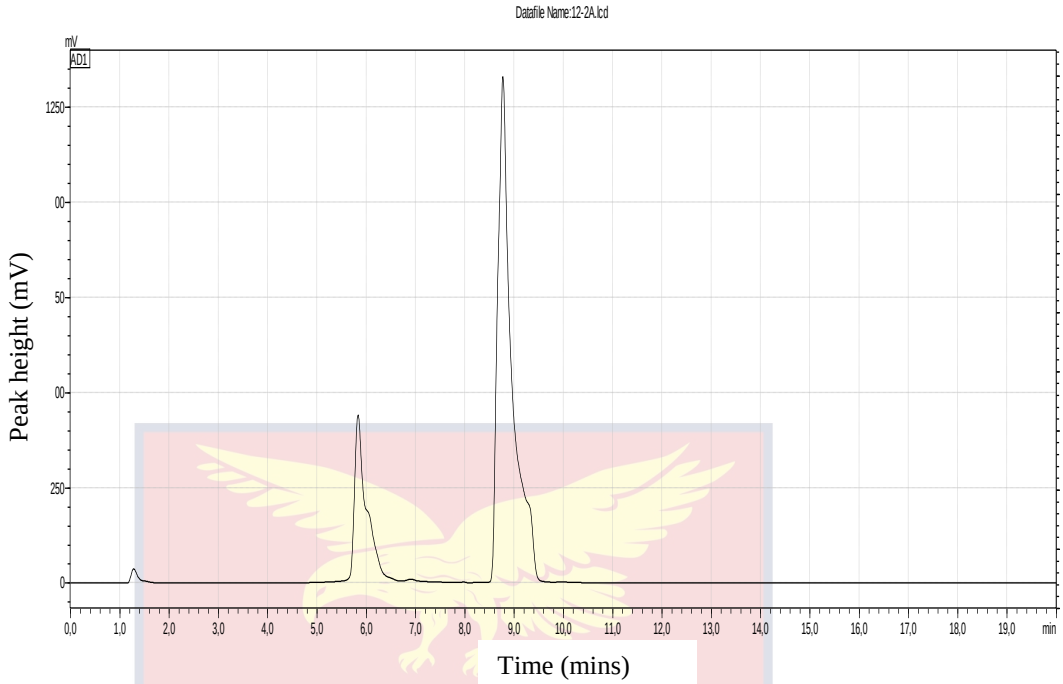
CHROMATOGRAMS OF MIXTURES USING DIFFERENT VOLUMES (10 µL, 20µL)



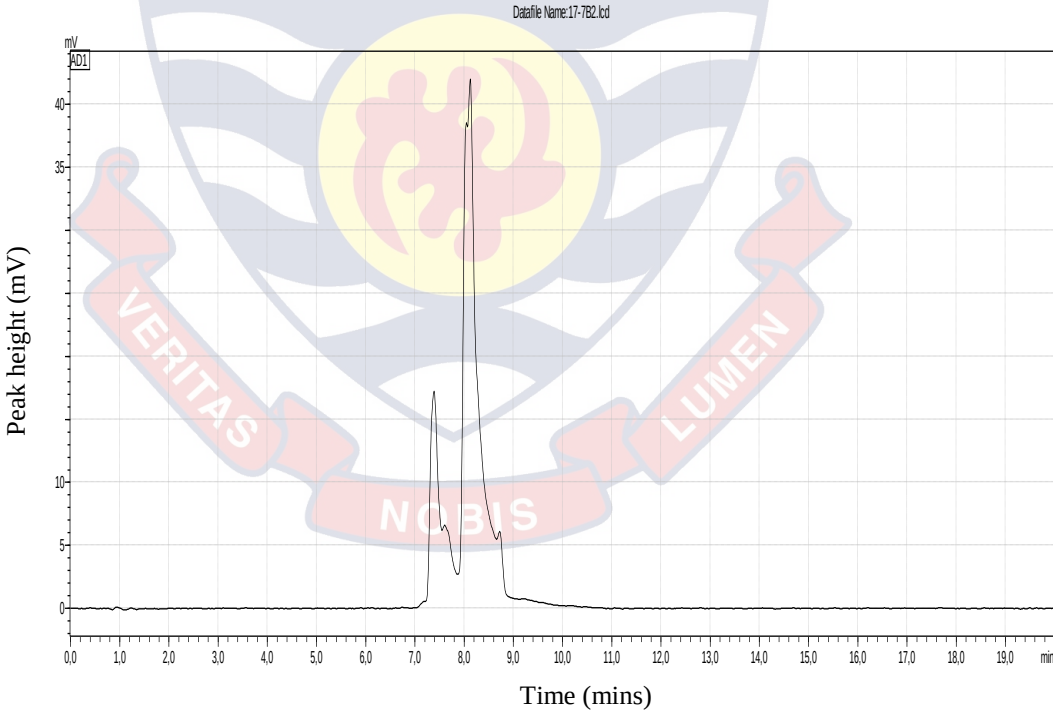
SARAFLOXACIN, ETHOXYQUIN (10 µL)



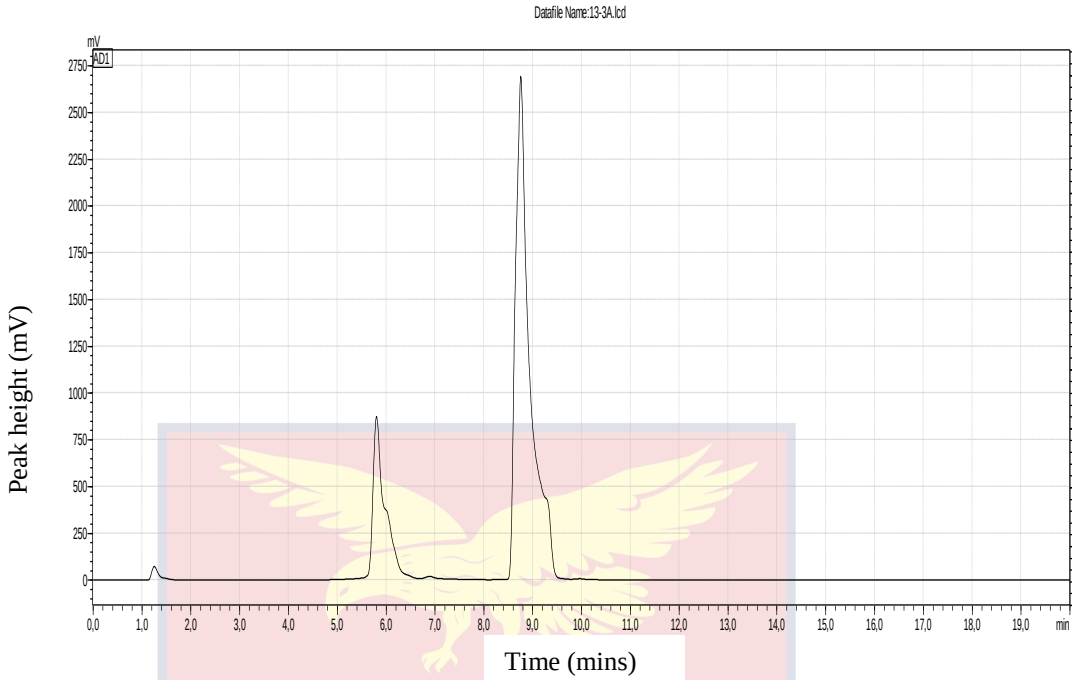
MALACHITE GREEN, CRYSTAL VIOLET (10 µL)



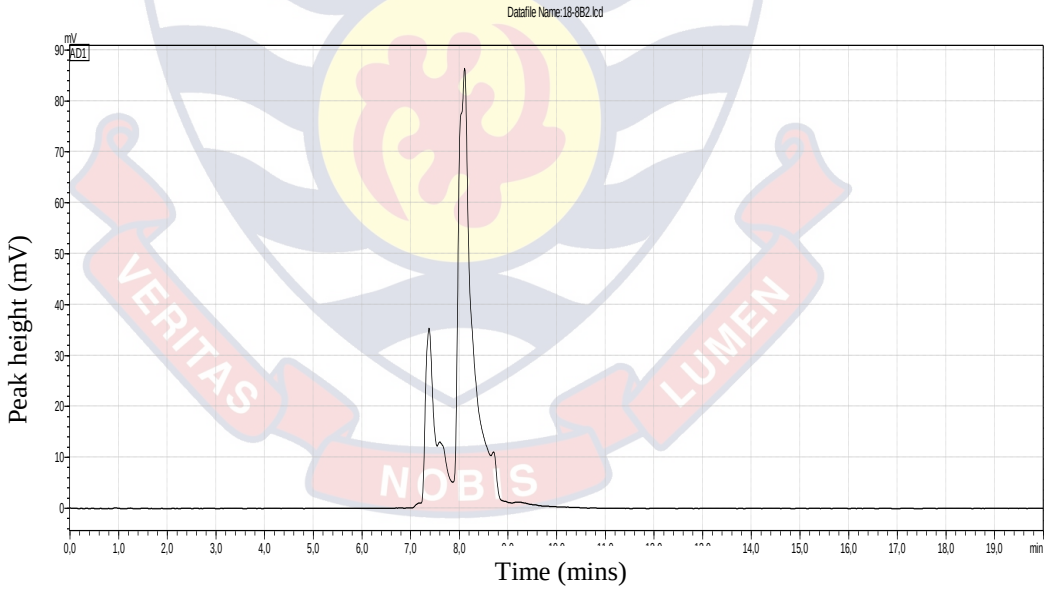
SARAFLOXACIN, ETHOXYQUIN (20 µL)



MALACHITE GREEN,CRYSTAL VIOLET (20 µL)



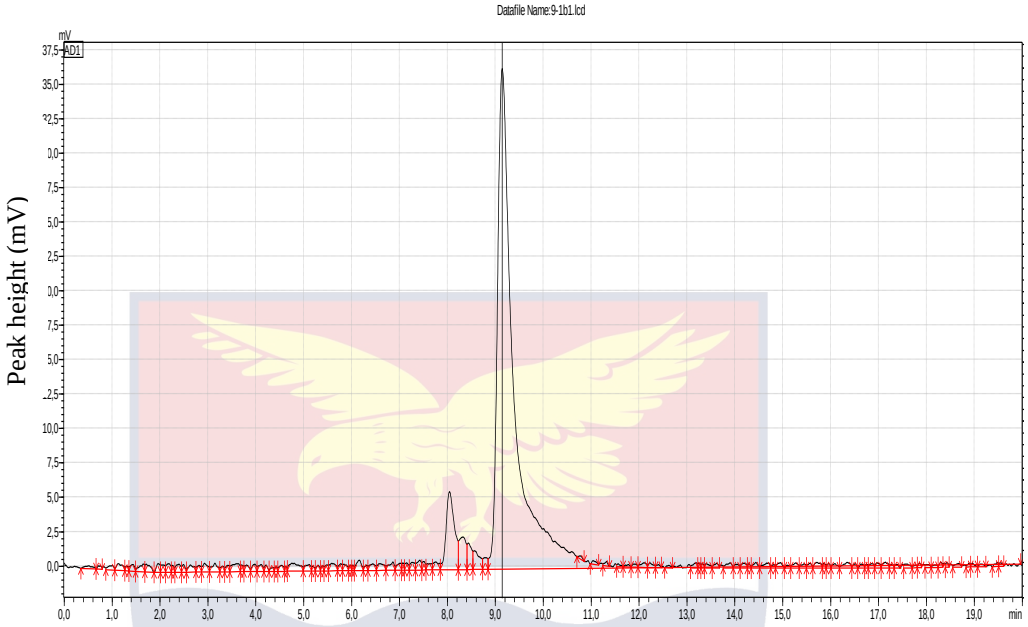
SARAFLOXACIN, ETHOXYQUIN (30 µL)



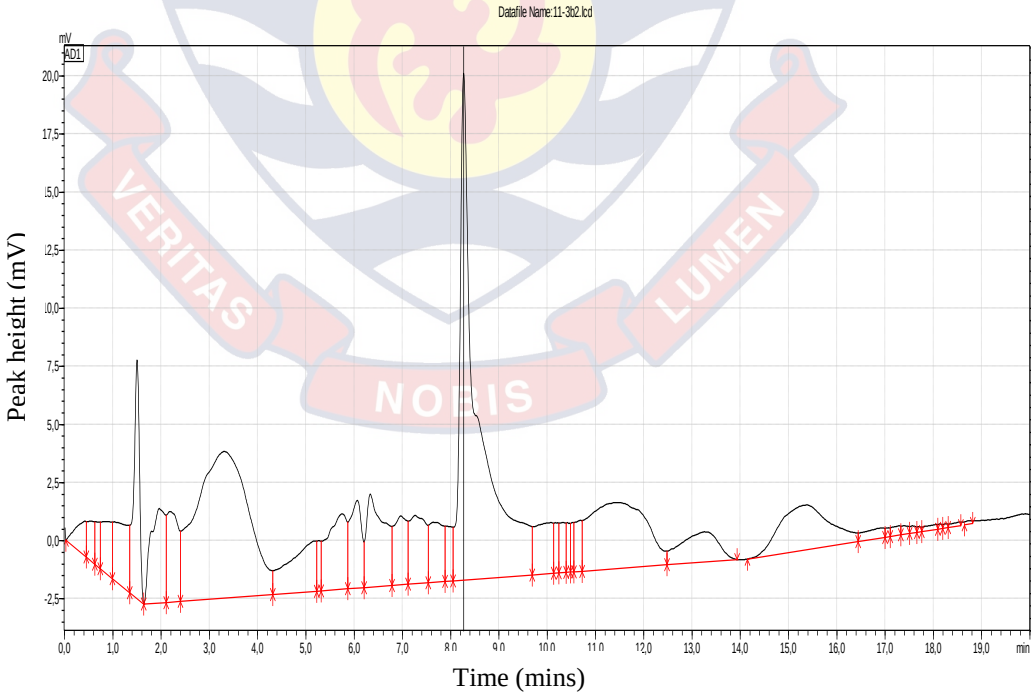
MALACHITE GREEN, CRYSTAL VIOLET (30 µL).

APPENDIX T

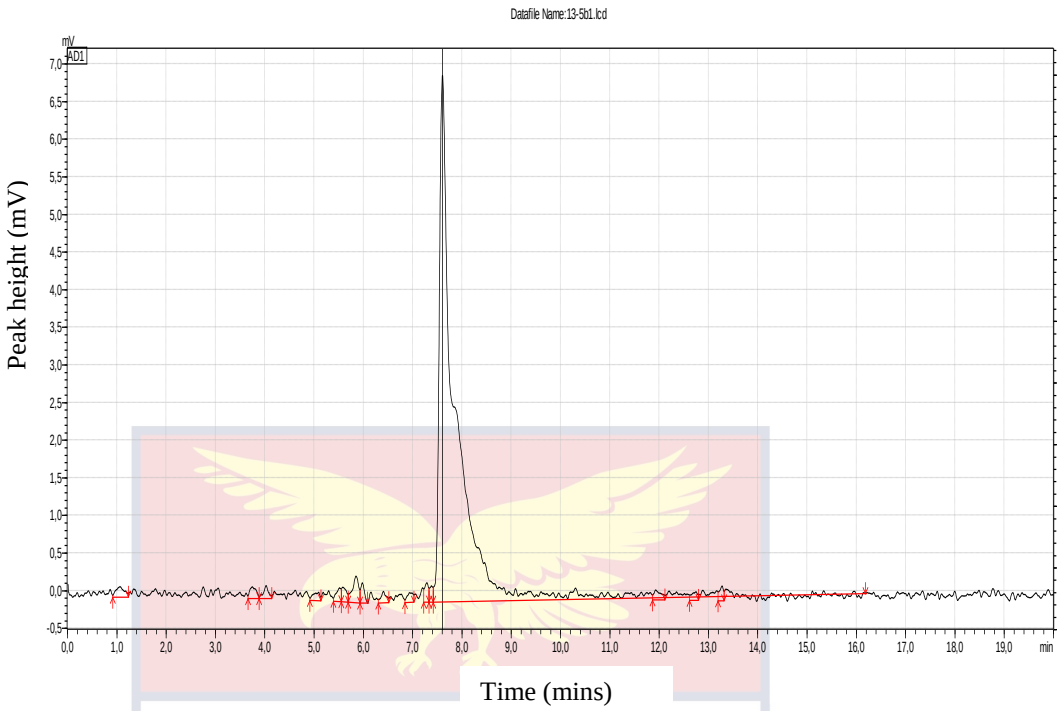
CHROMATOGRAMS WITH FLOW RATE OF 0.3 USING METHOD 14



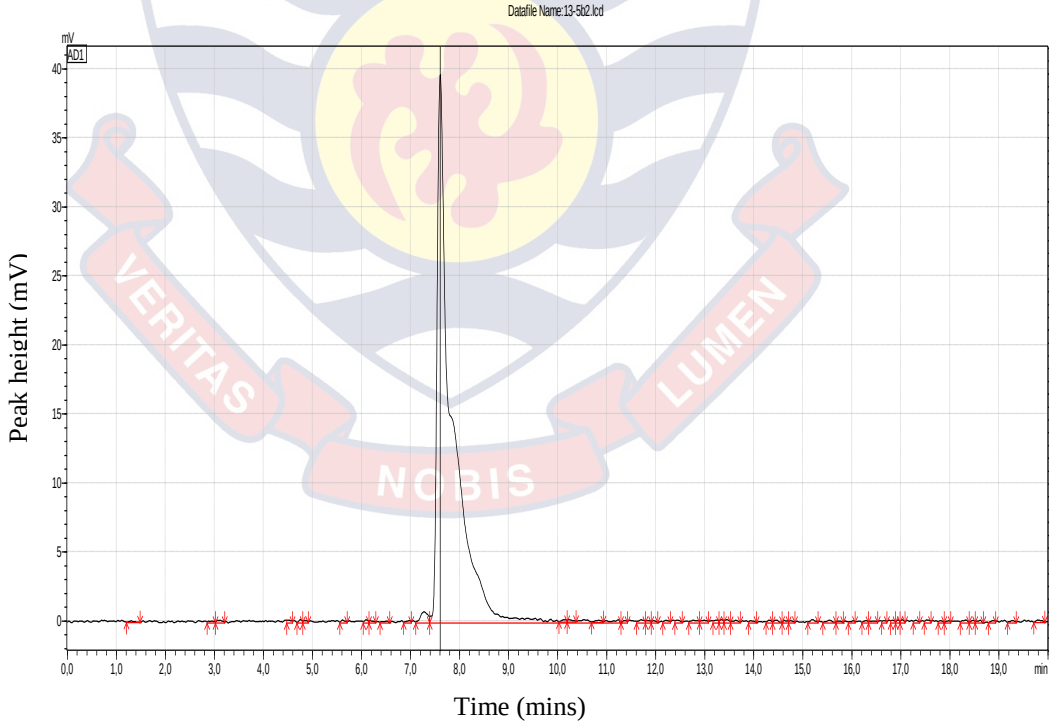
Crystal Violet



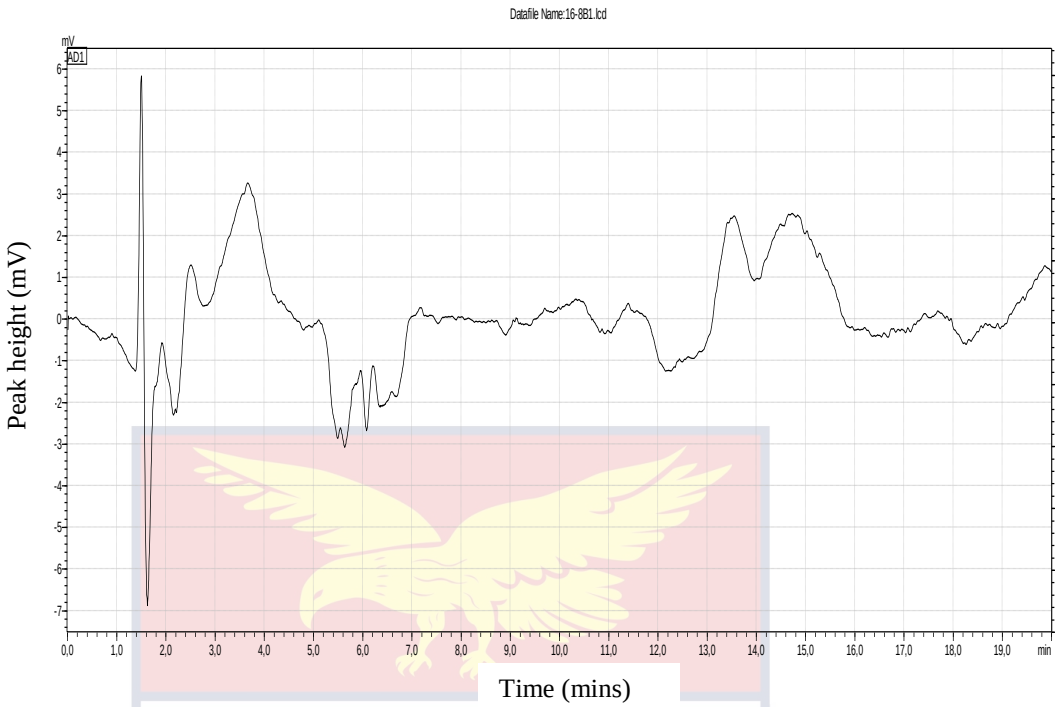
Diflubenzuron



Ethoxyquin



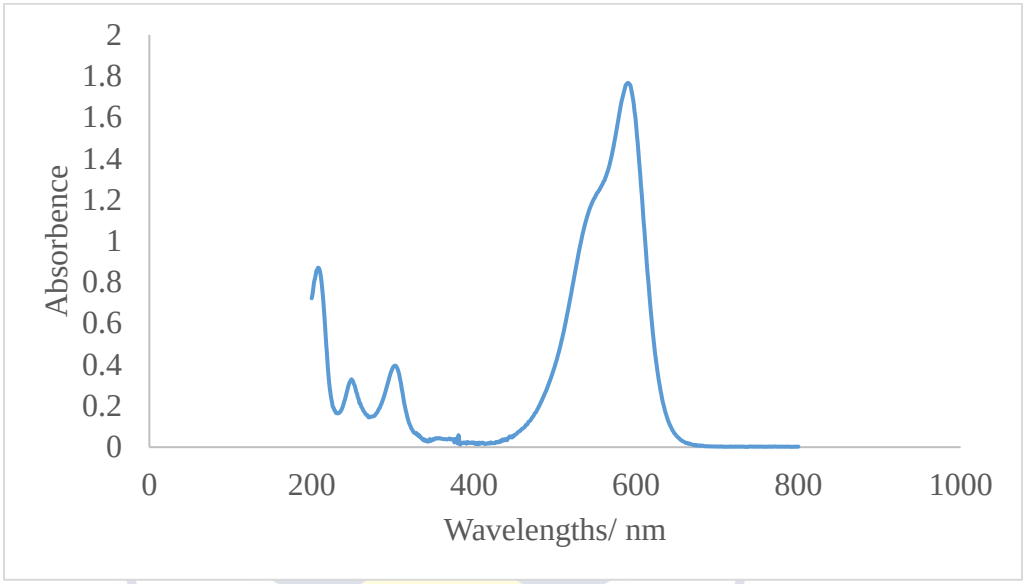
Malachite Green



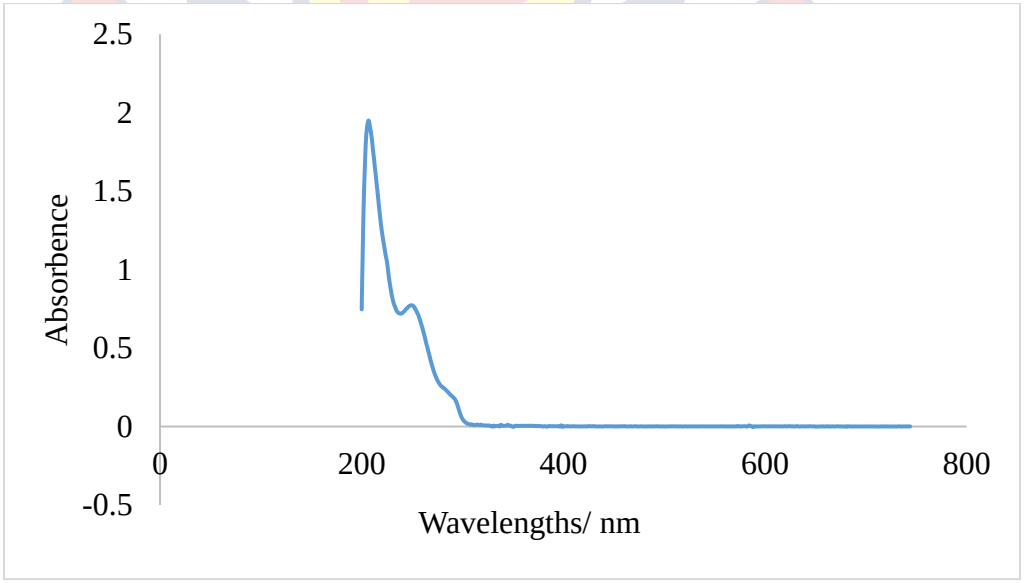
Teflubenzuron

APPENDIX U

ABSORPTION SPECTRA



Crystal Violet



Teflubenzuron

APPENDIX V

CONCENTRATION OF STANDARDS USED IN CALIBRATION

Concentration (ppm) Standards	0.01	0.05	0.07	0.1	0.5	1.5	3	8	10	15	20
						Peak area					
Sarafloxacin	2908	15542	20358	29084	125420	436262	872524	2326745	2908415	4362660	5816830
Ethoxyquin	7993	39969	55956	79938	399671	1199073	2398147	6395040	7993825	11990739	15987650
Diflubenzuron	135	650	948	1155	6755	20325	40651	108404	135505	203259	271010
Teflubenzuron	46.3	211	324	463	2315	6945	13890	37040	46300	69452	92600
Crystal violet	271	1356	1899	2713	13547	40702	81405	217080	271350	407027	542700
Malachite Green	181	909	1272	1818	9072	27276	54552	145472	181840	272762	363680

Source: Laboratory Analysis (2019)

APPENDIX W

PERCENTAGE RECOVERY OF RESULTS SPIKED 0.5 OF MRL, MRL, 2 MRLOF STANDARDS

	No. of runs							% Rec.
Analyte	1	2	3	4	5	Average	Standard	
Sara	107716	107724	107720	107718	107722	107720	109065	98.76
Etho	27905314	27905316	27905312	27905317	27905311	27905314	29976684	93.09
Diflu.	137115	137111	137113	137114	137112	137113	169381	80.95
Terf.	27189	27189	27189	27189	27189	27189	28937	93.96
Cry.	569	569	569	569	569	569	678.375	83.9
Mala	389	389	389	389	389	389	454.6	85.72

0.5 of MRL

Analyte	1	2	3	4	5	Average	Standards	% Rec.
Sara	215429	215423	215427	215427	215424	215426	218131	98.76
Ethox	55452088	55452088	55452084	55452089	55452086	5545208	59953686	92.49
Diflu	273821	273821	273821	273821	273821	273821	338762	80.83
Teflu	54379	54379	54379	54379	54379	54379	57875	93.25
Cry	1129.3	1129.2	1129.3	1129.1	1129.1	1129.2	1356.75	83.22
Mala	777.2	777.2	777.2	777.2	777.2	777.2	909.2	85.50

Source: Laboratory Analysis (2019)

Sara = Sarafloxacin, Etho. = Ethoxyquin, Diflu. = Diflubenzuron, Terf = Terflubenzuron

Cry. = Crystal violet, Mala. = Malachite green

MRL

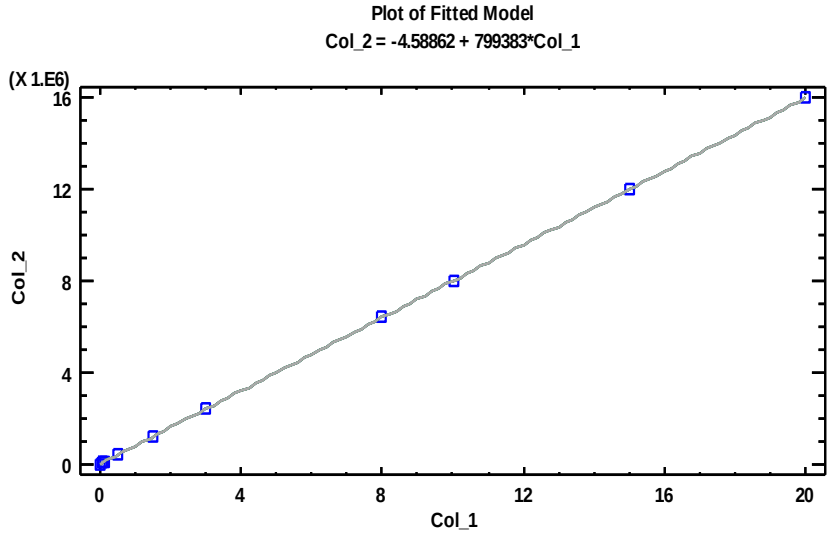
Analyte	1	2	3	4	5	Average	Standards	% Rec.
Sara.	430853	430853	430853	430853	430853	430853	436262	98.8
Ethox	111611 778	111611 776	1116117 72	1116117 75	1116117 76	1116117 73	1199073 75	93.1
Diflu.	547642	547642	547642	547642	547642	547642	677524	80.8
Teflu.	107936	107936	107936	107936	107936	107936	115750	93.3
Cry.	2258	2258	2258	2258	2258	2258	2714	83.2
Mala.	1544.6	1545.6	1545.6	1546	1545.5	1545.6	1818.4	85.0

Source: Laboratory Analysis (2019)

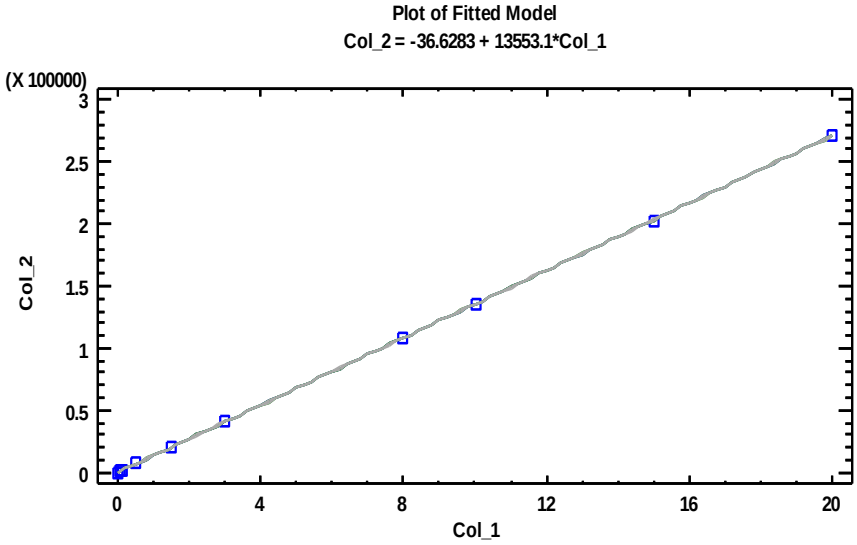
2 of MRL

APPENDIX X

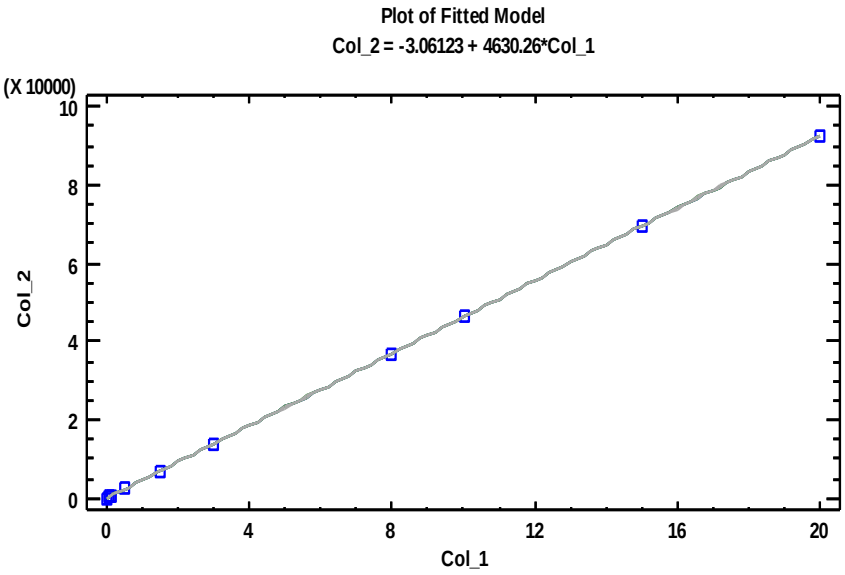
CORRELATION GRAPHS



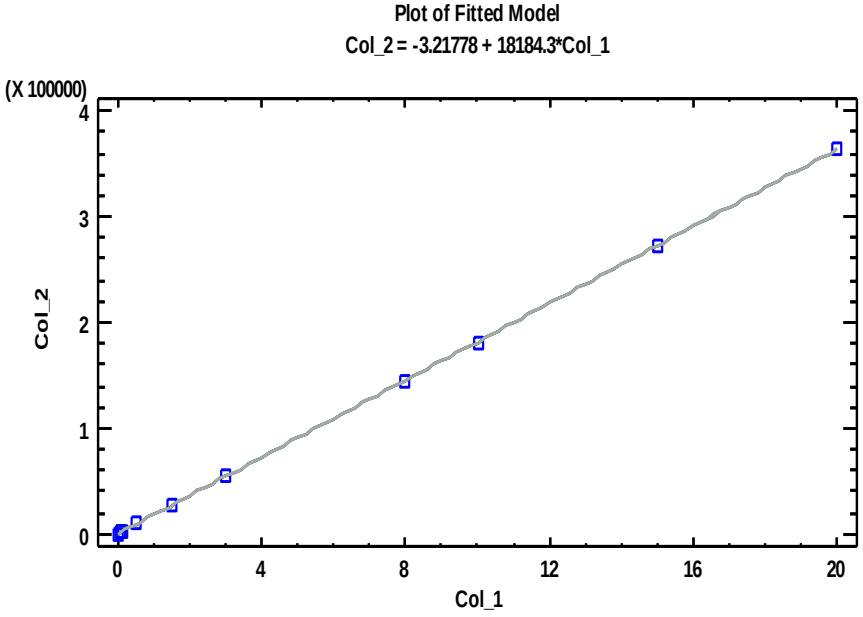
Ethoxyquin



Diflubenzuron



Teflubenzuron



Malachite Green

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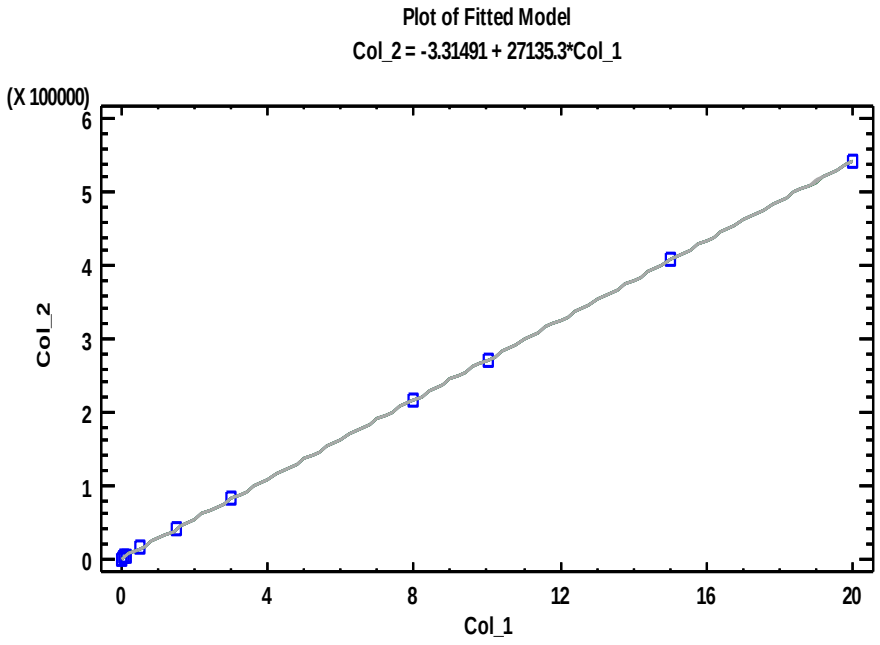


Crystal Violet

APPENDIX Y

AVERAGE RECOVERY STUDIES OF EXTRACT USING 0.5 ML, 1 ML OF ELUENT AT pH 2.5 AND pH 3

Standards	Conc.(ppm)	Experiment 1	Experiment 2	Experiment 3
Sarafloxacin	15	13.50	13.51	13.52
Ethoxyquin	15	2.13	2.14	2.15
Diflubenzuron	15	6.37	6.38	6.39
Teflubenzuron	15	4.75	4.76	4.77
Malachite green	15	5.55	5.56	5.57
Crystal violet	15	10.98	10.99	10.99

Source: Laboratory Analysis (2019)

Conditions pH 2.5, 0.5 mL Eluent

Standards	Conc.(ppm)	Experiment 1	Experiment 2	Experiment 3
Sarafloxacin	7.5	9.00	9.00	8.99
Ethoxyquin	7.5	7.00	6.99	7.00
Diflubenzuron	7.5	7.15	7.15	7.14
Teflubenzuron	7.5	7.38	7.37	7.38
Malachite green	7.5	7.35	7.34	7.35
Crystal violet	7.5	7.81	7.80	7.80

Source: Laboratory Analysis (2019)

Conditions pH 2.5, 1 mL Eluent

Average Recovery Studies of Extract Using 0.5 ml Eluents at Buffer pH 3.0

Standards	Conc.(ppm)	Experiment 1	Experiment 2	Experiment 3
Sarafloxacin	15	9.60	9.61	9.62
Ethoxyquin	15	4.98	4.99	4.99
Diflubenzuron	15	3.00	3.01	3.02
Teflubenzuron	15	4.34	4.35	4.36
Malachite green	15	11.25	11.26	11.27
Crystal violet	15	9.08	9.09	9.09

Source: Laboratory Analysis (2019)

Conditions pH 3, 0.5 mL Eluent

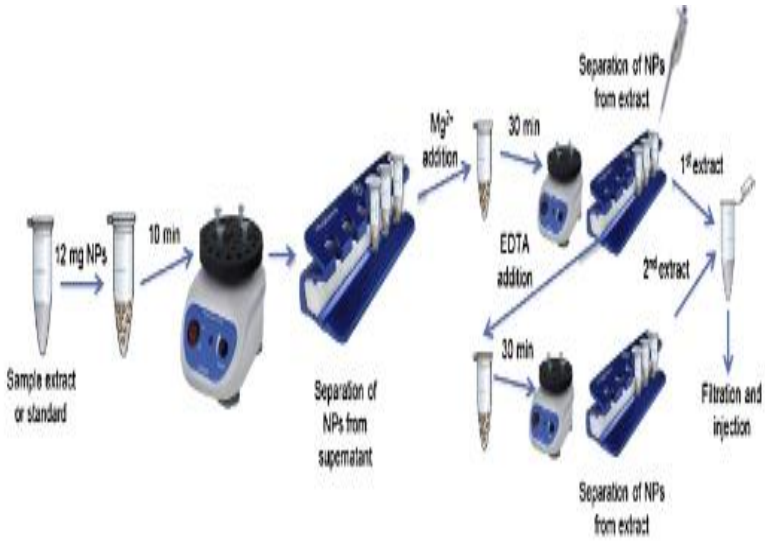
Standards	Conc.(ppm)	Experiment 1	Experiment 2	Experiment 3
Sarafloxacin	7.5	6.23	6.24	6.25
Ethoxyquin	7.5	4.67	4.68	4.69
Diflubenzuron	7.5	1.91	1.90	1.91
Teflubenzuron	7.5	3.23	3.24	3.23
Malachite green	7.5	6.43	6.44	6.43
Crystal violet	7.5	5.77	5.78	5.79

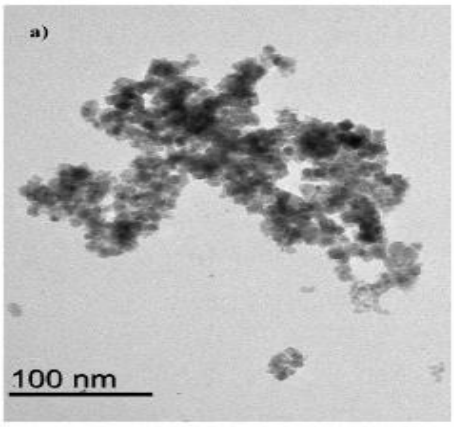
Source: Laboratory Analysis (2019)

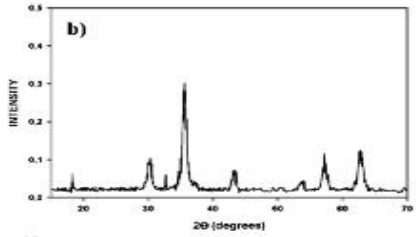
Conditions pH 3, 1 mL Eluent

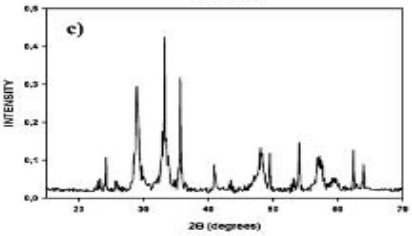
APPENDIX Z

.DIAGRAM OF DISPERSION SOLID PHASE EXTRACTION (DSPE) OF SARAFLOXACIN EDS AND TEM IMAGE OF MAGNETIC NANOMATERIAL









APPENDIX ZA

EXTRACTION OF SPIKED FISH USING EXTRACTION METHOD 1,2 AND 3

Standards	Conc.(ppm)	Experiment 1	Experiment 2	Experiment 3
Sarafloxacin	2	0.249	0.249	0.249
Ethoxyquin	2	0.065	0.064	0.066
Diflubenzuron	2	-	-	-
Teflubenzuron	2	0.286	0.286	0.286
Malachite green	2	0.408	0.408	0.408
Crystal violet	2	0.357	0.358	0.356

Source: Laboratory Analysis (2018)

METHOD 1

Standards	Conc.(ppm)	Experiment 1	Experiment 2	Experiment 3
Sarafloxacin	2	0.21	0.22	0.20
Ethoxyquin	2	0.707	0.709	0.705
Diflubenzuron	2	0.1	0.1	0.1
Teflubenzuron	2	0.2	0.2	0.2
Malachite green	2	0.706	0.706	0.706
Crystal violet	2	0.602	0.602	0.602

Source: Laboratory Analysis (2018)

METHOD 2

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Standards	Conc.(ppm)	Experiment 1	Experiment 2	Experiment 3
Sarafloxacin	2	1.99	1.99	1.99
Ethoxyquin	2	1.87	1.89	1.85
Diflubenzuron	2	1.61	1.61	1.61
Teflubenzuron	2	1.93	1.94	1.92
Malachite green	2	1.72	1.72	1.72
Crystal violet	2	1.64	1.65	1.63

Source: Laboratory Analysis (2018)

METHOD 3

APPENDIX ZB

CONCENTRATION AND PEAK AREAS USED FOR LODS, LOQS AND LINEARITY

Concentration (Sarafloxacin)	Peak areas
0.01	2908
0.05	15542
0.07	20358
0.1	29084
0.5	125420
1.5	436262
3.0	872524
8.0	2326745
10.0	2908415
15.0	4362660
20.0	5816830

Concentration (Ethoxyquin)	Peak areas
0.01	7993
0.05	39969
0.07	55956
0.1	79938
0.5	399671
1.5	1199073
3.0	2398147
8.0	6395040
10.0	7993825
15.0	11990739
20.0	15987650

Concentration (Diflubenzuron)	Peak areas
0.01	135
0.05	650
0.07	948
0.1	1155
0.5	6755
1.5	20325
3.0	40651
8.0	108404
10.0	135505
15.0	203259
20.0	271010

Source: Laboratory Analysis (2018)

Concentration (Teflubenzuron)	Peak areas
0.01	46.3
0.05	211
0.07	324
0.1	463
0.5	2315
1.5	6945
3.0	13890
8.0	37040
10.0	46300
15.0	69452
20.0	92600

Concentration (Malachite Green)	Peak areas
0.01	181
0.05	909
0.07	1272
0.1	1818
0.5	9072
1.5	27276
3.0	54552
8.0	145472
10.0	181840
15.0	272762
20.0	363680

Concentration (Crystal Violet)	Peak areas
0.01	271
0.05	1356
0.07	1899
0.1	2713
0.5	13547
1.5	40702
3.0	81405
8.0	217080
10.0	271350
15.0	407027
20.0	542700

Source: Laboratory Analysis (2018)

APPENDIX ZC

PEAK AREA OF REPLICATES OF INTER-DAY PRECISION ANALYSIS OF STANDARDS

Compounds	Sara	Etho	Diflu	Teflu	Cry	Mala
Day 1	Peak Area					
1	4362660	11990739	203259	69452	407027	272764
2	4362660	11990739	203259	69452	407027	272763
3	4362660	11990739	203259	69452	407026	272763

Compounds	Sara	Etho	Diflu	Teflu	Cry	Mala
Day 2	Peak Area					
1	4362658	11990739	203258	69451	407027	272762
2	4362658	11990739	203259	69452	407028	272762
3	4362658	11990740	203259	69452	407028	272762

Compounds	Sara	Etho	Diflu	Teflu	Cry	Mala
Day 3	Peak Area					
1	4362658	11990739	203258	69451	407027	272762
2	4362658	11990739	203259	69452	407027	272762
3	4362658	11990740	203259	69452	407028	272762

Compounds	Sara	Etho	Diflu	Teflu	Cry	Mala
Day 4	Peak Area					
1	4362658	11990739	203259	69452	407027	272762
2	4362658	11990739	203259	69452	407027	272761
3	4362658	11990740	203258	69453	407027	272762

Source: Laboratory Analysis (2019)

Compounds	Sara	Etho	Diflu	Teflu	Cry	Mala
Day 5	Peak Area					
1	4362658	11990739	203259	69452	407027	272762
2	4362658	11990739	203259	69452	407027	272761
3	4362658	11990740	203258	69453	407027	272762

Source: Laboratory Analysis (2019)

APPENDIX ZD

PEAK AREA OF REPLICATES OF INTRA-DAY PRECISION ANALYSIS OF STANDARDS

Average Concentration (Sarafloxacin)	Peak Area
15	4362660
15	4362662
15	4362658
15	4362660
15	4362661
15	4362661
15	4362660
15	4362660
15	4362660
15	4362660

Average Concentration (Ethoxyquin)	Peak Area
15	11990740
15	11990739
15	11990739
15	11990739
15	11990739
15	11990740
15	11990739
15	11990739
15	11990739
15	11990739

Average Concentration (Diflubenzuron)	Peak Area
15	203257
15	203259
15	203259
15	203258
15	203259
15	203259
15	203259
15	203259
15	203259
15	203259

Average Concentration (Tflubenzuron)	Peak Area
15	69453
15	69452
15	69452
15	69451
15	69452
15	69452
15	69452
15	69452
15	69452
15	69452

Crystal Violet

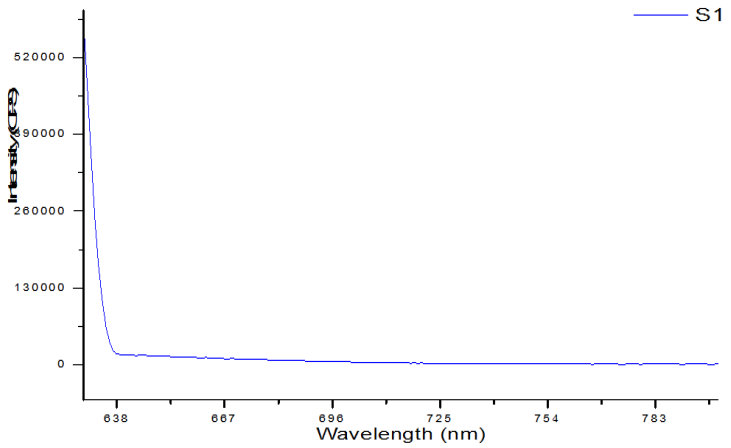
Average Concentration (Crystal Violet)	Peak Area
15	407027
15	407027
15	407027
15	407027
15	407027
15	407027
15	407027
15	407027
15	407027
15	407027

Average Concentration (Malachite Green)	Peak Area
15	272762
15	272762
15	272762
15	272762
15	272762
15	272762
15	272762
15	272762
15	272762
15	272762

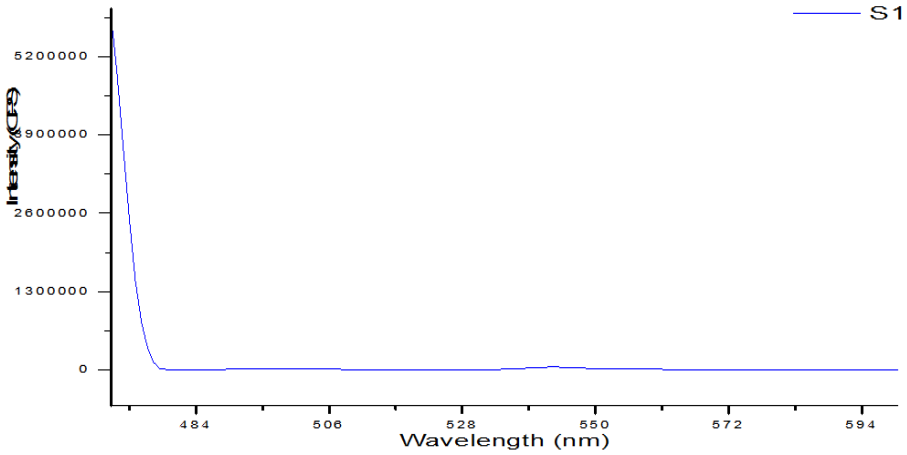
Source: Laboratory Analysis (2019)

APPENDIX ZE

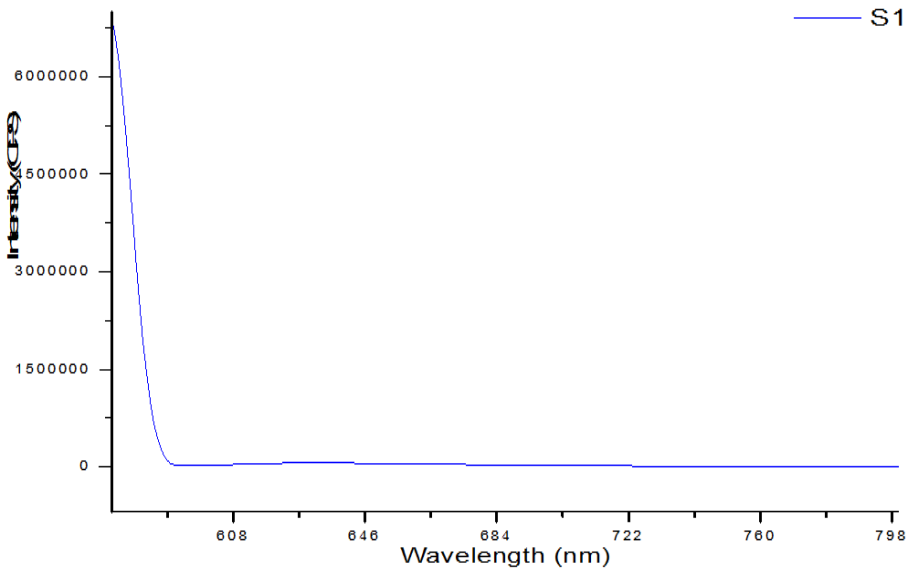
FLOURESCENE EMISSION OF STANDARDS



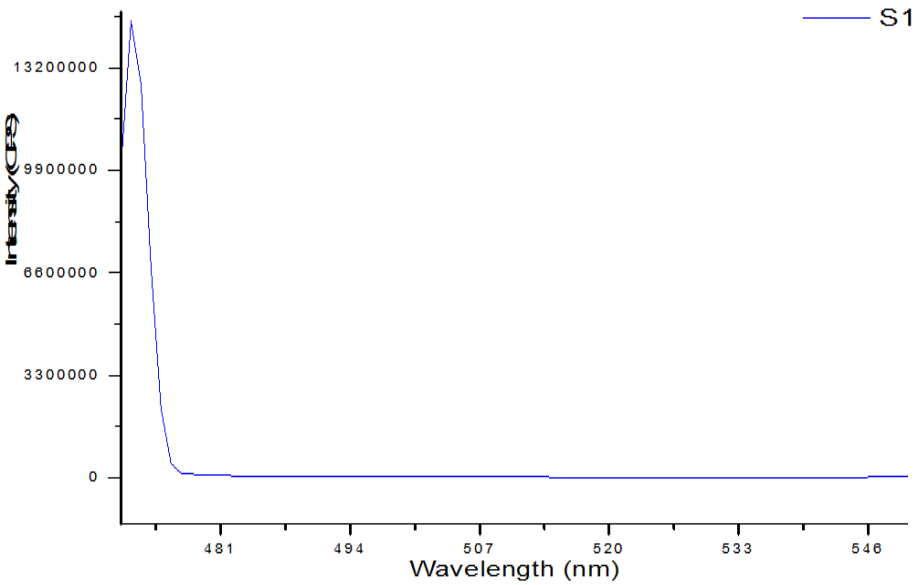
Crystal violet



Diflubezuron



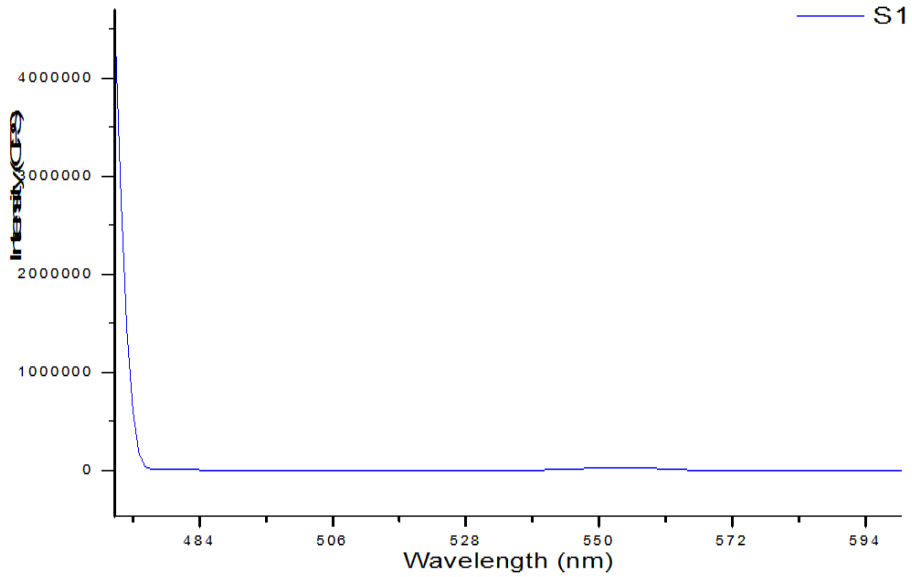
Malachite Green



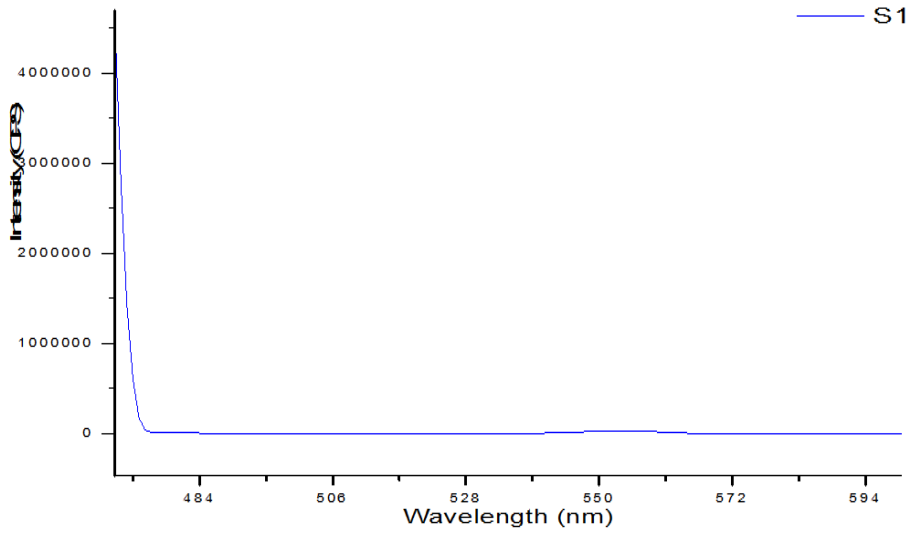
Teflubenzuron

APPENDIX ZF

EXCITATING THE BLANKS (SOLVENT) WITH THE EXCITATION WAVELENGTHS OF ETHOXYQUIN AND SARAFLOXACIN



Sarafloxacin



Ethoxyquin