Analysis of antibiotics and PFAS as potential emerging pollutants in the water bodies and their accumulation in food samples

By

Ntshani Gershom Mpela

(16022661)

A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Science in Chemistry Department of Chemistry School of Mathematics and Natural sciences Thohoyandou, Limpopo province South Africa

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Declaration

I, **Ntshani Gershom Mpela** (16022661), hereby declare that this research report entitled 'Analysis of antibiotics and PFAS as potential emerging pollutants in food and environmental samples' submitted by me to the Department of Chemistry, University of Venda, has not been submitted for examination at this or any other tertiary institution. This is my own work and design. The information derived from the literature has been duly acknowledged in text and list of references provided.

---------- 24/ 06/ 2021

Signature Date

List of publications

I. **Innovative liquid phase extraction based analytical extraction techniques of antibiotics as emerging pollutants from different water sources.**

Gershom Mpela Ntshani, Nikita Tawanda Tavengwa

Book chapter submitted to Emerging Freshwater Pollutants

II. **Dispersive liquid-liquid micro-extraction of perfluoroalkyl substances as emerging contaminants.**

Gershom Mpela Ntshani, Nikita Tawanda Tavengwa

Manuscript in preparation to be submitted to Trends in Analytical Chemistry

III. **Dispersive liquid-liquid micro-extraction of multi-class antibiotics in macadamia nuts prior to liquid chromatography coupled to quadrupole time-of-flight mass spectroscopy.**

Gershom Mpela Ntshani, Nikita Tawanda Tavengwa

Manuscript in preparation to be submitted to Chromatographia

IV. **Liquid-liquid micro-extraction of multiclass antibiotics using deep eutectic in green beans prior to liquid chromatography coupled quadrupole mass spectrometer analysis.**

Gershom Mpela Ntshani, Nikita Tawanda Tavengwa

Manuscript in preparation to be submitted to Journal of Pharmaceutical and Biomedical Analysis

V. **Dispersive liquid-liquid micro-extraction of PFAS in food packaging materials prior to liquid chromatography couples to quadrupole time-of-flight mass spectroscopy analysis.**

Gershom Mpela Ntshani, Nikita Tawanda Tavengwa

Manuscript in preparation to be submitted to Journal of Chromatography A

Contribution of the author

Paper I Principal author was involved in planning and drafting of the paper. Co-author was involved in supervision and review of the draft.

Paper II: Principal author was involved in planning and drafting of the paper. Co-author was involved in supervision and review of the draft.

Paper III: Principal author was involved in planning, sample preparation, pre-concentration and LC-MS/MS analysis, evaluation of results and writing of a paper. Co-author revised the manuscript and suggested improvement.

Paper IV: Principal author was involved in planning, preparation and characterisation of deep eutectic solvent, sample preparation, pre-concentration and LC-MS/MS analysis, evaluation of results and writing of a paper. Co-author revised the manuscript and suggested improvement.

Paper V: Principal author was involved in planning, sample preparation, pre-concentration and LC-MS/MS analysis, evaluation of results and writing of article. Co-author revised the manuscript and suggested improvement.

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Abstract

Antibiotics and perfluoroalkyl substances (PFAS) are groups of anthropogenic substances that are found in industries and consumer products, i.e., antibiotics are used in healthcare facilities, pharmaceutical companies, and agriculture, whereas PFAS are used in industries and households. These compounds find their way into the environment through emission, landfill disposal, agricultural run-offs, irrigations, leaching and wastewater spillage. Their residues are widely detected in almost every environmental compartment. Their detection in drinking water and food is one of the global concerns. Their effect in living organisms have been widely documented ranging from allergic reaction to carcinogenic effect, and in serious situations they can lead to death. In this project, the background review on the behaviour of antibiotics and PFAS in different environments are discussed in chapter two. This has been done by looking into their physico-chemical properties, distribution pathways, accumulation and toxicity in various environmental compartments through available literature. After understanding their behaviour in the environment two review papers, namely **Paper I** and **Paper II,** were written. **Paper I** was a book chapter which focused on the application of miniaturised liquid phase extraction techniques used for extraction of antibiotics in environmental water samples. In this book chapter, the use of liquid-liquid extraction techniques was discussed and the evolution of liquid-liquid extraction technique into miniaturised extraction technique was reviewed. **Paper II** was a critical review which focuses on the application of dispersive liquid-liquid micro-extraction of PFAS in different environmental samples. In this paper, the principles of dispersive liquid-liquid micro-extraction was explained. Later, its application for pre-concentration of PFAS was reviewed and the future trends were discussed. In chapter four, three lab-based papers, namely **Paper III**, **Paper IV** and **Paper V**, were written. In **Paper III,** for the first time a dispersive liquid-liquid microextraction technique was developed and applied for extraction of multi-class antibiotics in macadamia nuts prior to UHPLC-qToF-MS analysis. **In paper IV**, a novel deep eutectic solvent was synthesised and characterised using FTIR spectroscopy. For the first time the synthesised deep eutectic solvent was applied for extraction of multiclass antibiotics in green beans prior to UHPLC-qToF-MS analysis. In **Paper V**, for the first a dispersive liquid-liquid micro-extraction technique was developed and applied for extraction of PFAS in food contact materials prior to UHPLC-qToF-MS analysis. Owing to their ease of operation, efficiency and sensitivity, these techniques can also be applied for preconcentration of antibiotics and PFAS in different food, environmental and biological samples.

Keywords: Antibiotics, PFAS, extraction techniques, miniaturisation, chromatography, mass spectrometry, emerging pollutants

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List of abbreviations

AFFF	Aqueous film forming form
CE	Capillary electrophoresis
DAD	Diode array detector
DES	Deep eutectic solvent
DLLME	Dispersive liquid-liquid micro-extraction
DOC	Dissolved organic content
DSPE	Dispersive solid phase extraction
EF	Enrichment factor
GC	Gas chromatography
HFLPE	Hollow fiber liquid phase extraction
HLB	Hydrophilic lipophilic balance
HPLC	High performance liquid chromatography
IL	Ionic liquid
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid-liquid extraction
LLME	Liquid-liquid microextraction
LOD	Limits of detection
LOQ	limits of quantification
MRL	Maximum residue limit
MRM	Multiple reaction monitoring
MSPE	Magnetic solid phase extraction

PFAA	Perfluoroalkyl acid
PFAS	Perfluoroalkyl substance
QToF-MS	Quadrupole time of flight mass spectrometry
QuEChERS	Quick, easy, cheap, effective, rugged, and safe
SPE	Solid phase extraction
SPME	Solid phase micro-extraction
SUPRAS	Super molecular solvents
UHPLC	Ultra performance liquid chromatography
UV-Vis	Ultra-violence visible spectroscopy
WWTP	Wastewater treatment plants

Chapter one: Introduction

This chapter outlines the background of the study, problem statement, aims and objectives.

1.1. Background

Antibiotics and perfluoroalkyl substances (PFAS) are groups of anthropogenic organic compounds classified as emerging pollutants (Hoover et al., 2019). Referring to the definition of emerging pollutants, it means antibiotics and PFAS are not routinely monitored but they have the potential to be released into the environment and cause severe effects on human and environmental health (Geissen et al., 2015; Gogoi et al., 2018). These compounds are ubiquitous and have a long range of transportation (Zhao et al., 2020; MacInnis et al., 2019). Their source to the environment may include industrial and domestic emissions. For example, residues of antibiotics can be released from pharmaceutical companies, agriculture practices and healthcare practices (Chow et al., 2021), while PFAS may be released from metal plating industries, oil refining industries, electrical appliances industries, pulp and paper industries, aqueous film forming forms and households' detergents, and furniture (Meegoda et al., 2020). Once the antibiotic and PFAS residues are released into the environment, they are subjected to both biotic and abiotic compartments (Rodil et al., 2019; Joerss et al., 2019). Their distribution to various environmental compartments is through leaching, agricultural run-offs, and air particulates (Schulz et al., 2020). This distribution results in antibiotic and PFAS residues present in surface water, ground water and wastewater treatment plants. Wastewater treatment plants have been identified to be one of the point sources of antibiotics and PFAS (Rodriguez-Mozaz et al., 2020; Voigt et al., 2020; Yu et al., 2020).

Improper handling and misuse are some of the factors that increase the concentration of antibiotics and PFAS in the environment (Wang et al., 2019). Other factors contributing to the high concentration of antibiotics and PFAS is lack of proper sanitation and poor wastewater treatment plants (Kyriienko et al., 2018). Conventional wastewater treatment plants are not specifically designed for removal of antibiotics and PFAS; hence their residues are widely detected in wastewater effluents, wastewater slug and aquatic organisms (Dharupaneedi et al., 2019). Antibiotics and PFAS are bio-accumulative, and their accumulated residues found in sediments and aquatic organisms can lead to biomagnification through the food web (Liu et al 2017; Simonnet-Laprade et al., 2019). Higher concentrations of antibiotics and PFAS in wastewater effluents and slug can also result in their accumulation to the agricultural farmlands through irrigation. This is because wastewater effluents and slugs are used in farmlands to fertilise the soil. Inhalation, ingestion and dermal absorption of antibiotics and PFAS by living organisms have been associated with serious health threats which signify the importance of

guidelines and management strategies (Dharupaneedi et al., 2019). The only way to ensure water and food quality is through routing monitoring using analytical techniques.

Recently, sophisticated analytical instruments such as liquid chromatography coupled to UV or tandem mass spectrometers are routinely used for analysis of organic contaminants in environmental, biological and food samples (Agadellis et al., 2020; Müller et al., 2020; Bellouard et al., 2020). The use of high-resolution mass spectrometer such as quadrupole time-of-flight mass spectrometer (QToF-MS) have gained a lot of attention in the scientific community due to their robustness, sensitivity and accuracy in analysis (Alygizakis et al., 2019; Andra et al., 2017). These instruments have the advantage of combining the ultra-high performance liquid chromatography and multiple reaction monitoring (MRM) in mass spectrometers which can easily isolate the desired analytes from other compounds, even isomers (Kuo et al., 2020; Salvador et al., 2020). For example, structural isomers are easily separated in LC column due to their different interaction with the stationary phase, while compounds with similar mass are filtered using the MRM in the QToF-MS.

Effective as these instruments are, direct analysis of substances in complex matrices is difficult, especially when the analytes exist in trace concentration. To resolve this problem, analysis is accompanied by pre-concentration techniques. These techniques are performed before instrumental analysis to isolate the analytes from other contaminants and enrich them so that they can be detected by the analytical instruments. Traditionally, solid phase extraction (SPE) and liquid phase extraction (LPS) are routinely used for extraction and pre-concentration of analytes in complex matrices. Classical SPE and LLE (using ion pair reagents) are some of the most readily used extraction and pre-concentration techniques in PFAS (Kaiser et al., 2020; Zhang et al., 2020; Miaz et al., (2020). However, classical extraction techniques such as SPE and LLE are criticised based on its failure to comply with the principles of green analytical chemistry (Ma et al., 2021). Miniaturised-SPE and miniaturised-LLE are modern extraction techniques that have been developed to resolve the setbacks encountered in the classical extraction techniques (Moyo et al., 2018; Daniels et al., 2020). Some of these techniques have been applied in extraction and pre-concentration of antibiotics (Zhao et al., 2020; Wang et al., 2020; Mohebi et al., 2020; Khatibi et al., 2020). In contrast, their application in PFAS is limited, therefore, more study is still needed.

1.2 Problem statement

Lack of sanitation in most developing countries is a serious concern since most people and farmers rely on surface water from rivers and dams for drinking and vegetation growing. It is difficult to implement regulation and safety guidelines without knowing the point sources. The qualitative and quantitative results of these contaminants in different environmental compartments are scarce. Recently, there are many analytical instruments with the capability to perform multi analysis of antibiotics and PFAS in trace amounts. However, Direct analysis of compounds that exist in trace levels is challenging. The complex nature of environmental samples is associated with false positive and false negative results. These matrices comprise of a wide range of organic, inorganic and biological compounds which might cause ion depression or ion enhancement during analysis. Thus, it is recommended to isolate the desired analytes as much as possible from the rest through extraction techniques. The problem is that most extraction techniques that are routinely used for environmental monitoring are less effective, time consuming, expensive, inaccurate and not environmentally friendly. Another setback is that most traditional techniques are only limited for one analyte or single class of compounds. There are many hazardous compounds that are released into the environment daily. Thus, using these techniques to analyse compound by compound or class by class is time consuming, expansive and environmentally unfriendly.

The aim of this project is to develop an innovative extraction technique for extraction and pre-concentration of multiple residues of antibiotics (sulphonamides, tetracyclines and fluoroquinolons) and of PFAS (PFCA and PFSA) in food samples and food contact material prior quadrupole time of flight liquid chromatography mass spectrometry analysis. These analytes were chosen based on their reported toxicity, wide usage and limited information of their occurrence especially in rural areas. The developed extraction techniques will improve some of the setbacks associated with conventional extraction techniques, and they will be applied for the assessment of antibiotics and PFAS in food samples and food contact materials. The assessment of contaminants will help in increasing the data and knowledge on the distribution and concentration of antibiotics and PFAS in the water and food, development of the regulation and management techniques on these contaminants, strengthening water and food security and ensuring a fair trade of good quality product to other countries.

1.3 Aims and objectives

1.3.1 Aims

- To develop DLLME and LLME extraction techniques for pre-concentration of antibiotics in food samples and food contact material prior to LC-MS/MS analysis.
- To develop DLLME extraction for pre-concentration of PFAS in food samples prior to LC-MS/MS analysis

1.3.2 Specific objectives

1.3.2.1 Extraction of antibiotics by DLLME

- Optimisation of the method by looking at factors such as effect of sample pH, effect of extraction solvent, effect of dispersive solvent, effect of volume of extraction solvent, effect of ionic strength and effect of vortex time which affect the enrichment factors.
- Validate the method by looking at the linearity of the method, LOD and LOQ, enrichment factors and the producibility of the method.
- Application of the developed method in macadamia nuts to evaluate its applicability.

1.3.2.2 Extraction of antibiotics by LLME

- Preparation deep eutectic solvent used as extraction solvent and characterisation using FTIR.
- Optimisation of the method by looking at factors such as effect of sample pH, effect of ionic strength, effect of type of DES, effect of ratio of DES, effect of volume of DES and effect of vortex time which affect the enrichment factors.
- Validation the method by looking at the linearity of the method, LOD and LOQ, enrichment factors and the producibility of the method.
- Applications of the developed method in green beans to evaluate its applicability.

1.3.2.3 Extraction of PFAS by DLLME

- Optimisation of the method by looking at factors such as effect of sample pH, effect of extraction solvent, effect of volume of extraction solvent, effect of dispersive solvent, effect of volume of dispersive solvent, effect of ionic strength and effect of manual shaking time which affect the enrichment factors.

- Validate the method by looking at the linearity of the method, LOD and LOQ, enrichment factors and the producibility of the method.
- Application of the developed method in food contact material to evaluate its applicability.

Chapter two: Literature review

Antibiotics and perfluoroalkyl substances have been reviewed looking at their physico-chemical properties, distribution, accumulation and their effect in the environment and human health. Chromatographic techniques that are used in analysis of organic compounds have been reviewed and discussed.

2.1 Emerging pollutants and their environmental behaviour

Emerging pollutants are substances that are not routinely monitored but have a potential to enter the environment and cause severe effects to human health and the environment (Gogoi et al., 2018; Hoover et al., 2019). This includes a group of persistent, toxic and high accumulative chemicals that are released into the environment through industrial emissions, agricultural run-offs, landfill leachate and wastewater treatment plants (Dharupaneedi et al., 2019; (Rasheed et al., 2019). Routine analyses are primarily focused on metal ions, and there are few regulations on organic compounds (Polyakova et al., 2018). This section discusses the characteristics and environmental behaviour of two groups of organic emerging pollutants, namely antibiotics and perfluoroalkyl substances.

2.1.1 Antibiotics

2.1.1.1 Background

Antibiotics are a group of antimicrobials classified into natural (have been discovered in nature), semi-synthetic (derived from the natural antibiotics) and synthetic compounds (Bena et la., 2019; Idowu et al., 2017). Their main purpose is to kill or inhibit the growth and production of bacteria and fungi; hence they are used for treatment of infectious diseases (Manyi-Loh et al., 2018). They are less effective against other infections such as archaea, protozoa, microalgae and viruses. Antibiotics are also used in agriculture for treatment, improving animal production and protecting animals from diseases (Wu et al., 2016; Li et al., 2020; Zhao et al., 2020). Studies have shown that approximately 50% and 36% of antibiotics have been used in agriculture and human's treatment, respectively, by the year 2000 to 2010 (Danner et al., 2019). While antibiotics are very effective in human treatment and agricultural practices, their presence in the environment is associated with severe health effects in humans and the environment. This is because antibiotics have the capability to induce selective pressure that will result in development of bacterial resistance genes (Osińska et al., 2020; Liu et al., 2020; García et al., 2020). These antibiotic resistance genes compromise the effectiveness of the already used antibiotics in treatment, and the ecological processes. Ingestion of antibiotics is also associated with severe health effects such as carcinogen effect, drug hypersensitivity, mutagenic effects and disruption of normal intestinal flora (Sun et al., 2020; Li et al., 2020).

Antibiotic residues in the environment have different half-life that range from a few hours to hundred days (Wang et al., 2019); Bena et al., 2019). Their degradation processes depend on the concentration discharged, solubility in water, the environmental conditions such as

temperature and pH and the environmental compositions such as the total organic matter. For example, antibiotics have been found to exist in higher concentrations in winter compared to summer (Wang et al., 2019). This could be the fact that winter is the season where most people are affected by many diseases or lower degradation due to lower temperature. Antibiotics such as quinolones, sulphonamides and diaminopyridine, are more persistent in soil while others such as macrolides, fluoroquinolones and tetracycline are more persistent in almost all environmental compartments.

2.1.1.2 Physico-chemical properties

Antibiotics are divided into groups based on their chemical structures (Table 2.1) and their effect inside the microbial cell (Figure 2.1). The physico-chemical properties of any compound determine its distribution and accumulation behaviour, and its fate in the environment. Below, only three classes of antibiotics (fluoroquinolones, sulfonamides and tetracyclines) have been discussed since they are the main classes that will be studied in this project.

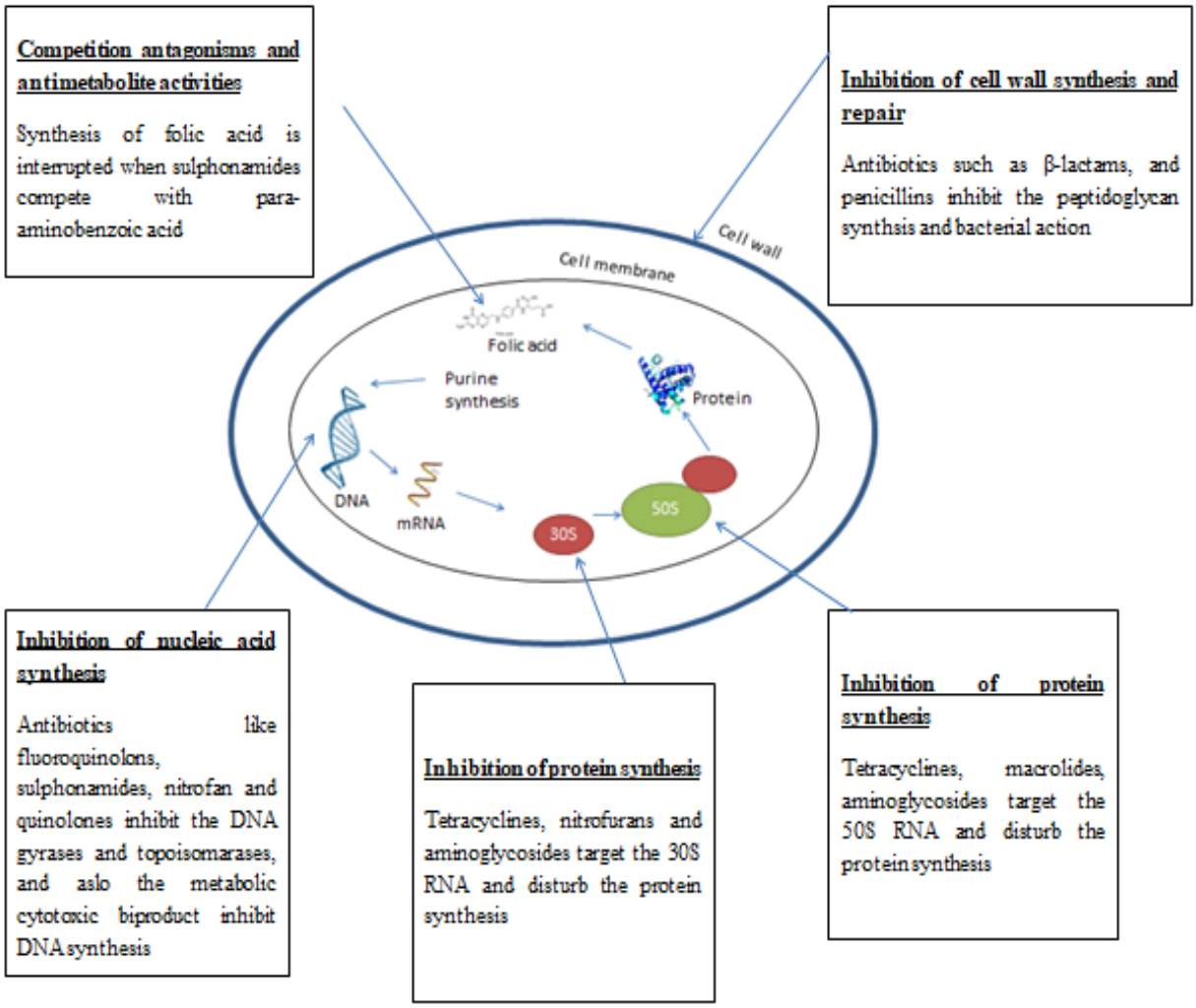


Figure 2. 1: The effect of antibiotics inside the bacterial cell

2.1.1.2.1 Fluoroquinolones

Fluoroquinolones are a group of antibiotics that disturb the DNA inside the cell of the microorganisms. These compounds evolved from the basic structure of quinolones. The first fluorinated structure of quinolones was flumequine, and the recent structures of fluoroquinolones (i.e., ciprofloxacin, enrofloxacin, ofloxacin) have been modified from it by adding functional groups such as nitrogen at position at C_1, carboxylic acid at C_3, ketone at C_4 and fluorine atom at C_6 to enhance their penetration into the tissues and increase its effectiveness (Figure 2.2) (Kocsis et al., 2016). Fluoroquinolones molecular structures normally have double pKa values due to carboxylic acid and piperazine ring, which enable them to interact differently in different environmental compartments (Table 2.1). Owing to their low Henry law constant and octanol-water partitioning coefficient, they are less

abandoned in the atmosphere and are more likely to absorb on the solid material when they are released (Riaz et al., 2018).

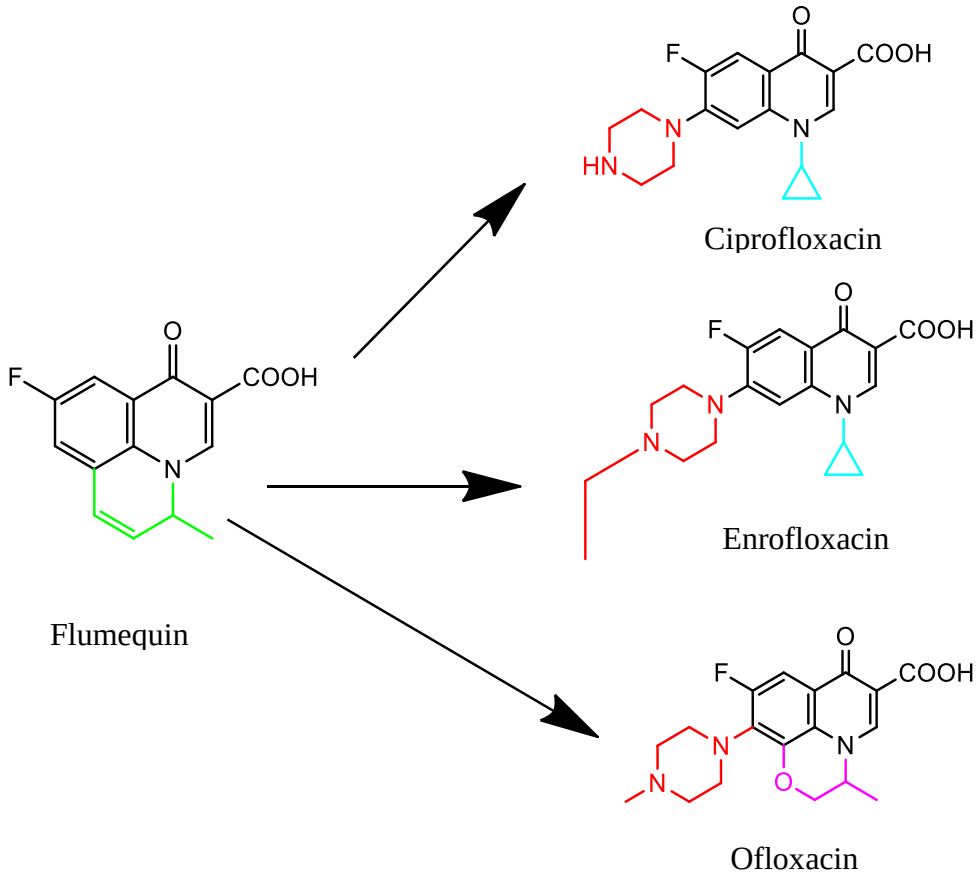


Figure 2. 2: The basic structure of fluoroquinolones

2.1.1.2.2 Tetracyclines

Tetracyclines are a wide group of antibiotics that inhibit protein synthesis inside the cells of microorganisms. These compounds arise from the parent compound tetracycline (Figure 2.3), in which the structural skeleton is made up of four ring cores that are attached to different functional groups (Chopra et al., 2001). The top side of the molecules is oxygen rich made up of enol, ketone and amide groups and the bottom site consists of dimethyl amino, hydroxide and methyl groups. These functional groups are very crucial for the compound's effectiveness on destroying the bacteria, especially the dimethyl amide and the oxygen rich side (Griffin et al., 2010). Tetracyclines are more likely to react with metals ions, especially metal ions such as Cu^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+} and Co^{2+}. Owing to the low Henry constant and K_{oa} values, the presence tetracyclines air is less compared to liquid and solid media.

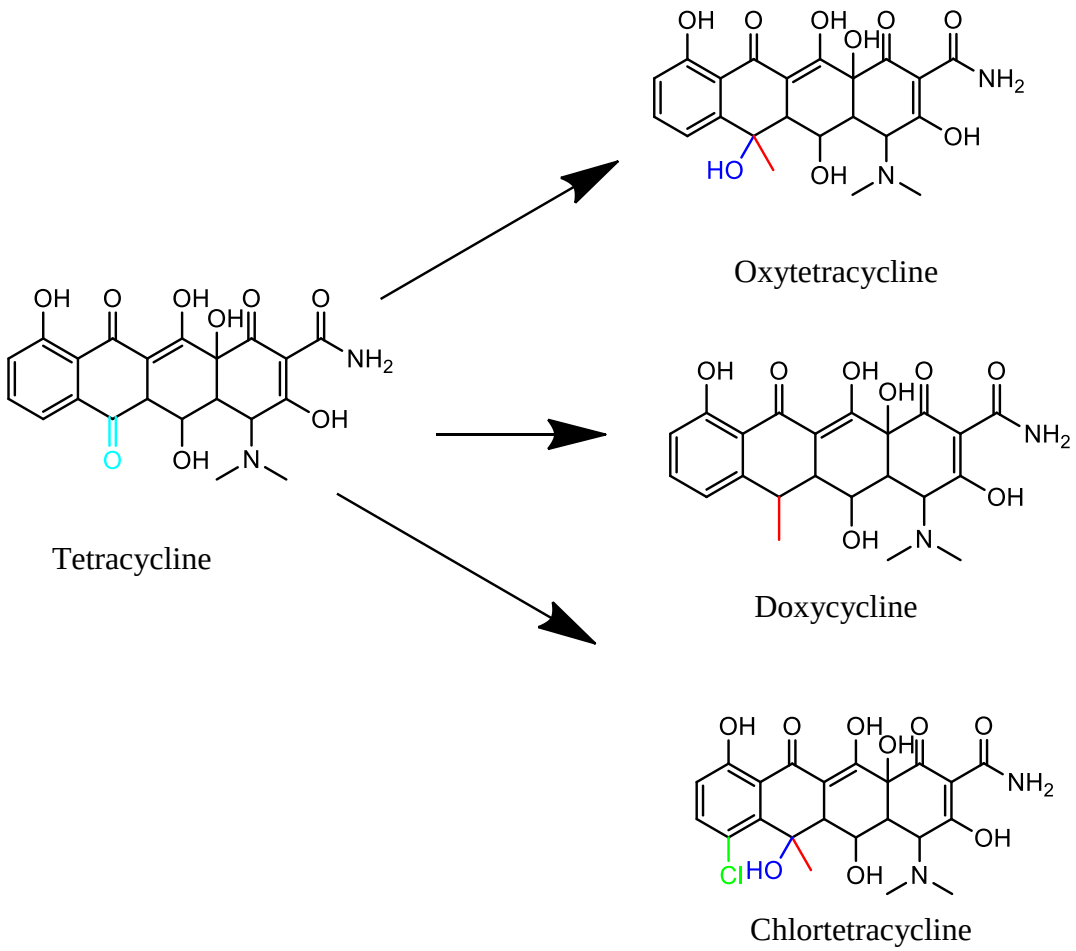


Figure 2. 3: The basic structure of tetracyclines

2.1.1.2.3 Sulphonamides

Sulfonamides are a group of antibiotics that disturb the production of dihydrofolic acid, thus they disturb the protein synthesis in the cell of a microorganism. The basic structure of these compounds consists of the aniline ring and SO_2-NH_2 group attached at position C_1 and C_4 of the ring (Figure 2.4). These recent sulphonamides are synthesised by replacing the hydrogen atom on the SO_2-NH_2 group with other structures. These compounds also follow the trends of poor air distribution and mostly found in the liquid and solid surfaces.

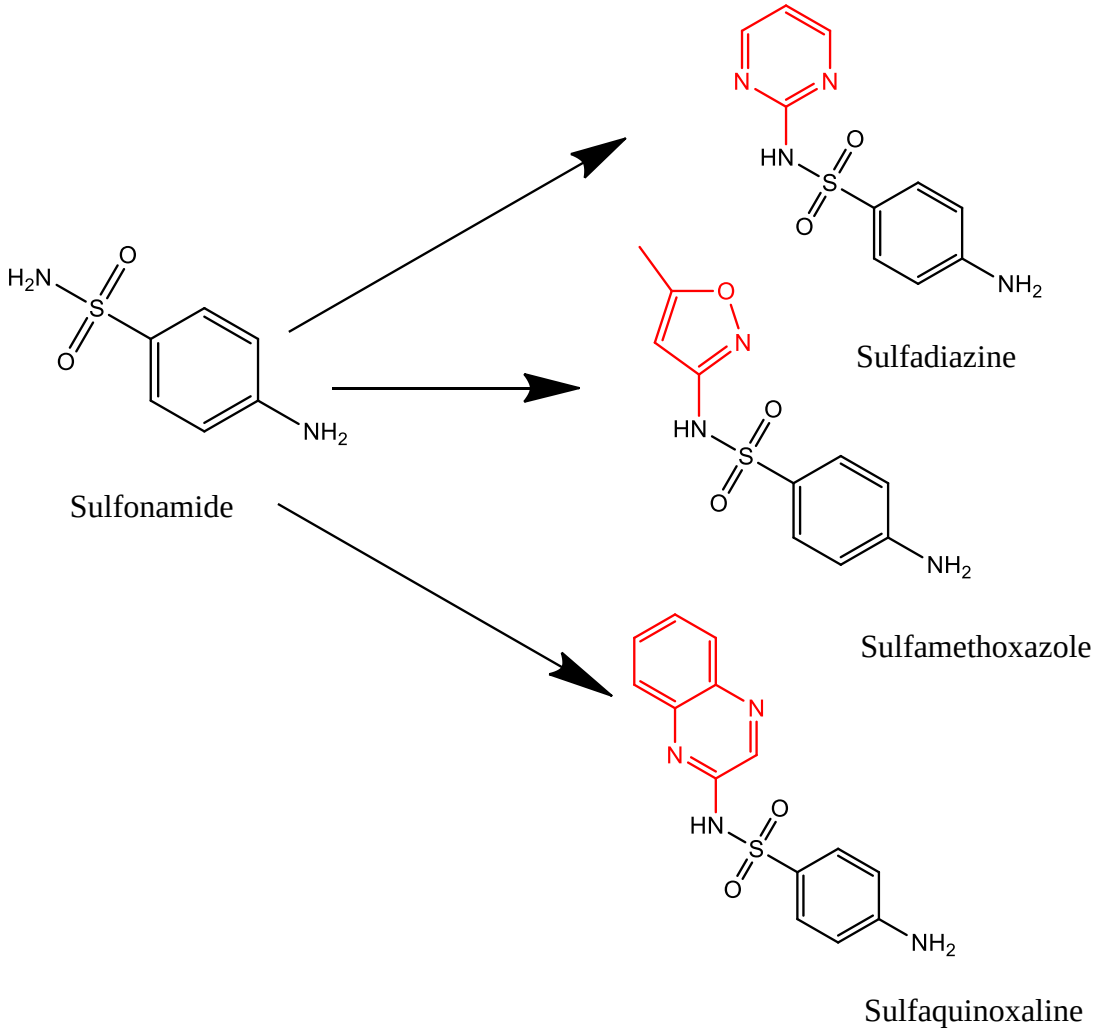
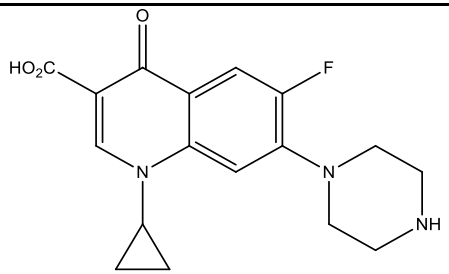
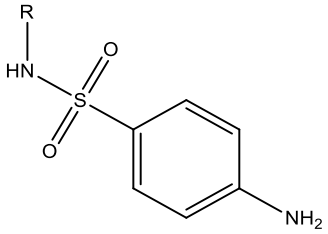
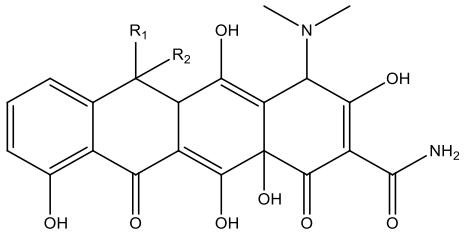


Figure 2. 4: The basic structure of sulfonamides

Table 2. 1: The physico-chemical properties of fluoroquinolones, tetracyclines and sulfonimines

Antibiotics group	Basic structure	Compound	pk_a values	K_{ow}	Water solubility (mg L^{-1})
Fluoroquinolone		Ciprofloxacin	6.09, 8.7	0.28	30
		Enrofloxacin	5.88 – 6.08 7.70 – 7.74	4.7	0.0539 – 0.146
		Ofloxacin	5.97, 9.28	-0.39	0.004
		Flumequine	6.5	1.6	2.190
Sulphonamide		Sulfamethoxazole	1.6, 5.7	0.89	610
		Sulfadiazine	6.36	-0.09	77
		Sulfaquinoxaline	1.6, 6.36	0.89	0.719
		Sulfachloropyridazine	1.6, 5.1	1.68	0.007

		Sulfamethoxypyridazine		0.32	0.000325
Tetracycline		Tetracycline	3.30	1.3	231
		Oxytetracycline	3.27, 9.5	-0.9	313
		Chlortetracycline	3.30	-0.62	0.000259
		Doxycycline	3.09	0.63	0.05
		Minocycline	2.85.0/7.8, 9.3	0.05	52000
		Methacycline	3.09	-0.3	7550

2.1.1.3 Distribution of antibiotics in the environment

Antibiotics applications are diverse, as mentioned above and their environmental distribution mechanisms are broad (Li et al., 2020). Their production and applications have increased over the years since their discovery (Roberts et al., 2020). Half of these antibiotics are used in agricultural practices such as livestock treatment, prophylactic and to enhance animal production. However, antibiotics are not completely absorbed or eliminated in the body, most are released into the environment in active form through excretion (Wang et al., 2020; Zhao et al., 2020); Danner et al., 2019). Therefore, most of them end up in municipal wastewater treatment plants. That is why wastewater treatment plants are regarded as a point source of antibiotics as high quantities of antibiotics are detected in wastewater influent (Table 2.2). Amongst all wastewater treatment plants, high quantities of antibiotics have been detected in the hospital wastewater treatment plants, out-pertinent clinics wastewater treatment plants, and veterinary clinics wastewater treatment plants (Szymańska et al., 2019; Azanu et al., 2018). Wastewater treatment plants are not designed to eliminate antibiotics, therefore, most of them are released into the surface water and eventually into the drinking water (Guo et al., 2017). The sludge and slurry obtained in wastewater treatment plants are used in agricultural farmlands as fertilisers to improve plant production. Hence, quantities of antibiotics have been detected in vegetables (Chen et al., 2020; Tadić et al., 2019). Antibiotics can also be directly introduced into the environment through emission in pharmaceutical industries, agricultural run-offs, and landfill leaching (Zhao et al., 2020; Esponda et al., 2009; et al., 2019). Different pathways of antibiotic dissemination are represented in Figure 2.5.

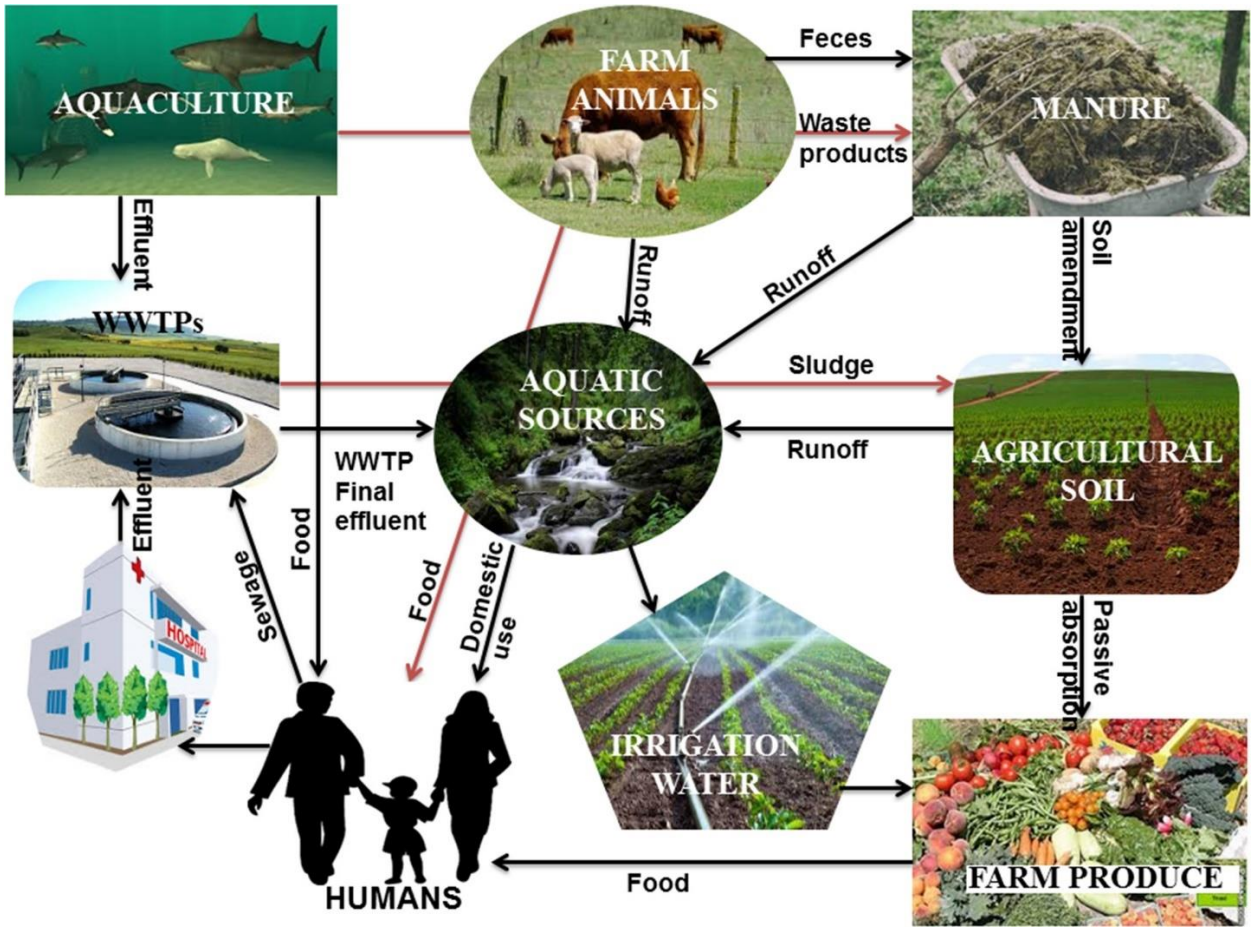


Figure 2. 5: Distribution of antibiotics in the environment

Dissemination of antibiotics in rural areas open water is expected to be much higher due to the lack of proper sanitation and adequate wastewater treatment plants (Dinh et al., 2017); Manyi-Loh et al., 2018). According to Meng et al. (2019), the presence of antibiotics in tap water coming from the rivers in Southern China which supply the population residing in rural areas were much higher compared to other reported data. Identifying the point source in a rural area is challenging due to the multitude of sources such as groundwater residues. Livestock treatment and misuse of antibiotics are some of the factors that contribute to the prevalence of antibiotics in rural areas (Gray et al., 2020). Most of them end up in open water where they bioaccumulate in both biotic and abiotic compartments. These compromises the health of both human and ecosystem as most people rely on water for drinking and vegetation. In most cases, the vegetation is planted near the river for easy access to water and when it rains these vegetables are flooded with contaminated water.

From the explanation above, it is worth saying that antibiotics residues end up in the drinking water and food. In food, antibiotics are introduced in different ways depending on the type of food. In meat, antibiotics are introduced to animals through treatment and growth promoting agents (Bacanli et al., 2019). In milk, antibiotics are introduced in dairy production and dairy livestock as treatment agents. In eggs, improper treatment can result in hens laying eggs with trace amounts of antibiotics. In vegetables and grains, the use of manure with antibiotics residue is the primary source of contamination since the use of organic fertilisers has been banned in most countries (McGuire, 2017). However, certain antibiotics such as streptomycin, combination of streptomycin-oxytetracycline, tetracycline and aminoglycosides are used for the treatment of plant diseases, and some antibiotics are used in genetically modification of food such as vancomycin (Bacanli et al., 2019). In fish and shrimps, antibiotics are introduced through oral administration in aquaculture and in trace amounts from water and sediments.

2.1.1.4 Accumulation of antibiotics

Antibiotics are released and accumulate in the environmental compartments and biomagnify through the food chains. Their accumulations into the environment depend on their physico-chemical properties, climate condition and content of organic matter (Muhammad et al., 2020; Gray et al., 2020). For example, sulfonamides are highly soluble in water, therefore they have higher mobility and higher accumulative potential in plants compared to tetracyclines which have a higher affinity for organic matter (Guo et al., 2017; Pan et al., 2018). WWTPs are the primary source of antibiotic residues and some of them can further be detected in WWTPs effluents. As can be seen in Table 2.1, Teglia et al. (2019) have detected four fluoroquinolones at concentrations ranging from 1.0 – 14.2 μg L^{-1} at WWTPs found in five provinces in Argentina. Sabri et al. (2020) have detected sulfamethoxazole, sulfapyridine, and trimethoprim in WWTP effluent at concentrations ranging from 1- 275 ng L^{-1} in the Grote Beerze River, the Netherlands. Kortesmäki et al. (2020) have detected sulfadiazine, sulfamethoxazole, trimethoprim, clarithromycin, erythromycin, roxithromycin, cloxacillin, Carbamazepine in WWTP effluent at concentrations ranging from < LOD – 194 ng L^{-1} in Finland—Turku, Tampere, and Helsinki. The presence of antibiotic residues in rivers is due to their poor degradation in the WWTP. Fernandes et al. (2020) have demonstrated that antibiotics are more prevalent in the water near the WWTPs than the water which is further away with a magnitude 12 times higher. Similar results have been depicted by Guo et al. (2017) and Dinh et al. (2017). These antibiotics accumulate in WWTP sludge which is used as fertilisers in agricultural farmlands. Studies have shown that the accumulation of antibiotics in the protected vegetation

soil is much higher than the traditional open cropland. This might be due to the continuous administration of fertilisers in the soil for long periods which resulted in their high persistency (Zhang et al., 2016). The presence of antibiotics in vegetation has been reported, and in most cases, their accumulation is in trace amounts which might not might not have a meaningful impact to the ecosystem and human health. Their accumulation depends on the properties of the soil, the properties of water, the physico-chemical properties of antibiotics and the concentration of antibiotics (Azanu et al., 2018). Antibiotic residues with high affinity with soil will not or less be detected in vegetation compared to those that are more soluble in water or aqueous soil (Pan et al., 2017). The amendment of soil can increase the availability of antibiotics in soil and aqueous samples, especially those that are strongly held to soil (tetracyclines and fluoroquinolones) and enhance the absorption of those that have less affinity with soil (sulphonamides). According to Albero et al. (2018), this is due to the increase in dissolved organic content in the soil aqueous phase (through soil amendment) which control the sorptive behaviour of antibiotics through accumulative sorptive or co-sorptive, and the mobility of antibiotics might be due to competition or co-transportation.

In aquaculture, the exposure of antibiotics to aquatic organisms can be directly through administration or indirectly through the presence of antibiotics in water. Guidi et al. (2017) detected enrofloxacin in Brazilian fish farm at a concentration of up to 50 μg kg^{-1}. The livestock manure in agriculture accumulates high content of antibiotics since they are poorly metabolised through their gut. Through their special distribution, these contaminants have been detected in air particulates, and even wild vegetation is exposed to these contaminants. This leads to their biomagnification through the food web.

Sediments and soil are the main sinkers of antibiotics which accumulate antibiotics as they are distributed through air and run-offs. The accumulation of antibiotics depends on the physico-chemical properties of the analytes and the properties of the soil texture. The pH of the soil controls the ionic state of the analytes which then determines their accumulation in soil or sediments. Most ionic antibiotics are more likely to be attained in the soil than the neutral analytes due to their interaction with soil such as cation exchange, surface complexation and cation bridging sorption mechanisms (Pan et al., 2018). The analytes that are poorly retained in the soil are more likely to be detected in the groundwater and plants. The accumulation of antibiotics in soil decreases in this way: tetracyclines > fluoroquinolones > sulphonamides > macrolides > beta-lactams. In most cases, the topsoil is sandy with low content of organic matter to slow the infiltration of the analytes into the bottom soil. The presence of antibiotics on the topsoil might be due to their recent introduction (Fernandes et al., 2020).

Table 2. 2: The accumulation of antibiotics in different locations in the environment

Matrix	Antibiotics	Location	Concentration	Reference
Wastewater effluent	Sulfamethoxazole, sulfapyridine, and trimethoprim	Grote Beerze river, the Netherlands	1- 275 ng L^{-1}	Sabri et al., 2020
Wastewater effluent	Doxycycline, amoxicillin, sulfamethoxazole, trimethoprim, ciprofloxacin and norfloxacin	Machakos (WWTP1), Gateei in Nyeri (WWTP 2), Meru (WWTP 4), and one trickling filter treatment plant in Kangemi (WWTP 3) Nyeri County, Kenya	0.3 - 8.5 µg L^{-1}	Kairigo et al., 2020
Wastewater effluent	lincomycin, cefazolin, spiramycin, enrofloxacin, sulfapyridine, ciprofloxacin, azithromycin, sulfamethoxazole, lincomycin, trimethoprim, ampicillin and cephalexin	Southern Sfax WWTP, Tunisia	7.5 – 37.04 ng L^{-1}	Harrabi et al., 2018
Wastewater effluent	Sulfadiazine, sulfamethoxazole, Trimethoprim, clarithromycin, erythromycin, roxithromycin, cloxacillin, Carbamazepine	WTTPs in Finland—Turku, Tampere, and Helsinki	< LOD[a] – 194 ng L^{-1}	Kortesmäki et al., 2020

Wastewater influent	lincomycin, cefazolin, spiramycin, enrofloxacin, sulfapyridine, ciprofloxacin, azithromycin, sulfamethoxazole, lincomycine, trimethoprim, ampicillin and cephalexin	Southern Sfax WWTP, Tunisia	23.30 – 690 ng L^{-1}	Harrabi et al., 2018
Wastewater influent	Sulfadiazine, sulfamethoxazole, Trimethoprim, clarithromycin, erythromycin, roxithromycin, Carbamazepine	WTTPs in Finland—Turku, Tampere, and Helsinki	20 – 1069 ng L^{-1},	Kortesmäki et al., 2020
Wastewater influent				
River water	Enrofloxacin	San Luis, Santa Fe, Córdoba, Argentina	0.97 µg L^{-1}	Teglia et al., 2019
River water	azithromycin, ciprofloxacin, clarithromycin, moxifloxacin, ofloxacin, trimethoprim, carbamazepine, citalopram, diazepam, fluoxetine, sertraline, trazodone, carbamazepine, fluoxetine and venlafaxine	Douro river and Leça river, North of Portugal	1, 87 – 2,82 ng L^{-1}	Fernandes et al., 2020

River water	Sulfadiazine, sulfamethoxazole, ciprofloxacin, ofloxacin, tetracyclines tetracycline oxytetracycline, erythromycin, and roxithromycin	Xiangshui River, megacity Beijing, China	715 – 2850 ng L^{-1}	Guo et al., 2017
River water	Sulfadiazine, sulfamonomethoxine, sulfaquinoxaline, norfloxacin, ciprofloxacin ofloxacin, amoxicillin, cefalexin, penicillinG, penicillin, tylosin, erythromycin, oxytetracycline, tetracycline, doxycycline, vancomycin and lincomycin	Taipu River and Jinze Reservoir, Shanghai, China	ND[b] – 385.8 ng L^{-1}	Li et al., 2020
River water	Doxycycline, amoxicillin, sulfamethoxazole, trimethoprim, ciprofloxacin and norfloxacin	Mitheu river, Mwania river, Sagana river, Chania river and Kanyuru river	ND – 49.7 µg L^{-1}	Kairigo et al., 2020
Agricultural run-offs	chlortetracycline, lincomycin, and tiamulin	University of Nebraska-Lincoln (UNL) Rogers Memorial Farm, UK	0.044 – 0.597 µg L^{-1}	Barrios et al., 2020
Farm wastewater	ciprofloxacin, enrofloxacin, ofloxacin, enoxacin, and difloxacin	San Luis, Santa Fe, Córdoba, Argentina	1.14 – 22.1 µg L^{-1}	Teglia et al., 2019

Soil	nine macrolides, sixteen quinolones, fifteen sulfonamides and four tetracyclines	Haihe River Basin (Beijing and Tianjin); Yangtze River Basin, particularly surrounding Shanghai City; Yellow River Basin surrounding Shanxi; Pearl River basin, surrounding Guangzhou; and South eastern Rivers Basin, surrounding Xiamen city, Eastern China	8.10 – 609 µg kg^{-1}	Lyu et al., 2020
Sediments	azithromycin and sulfamethoxypyridazine	Douro river and Leça river, North of Portugal	< MDL^{c} – 43.2	Fernandes et al., 2020
Sediments	Sulfathiazole, sulfapyridine, sulfamerazine, sulfamethazine, sulfamethiazole, sulfachloropyridazine, sulfamethozazole, sulfisoxazole, sulfadimethoxine, trimethoprim, oxolinic acid, enrofloxacin, tetracycline and oxytetracycline	Polish coastal zone in the southern Baltic Sea	< MDL – 449 ng g^{-1}	Siedlewicz et al., 2018

Muscle of fish	enrofloxacin and ofloxacin	Karakaya Dam Reservoir, Turkey	0.0034 – 0.0073 mg kg^{-1}	Varol and Sünbül ., 2019

a = limit of detection
b = non detected
c = minimum detection limits

2.1.1.5 The Negative effects of antibiotics on the ecosystem

The effect of antibiotic residues in the environment has been done looking at their toxicity on different organisms. Routinely, bioassays such as EC_{50} (concentration giving half of the maximum respond), LC_{50} (concentration giving half lethality), IC_{50} (concentration giving half inhibition), NOEC (concentration with no observed effect) and LOEC (concentration giving lowest observed effect) are measured to test the toxicity of antibiotics in the environment (Välitalo et al., 2017; Santás-Miguel et al., 2020; Liu et al., 2018). The environmental concentration of antibiotics range from parts per billion to parts per trillion as described. Most bioassay studies indicate that at these low concentrations, antibiotics show less negative impact in most living organisms (Sönmez and Sivri 2020). This is probably because these studies were done considering the acute toxicity (done less than 24 h). There is less information on the chronic toxicity (done in months or years) of antibiotics, but the studies which have been done so far indicate that longer exposure to antibiotics even in trace level can have an adverse consequence to any living organisms.

The environment consists of a diverse body of the microbial community. Most of these microbes play an important role in the environment, including climate regulations (e.g., the biological transformation of greenhouse gasses) and environmental quality regulations (Ngqwala et al., 2020). Since microorganisms are highly sensitive to antibiotics, the presence of antibiotic residues in the environment may cause adverse effects on them, and thus in turn, affecting the environment and human health. Antibiotic residues directly impact microorganisms when they are present in high concentrations. These high concentrations of antibiotics directly kill or inhibit their productions. The presence of a high concentration of antibiotic residues in the environment is caused by misuse of antibiotics or the flooding of antibiotics in hospitals through natural disasters. The presence of antibiotics at a concentration range of 10 - 100 times lower than that of its minimum inhibitory concentration (MIC) may cause an indirect impact by the induction of the selective pressure on the microbial community resulting in the antibiotic resistance bacteria (Li et al., 2020; Bena et al., 2019; Danner et al., 2019). The presence of antibiotic resistant bacteria in the environment has gained a lot of attention in the scientific community. This is because bacteria tend to transfer their antibiotic resistance strains to other bacteria simply through horizontal gene transfer, and thus resulting in a wide array of resistant bacteria and resistant genes in the environment.

2.1.1.6 The negative effect of antibiotics in human health and other macro-organisms

The body of a human and other living organisms consist of a wide array of microorganisms. These microorganisms are mostly found in the gastro-international tract. More than 90% of these organisms are essential microorganisms and the rest are opportunistic and detrimental microorganisms. Continual antibiotic exposure through treatment, inhalation and ingestion can result in detrimental health effects (Li et al., 2020). Zhou et al. (2018) have demonstrated that the environmental concentration can impair the gut health of zebrafish after exposure to sulfamethoxazole and tetracyclines for six weeks. This is because continual consumption of antibiotics can result in destroying some of the essential bacteria in the body resulting in high colonisation of opportunistic and detrimental microorganisms. Accumulation of these organisms can result in pseudomembranous colitis and colorectal cancer, and intestinal ailments (Bilal et al., 2020). The selection of bacterial resistance and resistance to other microorganisms inside the gastrointestinal tract is a big issue (Kraemer et al., 2019). This is because bacteria and other deadly pathogens are unable to be treated using the available treatments, which results in the rapid multiplication of these organisms. The results of these effects are death due to the rapid increase of pathogens in the body and resistance to common treatments. Other human health associated with the presence of antibiotic residue in the body include drug hypersensitivity (penicillin and macrolides), carcinogenic effect (chloramphenicol, sulfamethazine, oxytetracyclines, furazolidone), disruption of normal intestinal flora (flunixin, tylosin, vancomycin, nitroimidazole and metronidazole), mutagenic effect (antibiotics that are alkalizing agents and analogues of DNA base), nephropathy (gentamicin) and teratogenic (benzimidazole) (Prajwal et al., 2017).

2.1.2 Perfluoroalkyl substances

2.1.2.1 Background

Perfluoroalkyl substances (PFAS) are a group of synthetic fluorinated organic compounds that were introduced to the world market around the 1950s (Kah et al., 2021). They are classified as amphiphilic compounds which is made of hydrophobic C-F alkyl chains with varying chain length, and hydrophilic functional group (carboxylic acid or sulfonic acids) into one compound (Fan et al., 2021). Perfluorocarboxylic acids and sulfonic acids are some of well-known of PFAS groups, but PFAS are diverse with an estimation of more than 20 groups and 5000 compounds (Table 2.2) (Wu et al., 2020). Looking at the hydrophobic part of the molecule, the bond between the carbon and the fluorine atom (C-F) is considered one of the strongest

covalent bonds which makes these compounds highly stable and degradation resistant in almost every environmental condition. The head of the compound is a highly reactive hydrophilic functional group. When the hydrophilic and the hydrophobic parts are combined, the resulting compound retains remarkable properties such as water, oil and stain resistance (Schulz et al., 2020). All these properties make PFAS suitable to be used in industries (e.g., paper, steel, petrochemical and electronics industry), household applications (e.g., non-stick cookware, detergents, carpets, polishes, waxes, rugs, and leather) and other materials such as aqueous film-forming forms.

The accumulation of PFAS and their presence in the environment is associated with detrimental effects on living organisms such as invertebrates and vertebrates. Some of the health effects include growth reduction, decrease in reproduction and production, size reduction in off-springs, change in heart rate, delay in hatching in other fish and reduction in off-spring survival (Bartlett et al., 2020). According to the review by Sinclair et al. (2020), more research is still needed specifically on the health effects associated with long-term exposure, a better ecotoxicological monitoring using biomarkers, and the effect of synergistic PFAS in the environment. PFAS are very persistent in the environment, and they have been estimated to persist in aqueous samples for more than five years (Ghisi et al., 2019). According to the review by Bartlett et al. (2020), the presence of PFAS even in low concentration can increase the bio-accumulation factor, and the longer the organisms remain in the polluted environment, the more the accumulation. Another study by Jantzen et al. (2017) confirmed that organisms that are exposed to low concentration (nano-gram range) of PFAS are also associated with an increase in PFAS serum.

2.1.2.2 Physico-chemical properties

PFAS are divided into perfluoroalkyl and polyfluoroalkyl groups (Wu et al., 2020). Polyfluoroalkyl substances are groups of compounds which some of the carbons are fluorinated, and the hydrophilic functional group is not directly attached to the C-F chain. Examples of poly-fluoroalkyl substances are fluorotelomer based substances polyfluoroalkyl phosphate monoesters (Table 2.2) (Hamid et al., 2020). Polyfluoroalkyl substances can easily degrade in the environment due to their low chemical stabilities. In contrast, perfluoroalkyl substances are a group of PFAS in which all the carbon atoms are fully fluorinated, except one carbon on carboxylic acid functional group, and the hydrophilic functional groups are directly attached to the C-F chain (Lee et al, 2020). Some examples of perfluoroalkyl substances are

perfluoroalkyl carboxylic acids (PFCA), perfluoroalkyl sulfonic acids (PFSA) and perfluoroalkyl phosphonic acids (Table 2.2). Because of their acidic moieties which are directly attached to C-F chain, they dissociate when they are in contact with aqueous solution at pH >5 to form perfluoroalkyl acids or PFAA (Table 2.3). PFAA are sometimes referred to as terminal compounds because of their high thermal and chemical stabilities. These stabilities arise from the bond between the carbon and fluorine atoms which is believed to be one of the strongest covalent bonds. The large atomic radii of a fluorine atom can hinder any interactions between C-C bond to make them resistant to any degradation processes.

Perfluoroalkyl acids are further divided according to their chain length, for example short chain PFAS are those with carbon chain less than C_8 for PFCA and less than C_6 for PFSA, and long chain PFAS are those with chain length more than C_7 for PFCA and more than C_5 for PFSA. These properties are very important because they describe their interaction with different matter. For example, long chains PFAS are less soluble in water because of the long hydrophobic chain compared to short chain perfluoroalkyl substances (Table 2.3). Short chain PFAS are more volatile due to their high vapor pressure compared to long chain. The toxicity, persistency and high accumulation behaviour of PFAS resulted in regulation and banning in most countries and environmental protection agencies. These regulations resulted in a production of novel compounds such as 6:2 chlorinated polyfluoroalkyl, 6:2 fluorotelomer sulfonates and perfluoro ether carboxylic acid, that have similar properties with the legacy PFAS, but they can easily be degraded and less toxic. However, other reports demonstrates that some of these novel PFAS are potential precursor of the terminal PFAA in the environment, and thus increasing the PFAA concentration in the environment (Figure 2.6) (Nguyen et al., 2019).

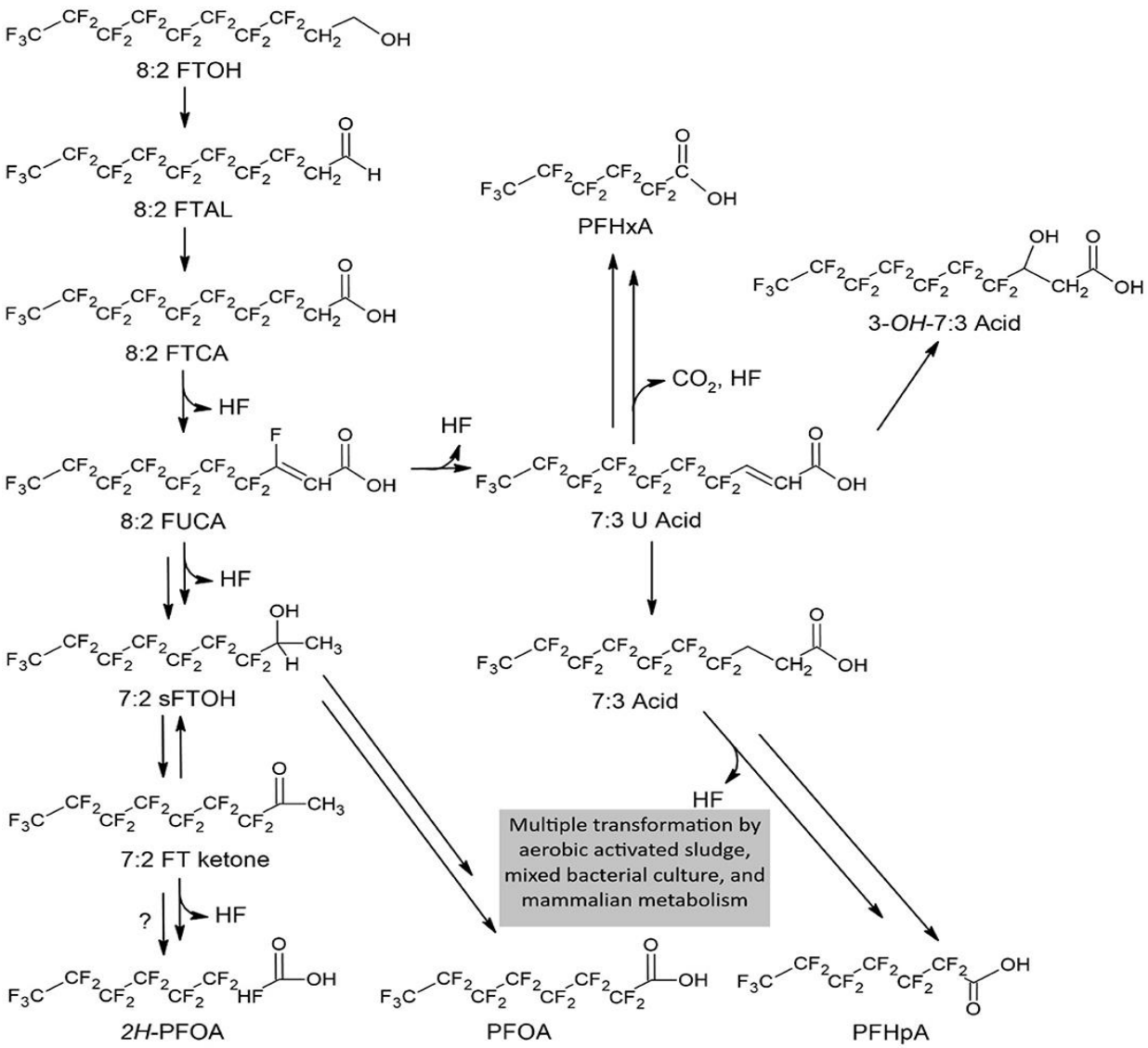
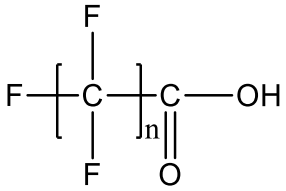
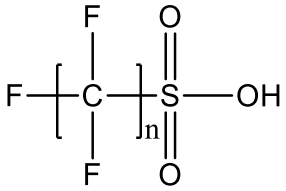
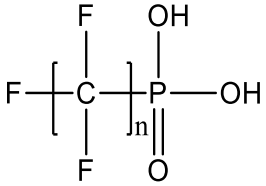
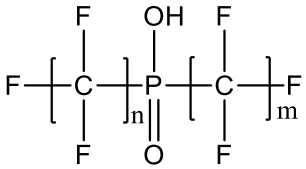
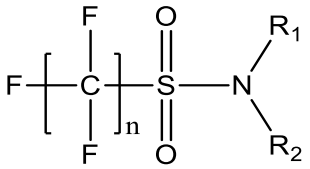
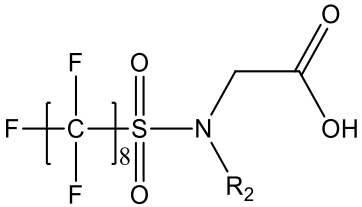
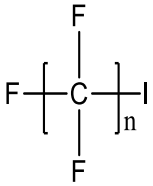
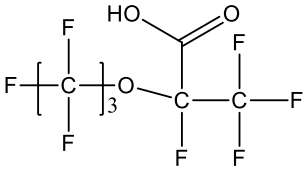
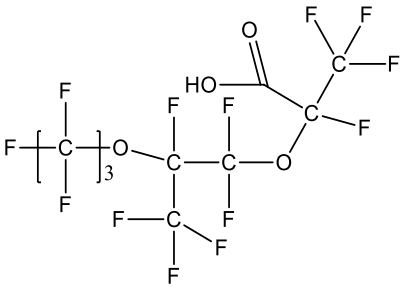
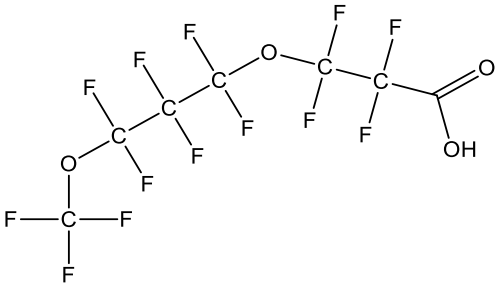
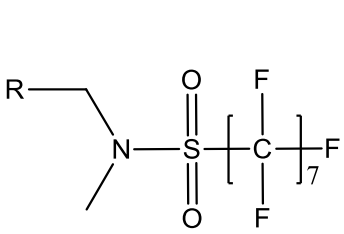
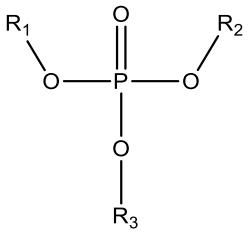


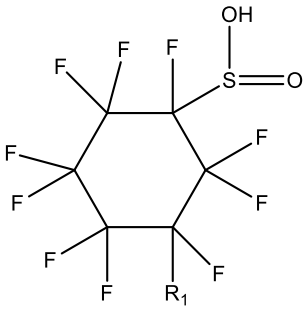
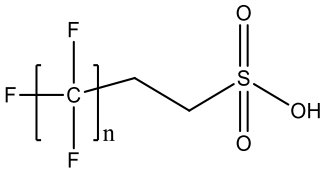
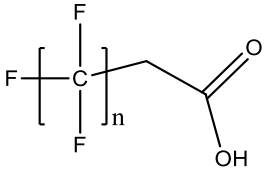
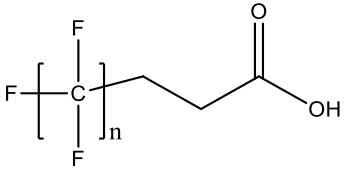
Figure 2. 6: The degradation of novel PFAS into legacy PFAS

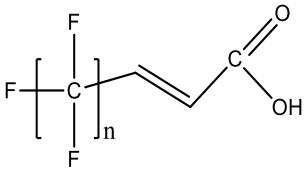
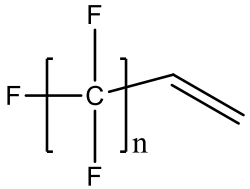
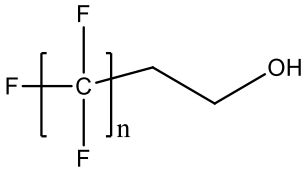
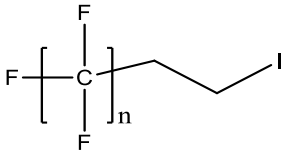
Table 2. 3 Different groups, classes and an example of PFAS with their basic structure

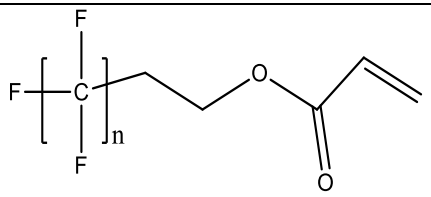
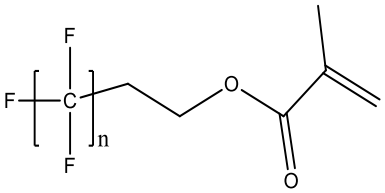
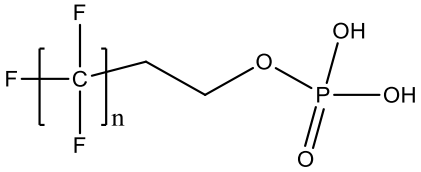
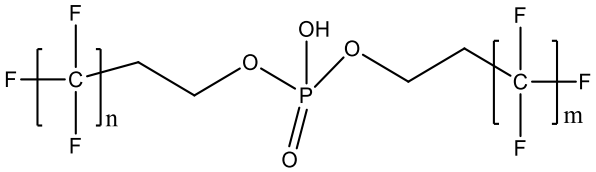
Group	Class	Basic structure	Example(s) of compound name
Perfluoroalkyl substances	Perfluoroalkyl carboxylic acid		Perfluorooctane carboxylic acid
	Perfluoroalkyl sulfonic acid		Perfluoroheptane sulfonic acid
	Perfluoroalkyl phosphonic acid		Perfluorohexane phosphonic acid
	Perfluoroalkyl phosphinic acid		6:6 perfluoroalkyl phosphonic acid

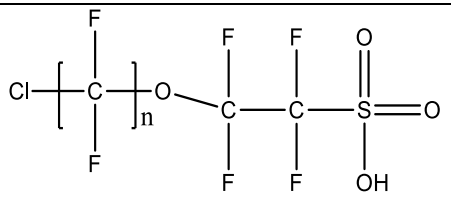
Perfluoroalkane sulphonamides	 R_1 = H, methyl or ethyl R_2 = H	Perfluorooctane sulfonate (n = 8; R_1 = H and R_2 = H) N-methyl fluorooctane sulphonamide (n = 8, R_1 = methyl and R_2 = H
N-alkyl perfluoroalkane phosphonamide acetic acid	 R_2 = H, methyl or ethyl	Perfluorooctane sulfonamido acetic acid (R_2 = H) N-ethyl fluorooctanamido acetic acid (R_2 = ethyl)
Perfluoroalkyl iodide		Perfluorodecyl iodide

Perfluoroether carboxylic acid			Hexafluoropropyiene oxide dimer acid (trade name: Gen X)
			Hexafluoropropyiene oxide trimer acid
			3.8-dioxa-3H-perfluorononanpic acid (ADONA)
Perfluorooctane sulfanamido ethanol-based phosphate esters			Phosphate diester of N-ethyl perfluoro sulfonamido ethanol (R_1 = R, R_2 = R, R_3 = H) Phosphate triester of N-ethyl perfluoro sulfonamido ethanol (R_1 = R, R_2 = R, R_3 = R)
	R_1 = R, R_2 = R, R_3 = H		

		R_1 = R, R_2 = R, R_3 = R	
	Cyclic perfluoroalkyl sulfonic acid		Perfluoromethylcyclohexane Sulfonic acid (R_1 = methyl)
		R_1 = methyl or ethyl	
Polyfluoroalkyl substances	Fluorotelomer sulfonic acid		4:2 fluorotelomer sulfonic acid
	Fluorotelomer carboxylic acid		6:2 fluorotelomer carboxylic acid
			5:3 fluorotelomer carboxylic acid

Fluorotelomer unsaturated carboxylic acid		6:2 fluorotelomer unsaturated carboxylic acid
Fluorotelomer olefins		8:2 fluorotelomer olefins
Fluorotelomer alcohols		8:2 fluorotelomer alcohol
Fluorotelomer iodide		4:2 fluorotelomer iodide

Fluorotelomer acrylates		6:2 fluorotelomer acrylate
Fluorotelomer methacrylates		6:2 fluorotelomer methacrylate
Polyfluoroalkyl phosphate monoesters		6:2 polyfluoroalkyl phosphate monoester
Polyfluoroalkyl phosphate diesters		4:2 polyfluoroalkyl phosphate diesters 4:2/ 6:2 polyfluoroalkyl phosphate diesters

	Chlorinated polyfluoroether sulfonic acid		6:2 chlorinated polyfluorinated ether sulfonic acid (trade name: F-53B)

n = number of C-F chain length

Table 2.3: The physico-chemical properties of PFAS

PFAS group	Compounds	K_{oa}	pK_a	Water solubility (mg L^{-1})
Perfluorocarboxilic acids	Perfluorohexanoic acid	3.48	-0.16	15700
	Perfluroheptanic acid	4.15	-2.29	3.65
	Perfluorooctanoic acid	4.81	0.5 to 4.2	2290 – 4340
	Perfluorononanoic acid	5.8	-0.21	625
Perfluoroalyl sulfonic acid	Perfluorobutane sulfonic acid	1.82	-3.31	344 – 510
	Perfluorohexane sulfonic acid	3.16	0.14	6.2
	Perfluorooctane sulfonic acid	4.49	> 1.0	32

2.1.2.3 Accumulation behaviour

The physico-chemical properties of PFAS and the environmental composition determine their accumulation behaviour in different environmental compartments. In many cases, the properties that are considered when determining the accumulation behaviour in the environment are their carbon chain length and partitioning coefficients. The length of the carbon chain affects the solubility of the compounds, since the structures of PFAS are composed of hydrophobic and the hydrophilic parts, as the chain of the molecule increases it becomes more hydrophobic making it less soluble in water and more soluble in other hydrophobic compartments such as solids and biological samples (Death et al., 2021). This was confirmed by high accumulation of long chain PFAS in invertebrates and other aquatic organisms in water (Van der Schyff et al., 2020). The chain of the molecule also determines its partitioning coefficient as reports have indicated that short chain PFAS are more volatile and more soluble in water.

The factors that affect the accumulation behaviour of PFAS are the total organic content which affect the accumulation behaviour in sediments, temperature affect the accumulation of PFAS

in humic acid, dissolved calcium and magnesium which are responsible for sorption enhancing salinity of PFAS (Groffen et al., 2018). In biological samples PFAS are more likely to bind to proteins than in other biological molecules. This can be explained by their high accumulations in a protein reach organ, such leaver and blood, and eggs.

2.1.2.4 Environmental distribution

As the world is shifting toward the novel and short chain PFAS, there has been a decrease in the concentration of PFOS, PFOA and other long chain PFAA in most countries. The recent publications reveal that when some of these conventional PFAS are released into the environment, they undergo degradation processes to form terminal ionic PFAS. This can be confirmed by continuous detection of ionic PFAS in almost every environmental compartment. The study by Lee et al. (2020) demonstrated that one of the primary sources of PFAS are the industrial facilities such as the semiconductor, paper-mill, automobile, and metal plating industries which the samples collected nearby showed high concentration of PFAS. Other PFAS point sources include their main production sites, municipal landfills, and the aqueous film forming forms. The route of transmission includes air and water. Since most conventional PFAS have high volatility, they are transmitted through air, especially in the production industries, into the environmental compartment such as water, soil, sediments, and suspended particulates. Another way of exposing PFAS into the environment is through the discharge of waste that contains residues of PFAS into environmental compartments such as landfills. The transmission of PFAS biological samples such as fish, plants and other mammals depends on the accumulation behaviour as mentioned in the section above.

There are no reports on the production of PFAS in South Africa and their distribution can be through electronic, metal plating, wax and oil industries, aqueous film forming forms or furniture that are imported with residues of PFAS. The available publications on the prevalence of PFAS in South Africa indicates the variation in concentration when comparing different locations. This might be due to different environmental properties as mentioned in the section above and industrialisation. Dividing South Africa in northern and southern regions, the southern region is known to be an industrial region compared to the northern region, and the main source of PFAS in the northern region could be through agricultural practices (from pesticides or insecticides). Mudumbi et al. (2014) have detected PFAS in the Western Cape Province, the concentrations of PFAS in river water ranged from 23 – 390 ng L^{-1} and sediment ranged from < LOQ – 28 ng g^{-1}. According to the author, some of the concentrations detected

are higher compared to other countries. Another study by Ojemaye et al. (2019), in the Western Cape in Kalk Bay Harbour, indicated that the concentration of PFAS ranged from 20.13 ng g^{-1} to 179 ng g^{-1}. Groffen et al. (2018) have found that the concentration of PFAS in abiotic samples in Vaal River were lower (< LOQ – 38.5 ng L^{-1}) compared to other countries, but those detected in biotic samples were higher (< LOQ – 34.0 ng g^{-1}) compared to other countries. Another study by Fredriksson et al. (2016) demonstrated that the source of PFAS in Vaal river could be Schoeman drift, which is connected to the most industrial and populated part of South Africa (Gauteng), and that PFAS are formed as metabolite of the fluorotelomer-based precursor. The variation of PFAS in different regions of South Africa was also demonstrated by Lesch et al. (2017) when comparing the amount of PFAS detected in dragonflies in the northern region and the southern region. Higher concentrations of PFAS were detected in the southern region compared to the northern region. Little is known about the point sources of PFAS in South African, especially the rural areas. More research is needed to access the concentration of PFAS in rural areas since many people rely on river water for animal and plant production and drinking in other areas.

Table 2. 4: The accumulation of PFAS in different locations in the environment

Matrix	Analytes	Location	Concentration	References
Wastewater influent	PFCAs (C_4 -C_{13}), PFSA (C_4 – C_8) and fluorotelomers (6:2 FTS and 8:2 FTS)	Municipality wastewater treatment plants, South Korea	ND[a] – 5560 ng L^{-1}	Kim et al., 2021
Wastewater influent	PFCAs (C_4 -C_{13}), PFSA (C_4 – C_8) and fluorotelomers (6:2 FTS and 8:2 FTS)	Industrial wastewater, South Korea	ND – 469000 ng L^{-1}	Kim et al., 2021
Wastewater influent	PFCAs (C_4 – C_{10}) and PFSAs (C_4, C_8 and C_{10})	Large wastewater, Australia	31 – 142 ng L^{-1}	Nguyen et al., 2019
Wastewater effluent	PFCAs (C_4 -C_{13}), PFSA (C_4 – C_8) and fluorotelomers (6:2 FTS and 8:2 FTS)	Industrial wastewater, South Korea	ND – 9190 ng L^{-1}	Kim et al., 2021
Wastewater effluent	PFCAs (C_4 -C_{13}), PFSA (C_4 – C_8) and fluorotelomers (6:2 FTS and 8:2 FTS)	Municipality wastewater treatment plants, South Korea	ND – 4450 ng L^{-1}	Kim et al., 2021

Landfill leachate	32 per-/poly-fluoroalkyl substances (PFAS)	Three landfill-WWTP pairs (landfill A-WWTP A, landfill B-WWTP B, landfill C-WWTP C) in Florida, USA	4800 – 8300 ng L^{-1}	Masoner et al., 2020
River water	PFCAs (C_4 -C_{13}), PFSA (C_4 – C_8) and fluorotelomers (6:2 FTS and 8:2 FTS)	Nakdong River, South Korea	ND – 218 ng L^{-1}	Kim et al., 2021
River water	PFOA and PFSA	Irrigated water, Nakdong River delta, South Korea	0.026 – 0.112 µg L^{-1}	Choi et al., 2021
River water	PFCAs (C_4 – C_{10}), PFSA (C_4, C_6 and C_8) and PFPrOPrA	Cape Fear River Watershed of North Carolina, USA	62 – 355 ng L^{-1}	Sun et al., 2016
Ocean	PFOA and PFOS	North Atlantic Ocean, Mid Atlantic Ocean, South Pacific Ocean and the Indian Ocean	5 – 439 pg L^{-1}	Yamashita et al., 2008

Soil	PFOA and PFSA	Agricultural solid Nakdong River delta, South Korea, South Korea	0.818 – 1.364 µg L^{-1}	Choi et al., 2021
Sediments		northern Bering Sea, Chukchi Sea and adjacent Arctic Ocean, China	0.00 – 10.23 ng g^{-1}	Kahkashan et al., 2019
Marine eggs	PFCAs (C_8 -C_{13}) and PFSA (C_6 and C_8)	St. Brandon's Atoll, Republic of Mauritius, Indian Ocean	0.93 – 2.3 ng g^{-1}	Van der Schyff et al., 2020

a = non detected

2.1.2.5 Human exposure

Beside the PFAS long-range of application, starting from the year 2000, long chain PFAS such as perfluoroalkyl carboxylic acid and perfluoroalkyl sulfonic acid and their precursors were considered as potential emerging contaminants because of their persistence, toxic and accumulative in the environmental compartments, and the adverse effects they impose on human health and the ecosystem. Since these substances end up in water and biomagnifies through food the web, humans accumulate these substances through food ingestion (especially seafood), drinking of water, inhalation, and dermal absorption (Forsthuber et al., 2020; Masoner et al., 2020). Food ingestion is considered the primary way for accumulation of PFAS, especially long chain PFAS. Short chain PFAS are introduced in the body through drinking of water (Domingo et al., 2019). However, the accumulation of PFAS sometimes depends on the environment people are living in. For example, high concentrations of PFAS have been detected in people working or living near the PFAS plants (Sharma et al., 2016). There are also studies that have suggested that the presence of PFAS in households can lead to the accumulation through dermal absorption and inhalation especially for PFAS that are more volatile.

2.1.2.6 Impact on human health

The studies based on *in vivo* and *in vitro* have confirmed that the presence of PFAS in the human body is associated with different adverse health effects. Most studies have been based on long chain PFAS. This is because short chain PFAS have less bioaccumulation coefficient in the body and most of them are excreted through urination due to their high-water solubility. However, recent studies have indicated that short chain PFAS is also associated with severe health issues such as decrease in cholesterol level, decrease in red blood cells and haemoglobin, delay development, changes in thyroid hormones, and changes in cells and weight increase in thyroid and liver (Li et al., 2020). There are different paths in which human beings are exposed to PFAS as mentioned above. Once they are in the body, they associate with different body components such as liver and kidneys (Stanifer et al., 2018). Studies have indicated that PFAS binds to fatty acid binding proteins because of their similar chemical and transport mechanisms. These mechanisms interfere with cholesterol level in living organisms. According to Forsthuber et al. (2020), long chan PFAS such as PFOS, PFOA, PFNA, PFHxS and PFDA are more likely to bind to the albumin fractions; however, they can also bind to low density lipoprotein and high-density lipoprotein. PFAS are associated with the change in protein level

and gene expression in the body due to epigenetic mechanisms acting on the transcriptional and post-transcriptional levels such as change in DNA-methylation or micro-RNA (miRNAs). The altered gene expressions (especially miRNAs gene expression) are used as biomarkers in understanding PFAS environmental exposure and their adverse effects. Xu et al. (2020) have found that the increased exposure to PFAS (dominated by PFOS and PFHxS) are associated with three downregulated miRNA (MiR-101-3p, MiR 144-3p and MiR-19a-3p) which are associated with cardiovascular diseases, Alzaimers and some type of cancer according to the *Silico* function analysis. Amongst all, the central nervous system has been found to be the most sennsitive target to PFAS. PFOS and PFOA are found to be associative with increased motor activity, decreased habituation, deficits in spatial learning and memory ability, developmental neurotoxicity, effect the pre- and postsynaptic processes and acutely affect the α_1 β_2 γ_{2L} $GABA_A$ receptor and spontaneous neuronal network function (Tukker et al., 2020). The liver is considered as the waste removal, but this is not the same for PFAS since long chain PFAS have high absorption affinity in the body, only few short chain PFAS will be released. According to the review by Stanifer et al. (2018), the accumulation of PFAS in the kidneys result in adverse health consequences including kidney functions, cellular derangement in the proximal tubules and dysregulated metabolic pathways linked to kidney diseases.

2.1.2.7 Impact on the ecosystem

PFAS have been proven to have a severe effect on living organisms on studies mostly based on invertebrates and vertebrates. Some of the effects include growth reduction, decrease in reproduction and production, size reduction, change in heart rate, delay in hatching in other fish and reduction in off-spring survival (Bartlett et al., 2020). According to the review by Sinclair et al. (2020) more research is still needed specifically on the health effect associated with the long-term exposure, a better ecotoxicological monitoring using biomarkers and the effect of PFAS synergy in the environment. The concentrations that have been used in monitoring the toxicity of PFAS in the laboratory are way too high (range from µg to mg) compared to those that are detected in the environment (mostly in ng) (Ankley et al., 2021). As can be seen in the section above, the concentrations that are detected in every environmental compartment ranges around nano-grams which are very low compared to those that are used for toxicity studies. PFAS are very persistent in the environment and they have been estimated to persist in aqueous samples for more than five years. According to the review by Bartlett et al. (2020) the presence of PFAS in low concentration can increase the bioaccumulation factor and the longer the organisms remain in the polluted environment the more the accumulation.

Another by Jantzen et al. (2017) confirmed that organisms that are exposed to low concentration (nano-gram range) of PFAS are also associated with similar adverse effects.

The effects of PFAS on plants have not yet been observed, but the fact that plants can accumulate almost every PFAS analyte possesses danger to the organism that feeds on them. Living organisms accumulate PFAS through nutrition as mentioned in the section above and biomagnifies through food the web. High concentrations of PFAS have been found in apex predators compared to other fauna organisms. The concentrations of PFAS increases among the fauna in this trend; mammals > birds > reptiles > fish > invertebrates (Rumsby et al., 2018). In microorganisms PFAS have been found to be associated with suppression of algae population, dysfunctional and physiological abnormalities in algae cell membrane, oxygen response stress and infect the size of the individuals and their eggs (these strains can later be passed to the offspring). In macro-organisms such fish, rodents and monkeys, the presence of PFAS is associated with damage of the oestrogen activities, disruption of thyroid hormones, suppression of the efflux transport protein, induction of tumour and damage in the liver, testosterone level decrease and increase in estradiol level (Liu et al., 2019).

2.1.2.8 Regulations on PFAS

Concerns have been raised about PFAS due to their toxicity, bioaccumulation, and persistency. Many regulatory authorities and industries aiming at restricting the production, uses and release of the long chain PFAS, especially C6 and C10 homologs and their potential precursors, have decided to eliminate the production of these compounds (Wang et al., 2019). Perfluorooctane sulfonic acids, its salts and perfluorooctane sulfonyl fluoride were added to the annex B of the Stockholm convention in 2009 to implement the restriction on their production and uses (Lockwood et al., 2019). Starting from 2012 to 2017 perfluorooctanoic acid and perfluorohexane sulfonic acid, C6 and C10 perfluoro carboxylic acids including their salts and precursors were added as the contaminants of the highest concern to the European chemical regulation. Consequently, industries have decided to produce short chain PFAS and novel PFAS such as 6:2 chlorinated polyfluoroalkyl, 6:2 fluorotelomer sulfonates and perfluoro ether carboxylic acid. These regulations have been found effective in limiting the amount of PFAS. For instance, Miaz et al. (2020), have observed a decline in PFAS from pooled serum level on first time mothers in Upssal (Sweden) from 1996 – 2017.

To ensure that everybody is living under a healthy environment and that the environment must be protected from hazardous chemicals is within the South African bill of rights. As mentioned

above, there are no reports on the production of PFAS in South Africa and the imported product may contribute to the distribution of PFAS in the country. To address these issues, in 1997, governments agreed that the way to manage the issue of contaminants was to bind international agreements and this legally binding instrument came into force in 2004. The South Africa government became the party of the Stockholm convention on the 4th of September 2001. The key aspect of the convention is to protect the human health and the ecosystem from persistent organic pollutants, which are intentionally and unintentionally produced and in stockpile and wastes, with the plan to limit or eliminate their production (Department of Environmental affairs., 2016). PFAS has been listed in the annex B of the Stockholm convention to be phased out with acceptable purpose and specific exemption Carlson et al., 2020).

2.2 Analysis of organic emerging pollutants

Environmental analysis of emerging pollutants may be challenging due to the nature of various matrices. Conventional analytical instruments used for analysis of organic compounds include spectroscopic methods such as ultra-violence visible photometry (Patle et al., 2020; Martelo-Vidal et al., 2014), diffuse reflectance-fourier transform infrared spectrometry (Kurrey et al., 2019) and enzyme linked immunosorbent assay (Wang et al., 2019). However, due to the complexity of environmental samples and the demand for fast and ultra-trace analysis of multiclass components, very sensitive and robust instruments have been invented. Ideally these instruments are composed of two components, namely separation (i.e., to enable multiple analyses of different compounds) and detection (i.e., for qualitative and quantitative) (Vijay et al., 2021). Examples of separation techniques are chromatography and capillary electrophoresis. Examples of detectors are fluorescence, UV-Vis, photodiode array, diode array and mass spectrometry.

The desire to analyse analytes in trace level with best selectivity and sensitivity have boosted the development of analytical instruments to the highest level. UV Vis, photodiode array, diode array and mass spectrometry are the mostly used detectors in trace analysis. However, the mass spectrometer stood out from the rest. Recently, mass spectrometric techniques such as triple quadrupole and quadrupole time of flight mass spectrometry have gained a lot of attention when it comes to trace analysis. All mass spectrometers have the same principles which is to ionise sample components and measure their mass to charge ratio. Traditionally, hard ionisation such as electrochemical ionisation was applied to ionise and fragment compounds. For analysis of environmental, soft ionisation such as electrospray ionisation has been routinely

used to protonate/deprotonate and fragment compounds to detect their precursor ions and its product ions. Modern mass spectrometers have the capability to scan all the analytes and impurities present in the sample in one run through multiple monitoring mode (MRM). MRM is both applicable in target and non-target analysis. This tool is very helpful in differentiating structural isomers.

2.2.1 Separation techniques

2.2.1.1 Chromatography

Chromatography is one of the strongest techniques that is used for separation, identification and purification of analytes. There are different types of chromatography, i.e., thin layer chromatography, liquid chromatography, gas chromatography and ion chromatography. However, liquid chromatography and gas chromatography are the instrument of choice through environmental surveillance (Richardson et al., 2021; Saxena et al., 2021; Ioime et al., 2021).

Liquid chromatography is a chromatographic technique where the analytes are separated on the separatory column. Liquid chromatography is mostly compatible with non-volatile compounds; however, it can also be used for separation of volatile compounds (Xia et al., 2021). Traditionally, separation requires a stationary phase to retain the analytes and mobile phase to move and separate the analytes. The choice of a stationary and a mobile phase is based on the type analytes that need to be separated. For example, non-polar compounds are better retained by a reverse phase and eluted by polar mobile phase (Vrancheva et al., 2021; Reyes-Garcés et al., 2021), while polar compounds are better retained by normal phase chromatography and eluted by non-polar mobile phase (Jandera et al., 2020). Recently, hydrophilic interaction liquid chromatography or HILIC stationary phase has been used to retain strong polar compounds, where water is mixed with the organic mobile phase to increase its polarity and enhance the separation of analytes (Reyes-Garcés et al., 2021; Serra-Mora et al., 2021). Modern liquid chromatography uses high pressure to effective and efficiently separate analytes smaller particle sized column to increase the number of theoretical plates. Examples includes high performance liquid (Wang et al., 2021) chromatography and ultra-performance liquid chromatography (Narváez et al., 2020). These instruments are normally coupled with diode array detector, photodiode array detector, mass spectrometer and tandem mass spectrometer for detection of analytes.

Gas chromatography is a chromatographic technique where the analytes are separated on the long thin column. Gas chromatography is only compatible with volatile compounds. However, derivatives can be added to the compounds through derivatisation methods to make them volatile so that they can simply be analysed by gas chromatography (Moldoveanu et al., 2019). In this case the mobile phase is an inert gas such as nitrogen or helium and the stationary phase is either a packed column or coated liquid. The compounds are retained and separated based on their polarity, volatility and their interaction with their stationary phase. GC instruments are coupled with diode array detector (Gras et al., (2017), mass spectrometer and tandem mass spectrometer (Ly et al., (2020) for detection of analytes.

2.2.1.2 Capillary electrophoresis

Capillary electrophoresis is a separatory technique where the analytes are separated on the capillary tube coated with silica particles. Capillary electrophoresis configuration is one of the simplest techniques which only has a capillary tube that consists of a viewing window. The viewing window of a capillary tube is aligned with a detector (Leach, 2005). The two ends of the capillary are directly immersed in a buffer solution. The buffer solution consists of two electrodes, i.e., the anode on one side and a cathode on the other side. The flow of buffer solution from one end to the other is totally controlled by the electric field inside the capillary wall and the voltage applied on the electrodes. For instance, the pH of the buffer ionises the silanol groups into negative charge inside the capillary wall, and the positive charge ions inside the capillary interact with this negative charge wall to form an electrical double layer. When the voltage is applied to the two electrodes, one side of the buffer solution becomes net positively charged and the other becomes net negatively charged. This enables the cations on the diffused passion of the double layer to migrate to the direction of the cathode in a motion called electroosmotic flow. Unlike chromatography where the analytes are separated through retention on the stationary phase based on their polarity, the analytes get separated capillary based on their size and charge. Another comparison between chromatography and capillary electrophoresis is that the motion of the fluid inside the capillary is driven by a wall which enables the entire flow to be at the same velocity. While the friction between the liquid-sorbent interface in chromatography, such as packing and the wall of the tubing, results in substantial pressure drop. This results in the high flow rate in the middle compared to the interface to create a non-uniform motion resulting in a lamina flow or parabolic flow. As a result, capillary electrophoresis has less peak broadening and peak tailing compared to chromatography. CE

are coupled with UV (Mehaffey et al., 2020) and mass spectrometers (Moreno-González et al., 2021) for detection of analytes.

2.3 Critical analysis of literature

In this section, two critical review have been written which focusses on the used of miniaturised extraction techniques for pre-concentration of antibiotics and PFAS different sample.

Paper I

This book chapter “I Innovative liquid phase extraction based analytical extraction techniques of antibiotics as emerging pollutants from different water sources” is submitted to Emerging Freshwater Pollutants. It describes the recent miniaturised liquid phase extraction techniques and the used of innovative extraction solvents.

Innovative liquid phase extraction based analytical extraction techniques of antibiotics as emerging pollutants from different water sources

Gershom Mpela Ntshani, Nikita Tawanda Tavengwa*

Department of Chemistry, School of Mathematics and Natural Science, University of Venda, Private Bag X5050, Thohoyandou, South Africa, 0950

*Corresponding author: nikita.tavengwa@univen.ac.za

Abstract

Antibiotics are a group of natural, semi-synthetic and synthetic compounds that have a wide range of application in treatment of human and animal diseases, prevention of incoming infections and improve animal growth in agriculture. The distribution of antibiotic residues in the environment results in accumulation in food and freshwater. Ingestion of food and drinking water contaminated with antibiotics is associated with a wide variety of health complications and sometimes can lead to death. Thus, routine analysis of antibiotics in the environment, especially freshwater is very important as a first step to ensure provision of clean water. Extraction techniques are very important for isolation, pre-concentration and detection of low concentration of antibiotics in aqueous solutions. Recently, miniaturised extraction techniques have attracted huge attention from the scientific community as they comply with the principles of green analytical chemistry. Miniaturised extraction techniques and the modern analytical instruments do not just solve the problem of exposing hazardous chemicals in the environment, but they also facilitate the detection of analytes present in ultra-trace level, reduce extraction time and cost, and the obtained results are reproducible and repeatable.

Keywords: Antibiotics, emerging contaminants, green analytical chemistry, advanced analytical techniques

1. Introduction

The life expectancy for human beings before the discovery of antibiotics was very short, with an estimation of 47 years. Diseases like smallpox, cholera, tuberculosis, diphtheria, pneumonia and fever were rampant (Adedeji, 2016). Infections caused by a cut or burn were fatal. The discovery of penicillin during World War II was one of the most profound discoveries in the

world (Gaynes, 2017). Penicillin is one of the naturally produced antibiotics which is produced by microbes or plants as defence against other microbes (Jiang et al., 2020). Recently, a lot of efforts has been made in laboratories to modify natural produced antibiotics to produce semisynthetic antibiotics such as doxycycline, metacycline, minocycline and other antibiotics that have unique structures or properties from naturally produced antibiotics called synthetic antibiotics (Chopra et al., 2001). The uses of antibiotics have been expanded to agriculture for treatment of diseases, prevention of incoming infections and improvement of animal production. During the midst of World War II, food supply was very crucial both for the citizens and the men and women on the forefront. For protection of livestock against diseases, different antibiotics were trialled against infectious diseases, and it was found that sub-therapeutic antibiotics could not just improve animal health, but the animal could also gain body weight (Kirchhelle, 2018). This was an important discovery because antibiotics in animal feed can act as growth promoter and was a very good substituent for the expensive vitamin B_{12} supplement. Until now, different antibiotics have been used in different agricultural practices and their estimated consumption is higher compared to human consumption (Manyi-Loh et al., 2018).

It is not a doubt that antibiotics have been a very useful tool for protection against deadly infections and to improve higher production of food in agriculture, but if care is not taken, they could also be deadly. For years, researchers have observed that antibiotics are not completely digested after they are consumed and their residues are being released in active form into the environment (Youngquist et al., 2016; Yin et al., 2019). High content of antibiotics has been detected in agricultural manure and wastewater treatment plants (Van Epps et al., 2016; Pham-Duc et al., 2020; Rodriguez-Mozaz et al., 2020). The use of irrigation in agricultural farmlands has resulted in residues of antibiotics in fruits and vegetables (Pan et al., 2017). More concerning is their release and detection in freshwater sources such as dams and rivers. This water is normally used by people for drinking and domestic uses in rural areas. Indirectly, people in urban areas are even more exposed because the wastewater treatment plants meant to remove emerging contaminants such as antibiotics are not 100% efficient as they are not originally designed to do so.

The ingestion of food and drinking of water contaminated with antibiotic residues is associated with severe health consequences including carcinogenic effect, and in a serious situation the results can be fatal (Bilal et al., 2020; Kraemer et al., 2019). The presence of antibiotics in the environment is also another challenge. This is because the main function of antibiotics is to kill

or inhibit the growth or metabolic activities of microorganisms (Kovalakova et al., 2020). While the environment is teemed with different kinds of microorganisms, some of these them are essential for the environmental regulation such as biodegradation and convention of the greenhouse compounds (Ngqwala et al., 2020). Antibiotic resistance bacteria and antibiotic resistance genes caused by selective pressure due to the presence of antibiotic residues in the environment is also one of the global concerns (Saxena et al., 2021). Antibiotic resistance genes can simply be transferred to other microbes without any physical contact among organisms through horizontal gene transfer (Bena et al., 2019; Danner et al., 2019). These results in a large pool of dangerous untreatable microbes in the environment, which will be devastating both economically and socially (Friedman et al., 2016).

Previously, analysis of organic compounds (Kim et al., 2020) and metal ions (Liu et al., 2020) have been a routine to protect the environment from hazardous chemicals and to ensure the quality of food and water security (Domínguez et al., 2020). Antibiotics were seldom analysed probably because the instruments that were used back then were incapable of detecting them. The recently developed analytical instruments or methods such as enzyme-linked immunosorbent assay (Wang et al., 2019), capillary electrophoresis (He et al., 2019), gas chromatography and liquid chromatography coupled to tandem mass spectroscopy or high resolution mass spectroscopy (Zhao et al., 2020; Li et al., 2020; Uddin et al., 2020) with the capability to detect and quantify analytes in parts per trillion had made it possible for analysis of trace antibiotic analytes. Trace analyses is very important not just for awareness but to open doors for researchers to determine their effect on human health and other living organisms (Rodriguez-Mozaz et al., 2020). These include the effect of the long-term exposure and the induction of selective pressure for bacterial resistance (Luo et al., 2020). Recently, it has been proposed that analytical techniques should adhere with the concept of green analytical chemistry (Billiard et al., 2020). The principle states that if possible, samples should be directly analysed (Armenta et al., 2018). However, due to the complex nature of environmental samples it is not simple to directly analyse samples. Environmental samples constitute a wide range of compounds including organic compounds, organometallic compounds and metal ions (Zhang et al., 2019). Thus, these contaminants can cause false positives or false negatives during analysis (Van den Meersche et al., 2016). Isolation of analytes is a very important step before analysis (Dugheri et al., 2020). To comply with green chemistry, miniaturised extraction techniques have been proven environmentally friendly, selective, sensitive and accurate (Soares da Silva Burato et al., 2020). Liquid phase micro-extraction (LPME) is one of the some

of the most widely used miniaturised extraction technique (Vakh et al., 2021; Ma and Row., 2021; Shahi et al., 2021). Compared to miniaturised-SPME techniques, miniaturised LPME solvents are easy to prepare, the solvents are cheap and widely available, and they are compatible with the chromatographic instruments. In contrast, SPE sorbents are associated with poor elution of analytes, and mostly their preparations are lengthy while most of them are not reusable (Farajzadeh et al., 2020).

The aim of this review is to discuss the use of LPME techniques in antibiotics as emerging contaminants of freshwater. The focus will also be on the advantages and disadvantages of the LPME techniques. The use of new extractive solvents to minimise the environmental burden of antibiotic residues inn freshwater will also be explored through recent miniaturise extraction techniques. Lastly, future trends are discussed looking into improving the extraction process in terms of reducing coat, time, labour and hazardous chemicals.

2. Liquid phase extraction

Liquid phase extraction is an extraction technique that is used to isolate or extract the desired analytes from a sample matrix through partitioning between two immiscible solvents i.e., water and an organic solvent such as dichloromethane (Smink et al., 2020). This technique has been used for extraction of a wide spectrum of antibiotics in different environmental samples. Antibiotics are classified as polar organic compounds, and they have more affinity to polar organic solvents, i.e., dichloromethane, chloroform, methanol and acetonitrile (Zhang et al., 2019). Conventional liquid-liquid extraction (LLE) technique requires 100% extraction efficiency, and this can be done through a series of repetitions until the ideal recovery is reached. This is because the extraction solvents have poor selectivity toward the desired analytes. The use of conventional LLE technique has also been criticised based on its failure to comply with the principles of green analytical chemistry (Dugheri et al., 2020; Dmitrienko et al., 2020). These shortfalls include high organic solvent consumption, high waste discharge, tedious and inability to be automated. A lot of effort has therefore been made over the past few years to improve the extraction process (Figure 1).

Recently, LLE techniques have evolved from a 500 mL separatory funnel (Peng et al., 2020) to a 10 mL centrifuge tube/ test tube and thus reducing the amount of extraction solvent and sample solution to about 1 to 5 mL and 10 mL, respectively (Figure 1) The volume of extraction solvent and the sample have been further reduced through the introduction of innovative extraction techniques called miniaturised liquid phase extraction techniques. Miniaturised

LLME have been introduced to reduce some the disadvantages encountered in conventional LLE technique by using low volume of organic solvent (in microliter) to improve the extraction efficiency, accuracy and to increase the enrichment factor. The enrichment factor is improved because when analytes are being extracted into low volume of organic solvent, its concentration will be enhanced. The use of low extraction volume does not only comply with green extraction technique but to be able to pre-concentrate the desired analytes. In this case, analytes that are present in trace level can be pre-concentrated into small volumes of organic solvents to increase their concentration so that they will be detected with instruments with slightly higher detection limits.

The organic solvent can be separated through alteration of temperature, sample pH or the ionic strength. The most used phase separation technique is the change of ionic strength (Farajzadeh et al., 2018). This has been made possible through the extraction technique called homogeneous salt assisted LLE or H-SA-LLE (Khatibi et al., 2020; Moreno-González et al., 2021). One of the requirements is that the type of salt added into the solution should have a partial solubility with the organic solvent e.g., sodium chloride, sodium acetate and ammonium acetate (Ahmed et al., 2020). The mechanism around this process is that, as salt molecules dissociate in water, some of the salt ions are attracted to the water molecules resulting in less interaction between water molecules and organic molecules, the process is called salting out effect (Li et al., 2020). Addition of salt is also beneficial to the extraction process because it increases the ionic strength in aqueous phase which facilitates the partitioning of analytes into the organic phase. But there is a precaution that need to be considered when adding salt into the mixture, because two much ionic strength can increase viscosity of the extraction solvent, which in turn reduce the analytes partitioning or extraction efficiency (Khatibi et al., 2020); (Rashidipour et al., 2019). H-SA-LLE have been recently applied for the extraction of antibiotics in milk (Mohebi et al., 2020), urine (Fikarova et al., 2021), honey (Thompson et al., 2019) and water (Chandrakar et al., 2020; Mokhtar et al., 2019) due to its high enrichment factor, percentage recovery and low limits of detection (Moreno-González et al., 2017). However, due to poor selectivity of the used polar extractive solvents mentioned above, this technique is coupled with other clean up techniques such as DLLME (Mohebi et al., 2020) or SPE (Fikarova et al., 2021). The use of acetonitrile of methanol is beneficial for DLLME because this solvent is in turn used as dispersive solvent (Mohebi et al., 2020).

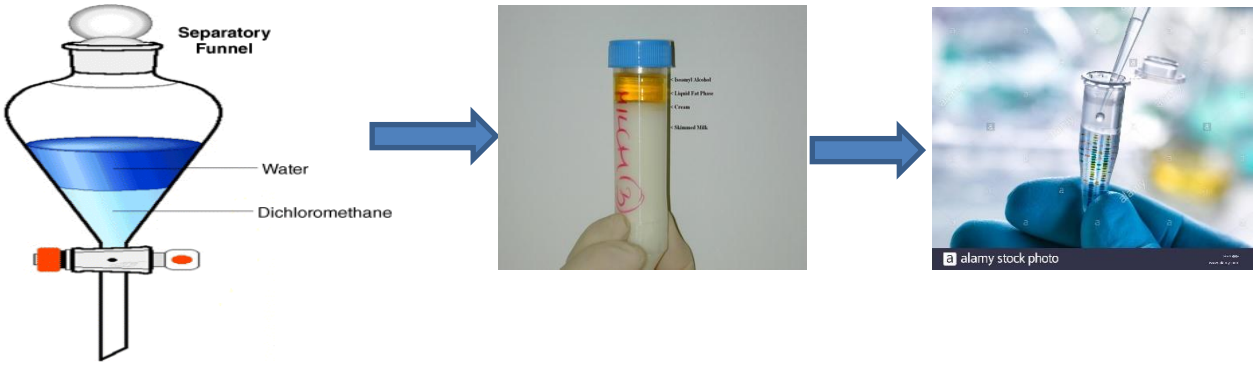


Figure 1: Transformation of the extraction from classical to modern extraction technique

Over the past few years, different innovative miniaturised extraction techniques have been studied for extraction of antibiotics in different environmental, biological and food samples. The first miniaturised LLME called single drop micro-extraction (SDME) was introduced by Jeannot and Cantwell (1996) for the extraction of 4-methylacetophenone using 8 µL of n-octane as extractive solvent. In this technique, a small droplet of an extractive solvent is held above the sample solution (head space-SDME) or directly immersed on the sample solution (direct immerse-SDME) using a micro-syringe for extraction of analytes. However, its application for extraction of antibiotics has recently dropped due to some limitations such as instability of the droplet and limited choice of the extractive solvents (Khatibi et al., 2020). To overcome such drawbacks, new techniques with the capability to store the extractive solvent in a porous hollow fiber membrane in the technique called hollow fiber liquid phase micro-extraction (HF-LPME) have been introduced. The recommendations are that solvents must be immiscible with the aqueous phase and should have higher affinity with the desired analytes. HF-LPME is a miniaturised liquid phase micro-extraction technique that applies porous hollow fiber membrane for isolation of desired analytes. In this extraction technique, an organic solvent and sometimes an ion pair reagent, usually 1-octanol and aliquat-336, is applied on the surface of the HF membrane to allow analytes to be extracted in the organic surface layer and then to the aqueous phase inside the membrane with an assistant of agitation. The use of ion pair reagent is one of the techniques used through HF-LPME and other techniques include pH gradient and electro-membrane extraction. The detailed procedures of miniaturised-LLME techniques are illustrated in Figure 2.

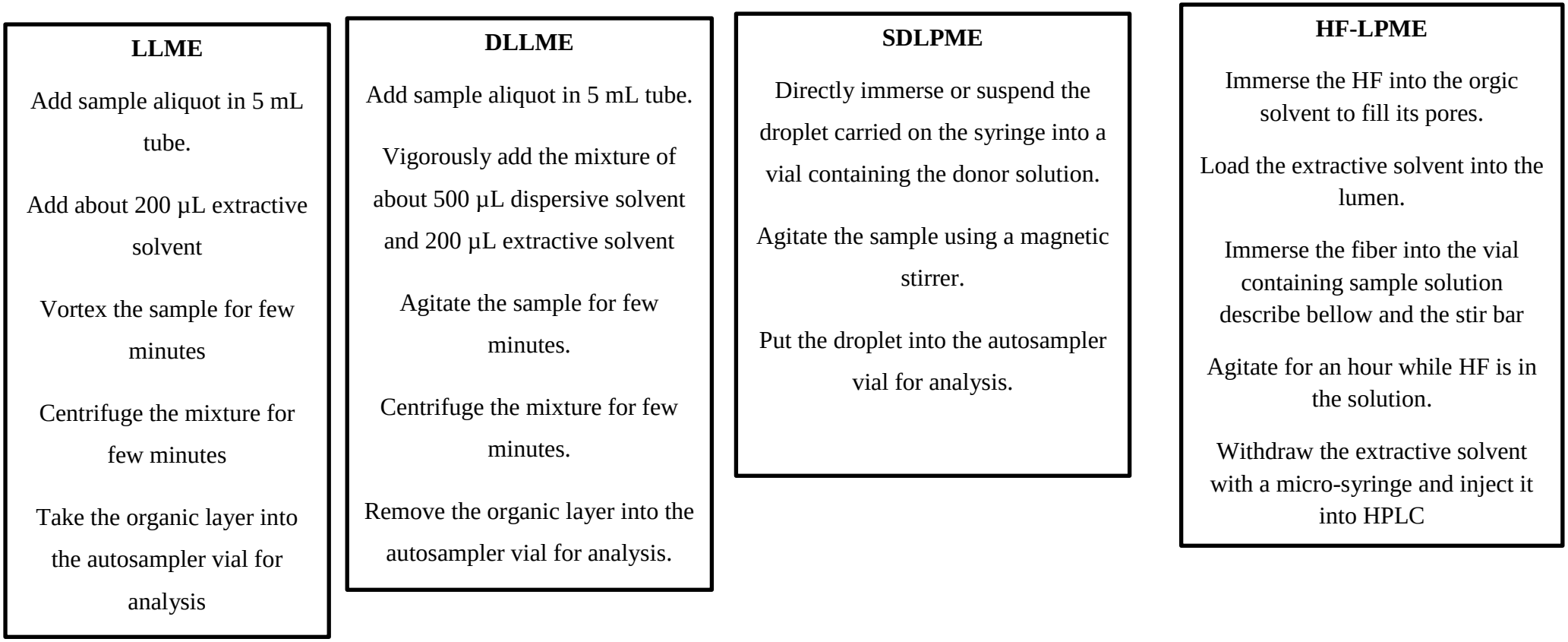


Figure 2: Miniaturised liquid phase extraction techniques

The interesting fact about miniaturised-LLME techniques are their flexibility to be automated, quick, inexpensive, selective and the use of environmentally friendly extractive solvents (Madikizela et al., 2020). Other innovative miniaturised extraction techniques are LLME and dispersive liquid-liquid micro-extraction (DLLME) and their procedure have been well outlined in Figure 1. These extraction techniques share the same concept of minimizing the extractive solvent and to comply with the principles of green analytical chemistry. Another benefit associated with these techniques include their ease of operation which do not require any intricate steps. LLME of antibiotics requires the optimum organic solvent, normally methanol and acetonitrile as in SALLE described above, and the optimum condition such as pH and ionic strength adjusted and the optimum vortex and centrifuging time. The difference between DLLME and LLME is that DLLME uses dispersive solvent to facilitate the movement of analytes into the extractive solvent but the procedures are similar with few additional steps in DLLME, see Figure 2 (a).

3. Extractive solvents

The extraction techniques mentioned above are regularly performed with the use of hazardous chemicals that are normally derived from petroleum and natural gases, and other hazardous reagents. Using water as an extractive or eluting solvent would be ideal since water is the best environmentally friendly solvent which is cheap in terms of production, transportation and disposal (Castro-Puyana et al., 2017). But water is not a solvent of choice when extracting antibiotics in normal conditions. However, the properties of water can be tuned by increasing the temperature and pressure until it is at the state called subcritical point (Esmaeelian et al., 2020). At this state, water behaves like polar solvents, such as methanol, and can be used to extract analytes through the technique called subcritical water extraction. Wang et al. (2008) have extracted three tetracyclines in animal feed with the method percentage recovery of 82.1% and 90.0%. Beside the high recovery of this method on extracting tetracyclines, subcritical water extraction is not suitable for extraction of other antibiotics. These disadvantages arise from using high temperature which might degrade some of thermolabile antibiotics. At these conditions, water can also be more soluble in such a way it loses its selectivity in complex matrices. Recently, large focus is on the use of biodegradable and environmentally friendly solvents for extraction and elution of antibiotics and other contaminants.

3.1 Super-molecular solvents

Super-molecular solvents (SUPRASs) are nanostructured liquids produced in colloidal solutions of amphiphilic compounds by spontaneous, sequential phenomena of self-assembly and coacervation (González-Rubio et al., 2020). SUPRASs are insoluble in water which makes them ideal for LLME with high extraction efficiency. Another unique feature is that when they are added into the solution, they self-assemble or aggregate into micelle or vesicles (Figure 3) depending on the type of the solution they are added (Torres-Valenzuela et al., 2019). Their density is usually less than that of the water, and after the extraction is completed, they can be withdrawn on top of the sample solution. The analytes can be extracted without the use of organic solvents, acids and heating (Seebunrueng et al., 2017). The mechanism which leads to better extraction of antibiotics is through hydrophilic and electrostatic interaction with the solvent (Gissawong et al., 2019; Selehle et al., 2019) have used the mixture of decanoic acid and tricaprylmethylammonium chloride as supper-molecular solvent base LLME for the extraction of three fluoroquinolones in wastewater prior to HPLC-DAD. The method had LOD and LOQ of 0.06 – 0.14 µg L^{-1} and 0.22 – 0.47 µ L^{-1} respectively. Gissawong et al. (2019) have used a mixture of didodecyldimethylammonium bromide and dodecyltrimethylammonium bromide based LLME for extraction of tetracyclines in milk and honey samples. The method had a high enrichment factor of 48 -198 and low limit of detection.

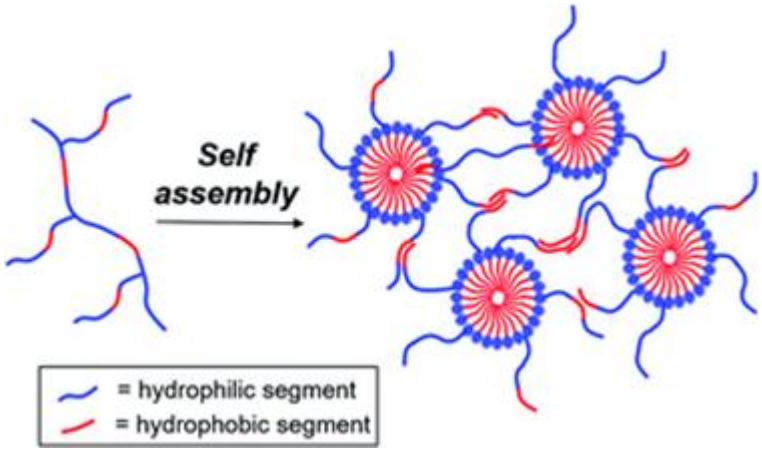


Figure 3: self-assembly of SUPRAS solvent

The disadvantage of the technique is that its effectiveness on biological samples may be poor. These arise due to the presence of hydrophobic parts in compounds which might interact with hydrophobic compounds such as lipids which might increase the matrix effect. While evaporation through the microwave could be effective but it could have some effect of some thermal sensitive antibiotics. In most cases for biological molecules, the method is coupled

with other extraction techniques such as DSPE and still the method has been proven to be environmentally compliant (González-Rubio et al., 2020).

3.2 Ionic liquids

Ionic liquids are classified as organic salts that constitute anionic and polyatomic inorganic anion (de los Ríos et al., 2013). These salts are characterised by being liquid below 100˚C, negligible vapour pressure, good thermal stabilities, high ionic conductivity and simple functionality (Vafaeezadeh et al., 2016). These properties are ideal for ionic liquids to be used as substituent of volatile solvents and other hazardous chemicals. Ionic liquids have also been coupled with adsorption particles that are used in miniaturised-SPME techniques to improve the extraction performance. da Silva et al. (2018) have developed a reproducible, simple, and fully automated online SPE–LC–ESI-TOF-MS for extraction and detection of sulphonamides in bovine milk samples using silica based ionic liquid. 1-butyl-3-methylimidazolium hexafluorophosphate as ionic liquid proved to be very effective for isolation of the desired analytes with lower LOD of 5 – 7.5 µg mL^{-1} and high percentage recovery of 74 – 93%. Lu et al. (2020) have also incorporated 1-aminopropyl-3-methylimidazolium chloride bis(trifluoromethane)sulfonimide lithium salt ([H_2Nmim] [NTf_2]) as an ionic liquid and Zr molecular (UiO-66-Br) organic framework to form [H_2Nmim] [NTf_2] @UiO-66-Br based DSPE for extraction of sulphonamides in water samples. The key interaction of the desired analytes and the nanocomposite, as demonstrated by the author were through electrostatic interaction, pi-pi interaction, and hydrogen bonding interaction. The method is a fast, sensitive, efficient, and economical method for detection of SAs with 270 enrichment factor and 90.5 – 101.9% recovery.

The use of ionic liquids has recently dropped because most of them are toxic. However, their application in antibiotics is found to be more effective through miniaturise-LLME. Wei et al. (2018) have effectively developed a simple LLME for extraction of five sulphonamides in blood samples using 1-hexyl-3-methylImidazolium chloride([C_6MIM] Cl), K_2HPO_4 ionic liquid as an extractive solvent. The solvent proved to be effective with LOD range of 2.45 – 4.13 µg mL^{-1} lower than MRL for sulphonamides and higher percentage recovery of 85.5 and 110.9%. Chatzimitakos et al. (2018) have applied magnetic ionic liquid based DLLME for extraction of sulphonamides and triazines in aqueous samples. The use of magnetic ionic liquid facilitated the extraction process and limited the extraction time. Clearly more research is needed for synthesis of ionic liquids that are compatible with the environment.

3.3 Deep eutectic solvents

Deep eutectic solvents are eutectic mixtures that are composed of two or more components whose melting points are lower compared to those of their individual components (Lee et al., 2020). They are liquid at room temperature and can be used as an extractive solvent. These solvents have been used as an alternative for ionic liquids to replace disadvantages associated with it (Liang et al., 2016). DESs are considered subclasses of ionic liquid which consist of hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA). These solvents share some common properties with ionic liquids, such as being liquid below 100˚C, negligible vapour pressure, good thermal stabilities and high ionic conductivity. There are different types of DES, and normally those that are used for antibiotics extraction are composed of a mixture of organic acid (as HBD) and the hydrogen bond acceptor (HBA) (Saei et al., 2020). The most common HBA used for sample extraction techniques is choline chloride. Saini et al. (2020) have synthesised DES using the combination of choline chloride as HBA and pivalic acid as HBD for extraction of penicillin G, oxytetracycline and tilmicosin in hamburger and cow liver through dispersive liquid-liquid microextraction and the method was very selective and have higher percentage recovery. However, choline chloride DES are normally water miscible and to break their miscibility a demulsifier such as tetrahydrofuran or acetonitrile is added (Lee et al., 2020). A technique called solidification of floating organic droplets such as the one used by Saei et al. (2020) can be used to separate the DES from aqueous sample. Other DES that are immiscible in water called hydrophobic DES have also been used for antibiotics extraction. Sereshtet et al. (2020) have synthesised the hydrophobic DES using octanoic acid as HBD and thymol as HBA for extraction of oxytetracycline, tetracycline, and doxycycline in milk through DLLME. The method proved to be very effective with high percentage recoveries. Mohebi et al. (2020) have synthesised DES (the combination of phosphocholine chloride, dichloroacetic acid and dodecanoic acid in a 1:1:1 ratio) prior to extraction of antibiotics in milk samples using the combination of salt assisted LLE and DLLME. The method solvent was also immiscible in water and have successfully extracted the antibiotics with 65 – 81% recoveries and 2.0 – 2.8 μg L^{-1} and 6.5 – 9.3 μg L^{-1} LOD and LOQ, respectively.

3.4 Other solvents

Supercritical fluid can be defined as fluid that is at the state higher than critical temperature and pressure. Of interest is the fact that these fluids can be tailored by varying the temperature and pressure into selective and extractive solvents (Pourmortazavi et al., 2014). CO_2

supercritical fluid is the widely used solvent for extraction of various analytes as it has low critical state.

Table 1: Solvent extraction of antibiotics from different water sources

Method	Solvent	Matrix	Analytes	LOD and LOQ	Recoveries	RSD (intra and inter) (%)	Reference
in situ hDES-SA-LLME-MS/MS	Thymol: heptanoic acid as DES	Surface water	Fluoroquinolones	3.0 ng mL^{-1} and 9.0 ng mL^{-1}	84.4 – 113.63	≤7.78 and ≤7.14	Li et al., 2020
pH-induced deep eutectic solvents based -HLLME-HPLC	Thymol: hexanoic acid as DES	Environmental water	Levofloxacin and ciprofloxacin	0.018 – 0.027 µg mL^{-1} and 0.06 – 0.09 µg mL^{-1}	94.52 - 110.8	≤ 4.21	Ma and Row, 2021
SUPRAS based LLME-HPLC-PDA	decanoic acid and tricaprylymethylammonium chloride	Wastewater effluent and influent	Ciprofloxacin, danofloxacin and enrofloxacin	0.06-0.14 µg L^{-1} and 0.22-0.47 µg L^{-1}	153 – 241 (PF)	3.4 – 4 and 4.1 – 5.0	Selahle et al., 2019

Magnetic ionic liquid-based-DLLME-LC-MS/MS	[P66614+][Dy(III)(hfacac)4−]	Lake water and effluent from a municipal WWTPs	Triazines and sulfonamides	0.034 – 0.091 µg L^{-1}	89 - 101	5.2 – 8.1	Chatzimitakos et al., (2018)
Temperature-Induced Ionic Liquids - DLLME-HPLC-UV	[Bmim]PF6	Environmental water	4-epitetracycline, 4-epichlortetracycline, doxycycline, chlortetracycline oxytetracycline, tetracycline, 4-epianhydrotetracycline and anhydrotetracycline	0.031 – 0.079 µg L^{-1} and 0.10 – 0.26 µg L^{-1}	55.1 – 94.5	-	Hou et al., (2011)
DLLME based on deep eutectic solvents doped with β-cyclodextrin- LC-UV	Thymol: octanoic acid (extractant) and choline chloride: ethylene glycol (disperser)	Well water, rainforest water, coastal sea water, Gardening water and mineral water	Oxytetracycline, tetracycline and doxycycline	1.37 – 4.38 µg L^{-1} 1.37–4.38 and 4.58–14.60 µg L^{-1}	74–113	2.9–4.6%	Sereshti et al., (2021)

Magnetic nanoparticles assisted DLLME – UV-Vis	Methanol (desorbing solvent) and decanoic acid (extractor)	Tap water and lake water	Chloramphenicol	16.5 and 50.0 µg L^{-1}	91–92.7	0.45–6.29	Saad et al., (2020)

4. Conclusion and future work

The presence of antibiotics in the environment is associated with a high negative impact on health and food and water security. Major action needs to be taken before it is too late. It is apparent from the literature that much attention on the presence of antibiotics is focused on developed countries and urban areas. Poor wastewater treatment plants, lack of sanitation and high prevalence of diseases in developing countries are the major sources of antibiotic exposure. Because antibiotics are ubiquitous, their continuous exposure can affect everyone. Thus, more attention is needed in all environmental compartment, especially freshwater sources.

The rise in pharmaceutical companies and the demand for new antibiotics increases the amount of antibiotics that can be found in freshwater systems. This is a problem since analysis of every antibiotic in the environment is time consuming, expensive and sometimes their standards are scarce. To solve such shortcomings, it is highly recommended to introduce innovative analytical methods with capabilities to extract and analyse different classes of antibiotics simultaneously through the method called multi-class analysis. Multi-class analysis is still an emerging concept in the field of analytical chemistry, and it has received much attention due to its advantages such as time saving and the ability to analyse a wide range of antibiotics in a single run. Thus, much effort is needed for this technique. The recent invention of chromatographic analytical instruments coupled to quadrupole time of flight mass spectroscopy (QToF/ MS) has been a success in the field of analytical chemistry. The advantages arise from its capability to perform a quick analysis and the ability to detect the mass of an analyte with highest possible precision and accuracy. The ability of high-resolution mass spectrometry to identify wide range of analytes that are found in a sample with high precision in a single can be used to analyse the presence of unknown hazardous antibiotics residues in the environment with the method called non-targeted analysis (Getzinger et al., 2020).

One can say that the miniaturisation of LLME has more advantages over the conventional extraction techniques. Extraction of analytes no longer requires a high quantity of samples, reagents and solvents. This achievement is very important to protect the environment against exposure from hazardous chemicals. It is also advisable to continue improving these extraction techniques until they are completely environmentally friendly. Such achievement can be

successful through innovation and much effort in research. As discussed above, more effort has been made to perform analysis using green solvents which replaces petrochemicals in miniaturised LLME techniques. Synthesis of adsorption particles that are reusable is another subject of interest in miniaturised SPME technique.

The uses of automation such as robots, flow techniques and microfluidic devices through analytical chemistry has been very useful in reducing some of labour-intensive work during sample pre-treatment and reducing the analysis time (Soares da Silva Burato et al., 2020). These innovations together with the use of powerful analytical instruments and environmentally friendly solvents are very effective through detection of ultra-trace analytes that are reproducible. The use of environmentally friendly analysis should be the responsibility of every analytical chemist, and for decades, a lot of effort has been made to achieve such goals. Agrochemical solvents, sorbents and reagents have been proven to be effective for extraction of analytes and much research needs to be conducted using these solvents.

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4.2 Paper II

This manuscript review article “Dispersive liquid-liquid micro-extraction of per and poly-fluoroalkyl substances as emerging contaminants” will be submitted to Trends in Analytical Chemistry. It describes dispersive liquid-liquid microextraction technique and its application in extraction of perfluoroalkyl substances.

Dispersive liquid-liquid micro-extraction of per and poly-fluoroalkyl substances as emerging contaminants

Gershom Mpela Ntshani, Nikita Tawanda Tavengwa*

Department of Chemistry, School of Mathematics and Natural Science, University of Venda, Private Bag X5050, Thohoyandou, South Africa, 0950

* Corresponding author: nikita.tavengwa@univen.ac.za

Abstract

Per- and poly-fluoroalkyl substances (PFAS) are a group of synthetic fluorinated organic compounds that have a wide range of applications in different industries and households' appliances. The presence of PFAS residues in the environment is associated with severe effects to the ecosystem and human health. Their distribution and accumulation into the environment are highly dependent on their physico-chemical properties. Understanding these properties, their trends and correlation in the environment can streamline their environmental monitoring. However, it is not simple to effectively analyse PFAS without proper extraction techniques. Solid phase extraction (SPE) is the common extraction technique used for extraction of PFAS in different matrices. Effective as it is, SPE is associated with downfalls such as high solvent consumption, high waist discharge, tedious and time consuming. SPE techniques are also associated with false negative results through their incapability to elute all the analytes after extraction. Recently, miniaturized LLE extraction techniques, such as DLLME, have been used for the extraction of PFAS in liquid and solid samples to resolve some of the shortcomings associated with SPE techniques. This review focuses on the use of DLLME as an alternative technique for extraction of PFAS. Conventional extraction techniques such as SPE and LLE have been discussed looking at their interaction with PFAS, their disadvantages and the way in which certain principles can be applied in development of DLLME.

Keywords: Perfluoroalkyl substances, solid phase extraction, green analytical chemistry, miniaturised liquid phase extraction, dispersive liquid-liquid micro-extraction

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1. Introduction

Perfluoroalkyl substances are a group of synthetic fluorinated organic compounds that were introduced to the world market around 1950s (Xiao, 2017; Goodrow et al., 2020). Unlike other compounds, their structures are classified as amphiphilic compounds because of the combination of the hydrophobic C-F alkyl chain and hydrophilic functional group such as carboxylic acid or sulfonic acids in one compound. Carboxylic acids and sulfonic acids are the popular groups of PFAS, but PFAS are diverse with an estimation of more than 20 groups and 5000 compounds (Wu et al., 2020; Sunderland et al., 2019). Looking at the hydrophobic part of the molecule, the bond between the carbon and the fluorine atom is considered one of the strongest covalent bonds which makes these compounds highly stable and degradation resistance in almost every environmental condition. The head of the compound is a highly reactive, hydrophilic, and when it joins with the hydrophobic part, the compounds retain remarkable properties such as water, oil and stain resistance (Kim et al., 2021; Sim et al., 2021). All these properties make PFAS suitable to be used in industries (e.g., paper, steal, petrochemical and electronics industry), household appliances (carpets, polishes, waxes, rugs and leather) and other materials such as aqueous film forming forms (Li et al., 2020).

The presence of PFAS residues in the environment have been confirmed to have severe effects on living organisms such as microorganisms (Zhang et al., 2020), invertebrates (Taylor, 2020) and vertebrates (Abercrombie et al., 2021). Some of the consequences include growth reduction, decrease in reproduction and production, size reduction in offspring, change in heart rate, delay in hatching in some fish and reduction in off-springs survival (Bartlett et al., 2020). In humans, PFAS can be associated with health effects such as gestational diabetes, childhood

obesity, preeclampsia, reduction in vaccine response and higher susceptibility in infectious diseases in children and foetal growth restriction (Szilagyi et al., 2020; Inoue et al., 2020; Goudarzi and Yamazak 2020). According to the review by Sinclair et al. (2020), more research is still needed specifically on the health effect associated with the long-term exposure, a better ecotoxicological monitoring using biomarkers and the effect of synergistic PFAS in the environment. PFAS are very persistent in the environment, and they are estimated to persist in aqueous samples for more than five years (Bulka et al., 2021). According to the review by Bartlett et al. (2020), the presence of PFAS even at low concentration can increase the bioaccumulation factor, and the longer the organisms remain in the polluted environment the more the accumulation. Another review by Jantzen et al. (2017) confirmed that organisms that are exposed to low concentration (nano-gram range) of PFAS are also associated with similar effects such as an increase in serum.

PFAS are present in trace levels in the environment. The recently developed chromatographic instruments coupled to tandem mass spectrometers, have enabled the detection of these compounds in parts per trillion. To improve the detection of analytes in the environmental samples and to isolate the analytes from complex matrices, extraction techniques such as liquid phase extraction (Wang et al., 2018; Vela-Soria et al., 2020; Backe et al., 2013; Guillette et al., 2020) and SPE (Zhang et al., 2020; Zhang et al., 2021; Sanan et al., 2020; Sznajder-Katarzyńska et al., 2020; Scordo et al., 2020) have been used. Compared to other contaminants in the environment, there is a limited knowledge based on the method development and optimisation of extraction techniques used for PFAS. However, most environmental monitoring is routinely performed using SPE (Groffen et al., 2018; Jin et al., 2020; Dauchy et al., 2019) and ion pair extraction (Meng et al., 2019; Guo et al., 2016; Pan et al., 2017; Fair et al., 2019). These extraction techniques are ancient and have been associated with more disadvantages such as inefficient, laborious, tedious, high solvent consumption and high waste discharge, and thus they are not environmentally friendly (Jalili et al., 2020; Huang et al., 2019). Recently, miniaturised extraction techniques such dispersive liquid-liquid micro-extraction (DLLME) have gained a lot of attention in the scientific community due to ease of operation, and the way it complies with green analytical chemistry (Soares da Silva Burato et al., 2020; Pena-Pereira et al., 2020). This review is focused on the recent use of DLLME for extraction of PFAS. Firstly, conventional extraction techniques will be reviewed, looking into their disadvantages and the need to focus on green extraction techniques.

2 Conventional extraction techniques of PFAS

As can be seen in Table 1, solid phase extraction has been the methods of choice for PFAS extraction. The technique uses an extraction cartridge filled with absorption particles or absorbents with the capability to selectively retain PFAS analytes and isolate them from the impurities. The conventional extraction cartridge used for PFAS extraction are the weak anion exchange or WAX (Zhang et al., 2021; Robey et al., 2020; Groffen et al., 2021) and hydrophilic lipophilic balance or HLB (Sanan et al., 2020; Gałęzowska et al., 2020). The adsorption particles in WAX cartridges are called weak anion polymeric resins which are made of primary amine modified divinyl benzene polymer (Fontanals et al., 2019). These sorbents are more desirable for PFAS extraction due to their ease of adsorption and desorption by changing the pH of the adsorbents through sample analysis (Zhang et al., 2020). The main interaction between the acid PFAS on the WAX adsorbents is through the acidic functional group of the PFAS, while other PFAS interact with the adsorbents through other mechanisms such as electrostatic and hydrophilic interaction. In case of poor recoveries for other PFAS, HLB has been used to improve the extraction (Kaiser et al., 2020; Sanan et al., 2020), however, HLB is rarely used for PFAS extraction.

To clearly illustrate the ability of SPE sorbents in extraction of PFAS, Sanan et al. (2020) have effectively developed online-SPE technique using WAX adsorbents which effectively extracted C_4 to C_{12} perfluorocarboxylic acids and C_4 to C_{10} perfluorosulfonic acids, as well as hexafluoropropylene oxide-dimer acid and three fluorotelomer sulfonates PFAS compounds in aqueous sample with high percentage recovery and the ability to tolerate matrix effect. However, the author did not explain the proper mechanism for the matrix tolerance, but the speculation was through the gradient elution of the method. Zhang et al. (2020) have also extracted 25 PFAS in aqueous samples using online-SPE technique also with higher recoveries. The use of online-SPE is the recent trend to improve performance, achieve reliable results and to reduce some of the tedious steps of manual SPE technique. Another important note is that there is a shortage of knowledge based on the application of other absorbents for extraction of PFAS using online-SPE. However, the carbon materials have been speculated to be effective in retaining PFAS, especially long chain PFAS, based on the adsorption affinity in wastewater treatment processes. Deng et al. (2018) have used charcoal for extraction of six PFAS (C_7 – C_{10} PFCAs, and C_6 and C_8 PFSAs) with the method exhibiting good repeatability, low LOD and high recoveries. Other absorption particles are styrene-divinylbenzene polymer resins used by Md. Al Amin et al. (2020) for extraction of perfluorooctanoic acid, perfluorooctane sulfonic acid and 6:2 fluorotelomer sulfonate.

Effective as it is, there are some downfalls associated with the conventional SPE technique such as the use of high organic content and high waste disposal (Wu et al., 2021; Jagirani and Soylak., 2020). The traditional SPE technique is more effective on extraction of short chained or hydrophilic PFAS (Ye et al., 2018). Recently, a lot of effort has been made to improve classical SPE techniques through miniaturisation. These recent innovations are implemented to overcome some of the weaknesses associated with the traditional SPE. For instance, SPE is a very lengthy and tedious technique, it suffers from analyte loss during manual extraction process, and the elution pH for WAX ranges above 8 which may not be compatible with most liquid chromatographic columns (Barreca et al., 2018). The presence of other anionic contaminants in the environmental samples is also a problem when it comes to the selectivity of the adsorbents that are routinely used for classical-SPE techniques. This problem rises from the retention of these compounds which might introduce matrix interference.

Liquid-liquid extraction is also applied for extraction of PFAS using methyl-tert-butyl ether as organic solvent and tetrabutylammonium hydrogen sulfonate as ion pair reagent through ion pair extraction technique (Liu et al., 2020) (Table 2). This technique has been applied in samples such as soil and sediments (Guo et al., 2016), mats and dust (Zheng et al., 2020), fish (Meng et al., 2019) and blood plasma (Pan et al., 2017). Compared to SPE techniques described above, these techniques are faced with more limitations. For instance, Kaiser et al. (2020) have compared three extraction procedures for biological samples using WAX-SPE, HLB-SPE and ion pair extraction. Through the comparison, the authors demonstrated that ion pair extraction is associated with more matrix effects compared to the other two methods. This arises from poor selectivity of ion pair reagents especially in complex matrices such as biological samples. Ahmadireskety et al. (2020) have also evaluated the accuracy of post-clean up strategies by comparing ionic pair method and ENVI-carb. The ENVI-carb had a better relative error of ≤ 20% compared to 51% relative error for the ion pair extraction technique.

Liquid-liquid extraction of PFAS, on the other hand, has another challenge of causing emulsification of organic solvents, especially when the PFAS are present in slightly higher concentrations. This is a challenge since most organic solvents that are used for PFAS extraction are immiscible in water. PFAS, as an emulsifier, will result in lowering the extraction rate and method efficiency. However, Zhang et al. (2021) have demonstrated that during the PFAS extraction the solvents should meet certain principles to effectively extract PFAS analytes. These principles include: (1) The extractant should form a complex structure with the desired PFAS to shift the equilibrium balance toward the organic phase. (2) The extractant

should suppress the emulsification during the extraction. (3) The extractant should be hydrophobic, high solubility in the organic phase and extremely low solubility in the aqueous phase. The author had used the ionic liquid called methyltrioctylammonium bis(trifluoromethylsulfonyl)imide or ([A336] [NTf_2]) which had more advantage over the extraction process, because the structure of the solvent comprises of long chain quaternary ammonium salt and hydrophobic anion [NTf_2]. The long chain strengthens its immiscibility in aqueous sample and the hydrophobicity increases the hydrophobic interaction of PFAS with the solvent, and thus suppressing the emulsification. But this does not resolve the fact that LLE is associated with high solvent consumption, high waste generation, time consuming, tedious, and not environmentally friendly as seen in SPE techniques (Wang et al., 2018; Dugheri et al., 2020).

3 Dispersive liquid-liquid microextraction

Dispersive liquid-liquid micro-extraction (DLLME) is a miniaturised liquid phase extraction technique. Unlike the conventional LLE technique, the analytes are extracted using micro litre solvents (Pacheco-Fernández et al., 2021; Karami et al., 2020; Ebadnezhad et al., 2021). This technique uses a ternary extraction solvent system i.e., a mixture of extractive solvent and dispersive solvent is rapidly injected into the aqueous solution. By rapidly injecting the mixture or simple agitation such as manual shacking creates a turbulence which is due to the formation of micro-droplets in the mixture which looks like a cloudy or milky solution (Sajid and Alhooshani., 2018). The formation of this turbulence is advantageous due to instantaneous partitioning of analytes into the organic solution. DLLME has been recently applies for isolation of both organic (Rashidipour et al., 2019; Dmitrienko et al., 2020; Karami et al., 2020; Hosseini et al., 2020) and metal ions (Sorouraddin et al., 2020; Machado and Tissot., 2020; Laosuwan et al., 2020) in environmental samples. However, it is difficult to directly apply DLLME technique in solid samples due to their complex nature. The solid samples are first subjected to a solid-liquid extraction such as ultrasound assisted (Ebadnezhad et al., 2021; Zhou et al., 2020), microwave assisted (Cui et al., 2020; Kin et al., 2020) or salt-assisted liquid-liquid extraction using methanol or acetonitrile as organic solvent and sodium chloride as salting-out agent (Mohebi et al., 2020; Soltanmohammadi et al., 2020. In most cases, these solvents can also be treated as dispersive solvents during the DLLME steps to facilitate the movement of analytes into the extractive solvent (Vela-Soria et al., 2021; Mohebi et al., 2020) or they can be dried completely using nitrogen gas. The principles are that the extraction solvent must have higher affinity with the desired analytes, immiscible in water and miscible with the dispersive

solvent. The dispersive solvent must be miscible to both the aqueous and organic phase of which acetonitrile and methanol are in most cases perfect candidates.

The application of DLLME for pre-concentration of PFAS is scarce. However, it is important to consider this technique for extraction of PFAS in future and extend its application, because it is easy to operate, cheap and it complies with green extraction technique (Soares da Silva Burato et al., 2020; Pena-Pereira et al., 2020). Looking into the literature, different solvents have been used for PFAS extraction through LLE as mentioned above. However, these solvents have poor affinity toward the analytes and result in poor recoveries, and the extraction must be repeated for two to three times which is tedious and not environmentally friendly. To resolve this, these solvents can be mixed with a dispersive solvent to form an emulsion or fine droplets to enhance extraction efficiency and reduce the volume of the extraction solvents. Vela-Soria have recently published two articles using the combination of SALLME and DLLME for extraction of PFCA (C_6 – C_{13}) and PFSA (C_6 and C_8) in placenta (Vela-Soria et al., 2021) and breast milk (Vela-Soria et al., 2020). The analytes were extracted using 1500 µL of trichloromethane as an extractive solvent and acetonitrile as a dispersive solvent. Other solvents used for PFAS extraction are fluorous affinity solvents (Backe et al., 2013; Wang et al., 2018). The mechanism around these solvents is unique and depends on non-covalent F-F interaction between highly fluorinated compounds (Jean-Marc Vincent, 2009). However, these solvents have low affinity towards short chain PFAS which are less fluorinated or more hydrophilic.

The factors that affect the extraction efficiency of PFAS in DLLME are sample pH, type and volume of extractive solvent, type and volume of dispersive solvent, ionic strength and the agitation time (Wang et al., 2018; Vela-Soria et al., 2020; Vela-Soria et al., 2021). It is very important to account for these factors during the method development to enable a complete partitioning of analytes into the acceptor phase. Acidic PFAS such as perfluoroalkyl carboxylic acid and perfluoroalkyl sulfonic acid are the most prevalent and most studied PFAS (EFSA Panel on Contaminants in the Food Chain (EFSA CONTAM Panel) et al., 2020; Bertanza et al., 2020; Eun et al., 2020). Due to their acidic moieties and the pKa values which range around -2,49 to 4.2, the optimum extraction pH condition ranges around 2 – 4 (Wang et al., 2021; Gagliano et al., 2020). This optimum pH is driven by the fact that analytes become neutral when their pH values equal to or around their pKa values, and neutral compounds are more likely to partition to the organic phase as compared to ionic compounds which have higher

affinity with water molecules (Khafri et al., 2019; Sharifi et al., 2016; Tajabadi et al., 2016). Similar effect has been observed by Wang et al. (2018) where the PFAS analytes were extracted at the optimum pH of 5 and (Vela-Soria et al. 2020; Vela-Soria et al. 2021), the optimum pH was 2. Due to limited data, more research is needed to back up these findings.

The choice of an extraction solvent and the dispersive solvent depend on their affinity with the analytes. PFAS interact with extractive solvents through the hydrogen bond interaction, hydrophobic interaction and F-F interaction. Different PFAS have different hydrophobicity due to their variation in chain length (Park et al., 2020). Liu et al. (2020) have demonstrated that the interaction of PFAS weakly polar solvents such as methyl-tert butyl ether depend on their dipole moment. The interaction increases as the dipole moment of the analyte increases or as the C-F chain length increases. Thus, the use of weakly polar solvent has lower recoveries on short chain PFAS. Fluorine containing solvents such as trifluoroethanol and perfluoro-tert-butanol have been explored for the extraction of PFAS (Backe et al., 2013; Wang et al., 2018). Their wide range of application is attributed to their fluoro affinity and selectivity toward fluorine containing analytes. The concept of using fluorine containing substance is also applied in sorbents for SPE techniques (Table 1). The increase of volumes of extraction solvents and dispersive solvents can both increase and the enrichment factor of the target analytes (Ye et al., 2018; Md. Al Amin et al., 2020). The enrichment factor increases due to enough space for analyte partitioning into the organic phase. However, when the equilibrium is reached, the concentration of analytes in the organic phase will decrease with an increase in volume of extraction solvent since concentration is inversely proportional to volume.

The use of salt and agitation facilitate the partition of analytes into the organic phase. The addition of salts such as sodium chloride increases the ionic strength in the solution, and enhances the partitioning coefficient. But this needs to be done precisely as it might cause theoretically higher results to other PFAS analytes (Liu et al., 2020). The increase in ionic strength is also associated with the increase in viscosity of the organic phase which will result in low partitioning of analytes (Afshar Mogaddam et al., 2020). Agitation such as vortex and ultrasound assistance increase the dispersion of analytes and the formation of micro droplets during DLLME. Normally, the agitation time is increased until an equilibrium is reached.

Table 1: Trends in PFAS extraction techniques

Analytes	Matrix	Method	Adsorbent	Solvent	LOD and LOQ	Recovery (%)	Reference
Carboxylate, sulfonate and sulphonamide PFAS (C_4 – C_{12})	Aqueous	Online SPE - UPLC-MS/MS	WAX (primary amine modified divinylbenzene polymer)	Distilled water containing 20 mM ammonium format and 1% ammonium hydroxide + methanol containing 4% ammonium hydroxide	-	88 – 107	Sanan et al., (2020)
PFOA, PFOSA and 6:2 fluorotelomer sulfonate	Water	SPE and fluoro-SPE-based smartphone detection	Styrene-divinylbenzene polymer resins and fluoro-GEL	Methanol	1 ng L^{-1}		Md. Al Amin et al., (2020)
PFCA (C_4 - C_{10}) and PFSA (C_4 – C_8)	Fats and oil	QuEChERS (LLE & dSPE)-HPLC-MS/MS	Styrene-divinylbenzene polymer resins	Acetonitrile	0.002-0.075 ng L^{-1}	72 – 104	Sznajder-Katarzyńska et al., (2020)

PFOA, PFNA, PFDoA	Water	MSPE and microwave assistance extraction	F_{12}-Fe_3O_4-@mSiO_2 composite	MSPE (Acetonitrile) and microwave assisted (Bis[trimethylsilyl]trifluoroacetamide-Trimethylchlorosilane)	0055 – 0.086 µg L^{-1} and 0.18 – 0.28 µg L^{-1}	93 – 107	Ye et al., (2018)
PFCA (C_4 - C_{13}) and PFSA (C_4, C_6 and C_8)	Environmental water	SPME-UPLC-MS/MS	50:50 C_{18} and aminopropyl silica	Methanol	< LOD to 898 ng L^{-1}	86 – 111	Lockwood et al. (2019)
PFCA (C_6, C_7, C_8, C_9, C_{10} C_{13}) and PFSA (C_4, C_6 and C_8)	Straw berry and olive fruits	QuEChERS and DSPE-LC-MS/MS	Graphitised carbon black (GCB)	Acetonitrile	2.6 – 393 pg g^{-1}	65 – 89	Scordo et al. (2020)
PFCA (C_4 - C_{12}) and PFSA (C_6 and C_8)	Environmental water	Stir bar sorptive extraction	poly(1-vinylimidazole-ethyleneglycol dimethacrylate)	MeOH containing 0.4% ammonia	0.06 – 0.40 ng L^{-1}	80.3 – 122	Yao et al. (2018)
PFCA (C_6 – C_{13}) and	Breast milk	SALLE – DLLME-	-	SALLE (acetonitrile) DLLME (tetrachloromethane)	And 20 pg mL^{-1}	85.9– 110.8	Vela-Soria et al. (2020)

PFSA (C_6 and C_8)		HPLC-MS/MS					
PFCA (C_6 – C_{13}) and PFSA (C_6 and C_8)	Placenta	SALLE – DLLME-HPLC-MS/MS	-	SALLE (acetonitrile) DLLME (tetrachloromethane)	0.02 ng g^{-1}	88.2 – 113.9	Vela-Soria et al. (2020)
PFCA (C_3 – C_{17}) and PFSA (C_4 and C_{10})	Tape and river water and urine	DLLME-HPLC-MS/MS	-	Acetonitrile (dispenser) and perfluoro-tert-butanol (extractor)	0.6 – 8.7 ng L^{-1}	80.6 – 121.4	Wang et al. (2018)
26 newly identified and 21 legacy PFAS	Aqueous film forming forms (AFFF) and groundwater	LLME-HPLC-MS/MS	-	10% trifluroethanol in ethyl acetate	0.71 – 67 ng L^{-1}	69 – 106	Backe et al. (2013)

- Not given

4. Conclusion and future work recommendation

The presence of PFAS in the environment is a serious concern because of its toxicity, persistence and high accumulation. Their distribution and accumulation behaviour seem to follow a specific trend based on their physico-chemical properties, i.e., the hydrophilicity, which is characterised by their chain length, and the functionality. Similarly, these physico-chemical properties are considered during their extraction in the environment. However, there is still a lag of research regarding this field. Most environmental surveillance is performed using classical extraction techniques such as SPE and ion pair extraction which are not environmentally compatible. Thus, more research is still needed in shifting to modern and environmentally friendly techniques. This can be achieved through synthesis and fabrication of extraction solvents and sorbents. These extractants must be capable of retaining a wide range of PFAS, including both short chain and long chain PFAS, and they must be selective toward the target analytes.

Dispersive liquid-liquid micro-extraction is a promising technique for extraction of PFAS. However, its applications on PFAS extraction have been explored recently and more research is still needed in future. This technique has advantages in complying with green extraction techniques, and that its application is easy and cheap compared to modern extraction techniques. DLLME were introduced in 2006 by Rezaee et al. (2006), and since then, many modifications have been made to increase its extraction effectiveness. Conventional solvents used for PFAS extraction are chloroform, 10% trifluoroethanol in ethyl acetate and perfluoro-tert-butanol. Recently, ionic liquids and deep eutectic solvents have been applied in extraction of analytes. However, the use of these solvents is scarce in PFAS analysis. Methyltrioctylammonium bis-(trifluoromethylsulfonyl)imide (Zhang et al., 2021) and choline chloride/ 1-(o-tolyl)-biguanide (Fan et al., 2021) are some of the modern solvents used in DLLME. What is common about these solvents is the presence of an N-H group which interacts with the analytes through N-H – F interaction, which is also beneficial for PFAS selectivity. Thus, more research is still for these solvents. The extraction efficiency can also be improved through optimisation of the experimental conditions such as the sample pH, type and volume of extraction and dispersive solvents, ionic strength and agitation time.

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Chapter three: Materials and methods

This chapter outlines all chemicals, materials and instruments that have been used during the project.

3.1 Chemicals and reagents

The list of all chemicals and reagents are found in chapter four.

3.2 General methodology

The general methodology used for analysis of antibiotics and PFAS are summarised in Figure 3.1 below. The first procedure outlines the DLLME extraction of antibiotics in macadamia nuts prior to UHPLC-qToF-MS analysis. The second procedure outline the LLME extraction of antibiotics in green beans prior to UHPLC-qToF-MS analysis. The last procedure outlines the DLLME extraction of PFAS in food contact material prior to UHPLC-qToF-MS analysis.

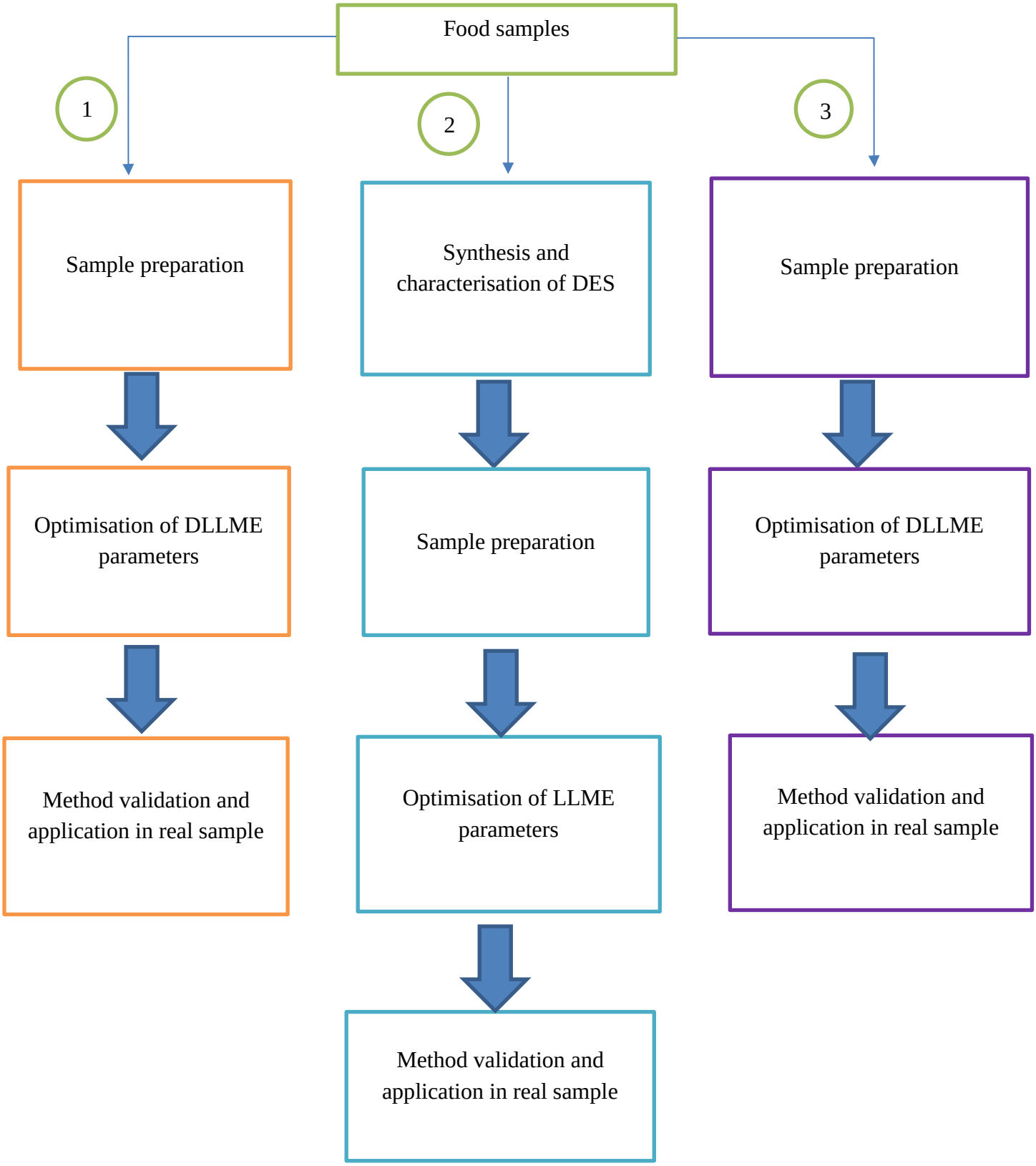

Figure 3. 1: General approach for sample of (1) DLLME of antibiotics in macadamia nuts; (2) LLME of antibiotics in green beans and (3) DLLME of PFAS in food contact materials

Chapter four: List of publications

This section gives the publications that were done during the duration of MSc program

Paper III

This paper “Dispersive liquid-liquid micro-extraction of multi-class antibiotics in macadamia nuts prior to liquid chromatography coupled to quadrupole time-of-flight mass spectroscopy” is a manuscript in preparation. It describes the development of DLLME for extraction of multiclass antibiotics in macadamia nuts. Factors affecting the extraction of analytes were optimised validated and applied in real analysis.

Dispersive liquid-liquid micro-extraction of multi-class antibiotics in macadamia nuts prior to liquid chromatography coupled to quadrupole time-of-flight mass spectroscopy

Gershom Mpela Ntshani, Nikita Tawanda Tavengwa*

Department of Chemistry, School of Mathematics and Natural Science, University of Venda, Private Bag X5050, Thohoyandou, South Africa, 0950

*Corresponding author: nikita.tavengwa@univen.ac.za

Abstract

The presence of antibiotic residues in food is associated with severe effects in human health. They are introduced into the food though agricultural irrigation with contaminated wastewater, uses of wastewater sludge as fertilisers and the treatment of livestock in agriculture. Since their concentration in the environment is in trace amounts and taking into consideration the complex nature of environmental samples, it is important to develop a pre-concentration technique prior to instrumental detection. In this study, a dispersive liquid-liquid microextraction have been developed for extraction of two fluoroquinolones, two sulfonamides and one tetracycline in macadamia nuts prior to ultra-high performance liquid chromatography coupled to quadrupole time of flight mass spectrometry (LC-qToF-MS) for separation and detection of analytes. The analytes were detected using a multiple reaction monitoring mode in the mass spectrometry. The method was optimised by looking at factors such as the effect of sample pH, type of extraction and dispersive solvent, volume of extraction solvent, concentration of NaCl and agitation time. The linearity of the method was found between 12.5 μg kg^{-1} – 800 μg kg^{-1} with an excellent coefficient of determination $r^2 > 0.99$. The LOD and LOQ range from 6.91 – 43.50 μg kg^{-1} and 20.75 – 130.73 μg kg^{-1}, respectively. The applicability and accuracy of the method was evaluated in terms of the relative recovery and the relative standard deviation which range from 59.12% – 131.25% and 1.03 – 6.36, respectively. Three antibiotics, namely, enrofloxacin, sulfadiazine and sulfamethoxazole were detected in macadamia nuts in the range of < LOD - 3.8 μg kg^{-1}. Enrofloxacin exhibited the lowest concentration while sulfamethoxazole exhibited the highest concentration.

Keywords: Antibiotics, DLLME, macadamia nuts, LC-QTOF/MS, green analytical chemistry, miniaturisation

1. Introduction

The use of antibiotics to treat diseases has been one of the most successes to living organisms. Over 60% of the antibiotics produced globally are used in agriculture to improve animal production, and treatment of bacterial and fungal infections (Danner et al., 2019). Because of their massive production, less regulation, poor metabolism and misuse, residues of antibiotics have been detected in wastewater effluent and influent and some in food and drinking water (Danner et al., 2019; Wang et al., 2020, Nilghaz and Lu., 2019; Gaudin., 2017). Besides their success in treatment and improving animal production, the presence of antibiotics residues in the living organisms is associated with deadly side effects ranging from allergic reactions on hypersensitive individuals to carcinogenic effects (Prajwal et al., 2017; Bilal et al., 2020). Another threat associated with antibiotic residues is the induction of antibiotic resistance on some of the deadliest microorganisms (Kraemer et al., 2019).

Wastewater treatments plants and agricultural wastes such as manure are the point source of antibiotics (Azanu et al., 2018; Szymańska et al., 2019). Antibiotic residues end up in sediments, soil, surface water, ground water and food through different pathways, including agricultural run-off, land irrigation and leaching (Guo et al., 2016). Food ingestion is one of the primary sources of antibiotic exposure to the human body (Ben et al., 2019). The contamination of food with antibiotics ranges from the use of wastewater for irrigation of crops (Gudda et al., 2020; Craddock et al., 2020), the use of wastewater or livestock sludge as compost (Boševski et al., 2020) and treatment of livestock in agriculture (Arun et al., 2020). Because of the adverse effects associated with antibiotics in human health, it is very crucial to monitor their presence in food samples. Analytical instruments which can detect antibiotics in trace amount are and LC techniques coupled to MS (Barco et al., 2020; Bellouard et al., 2020, DAD (Castillo-Aguirre et al., 2020; Li et al., 2020 and UV (Fage et al., 2020). The use of high-resolution MS techniques has gained most popularity in analysis of trace contaminants. Among these techniques is the qToF-MS which have the capability to detect the mass of compounds with four decimal places rendering for high precision and confidence in detecting compounds (Wang et al., 2020). With these techniques, the full chromatogram of the sample is analysed, and the exact analytes of interest are detected and isolated from the rest chromatographic peaks through a powerful tool called multiple reaction monitoring (Li et al., 2020). This will be beneficial in detecting multiclass analytes with different physicochemical properties.

Direct analysis of complex matrix such as macadamia nuts is difficult. This sample have higher quantities of diverse biological compounds, such as lipids, proteins and carbohydrates, and minerals, such as vitamins, magnesium, iron and phosphorus. These contaminants might reduce the sensitive during analysis by giving false positive or false negative results. To comply with the principles of green analytical chemistry, it is recommended to minimise as much as possible the reagents that are used in the laboratory (Abdussalam-Mohammed et al., 2020; Armenta et al., 2018). Sample preparation and pre-concentration consume almost 95% of the work done in the rest is analysis. Liquid-liquid extraction and solid phase extraction are some of the traditional techniques used for isolation and pre-concentration of analytes in the environment. Beside their effectiveness in extraction of antibiotics, these techniques are associated with some setbacks such as the consumption of high content of organic solvents, expensive and inability to be automated (Khatibi et al., 2020). Thus, it is important to limit these downfalls and be compliant with green analytical chemistry. Dispersive-LLME has gained a lot of popularity since its introduction. This is a ternary extraction technique which requires an extractive solvent, dispersive solvent and water for extraction of analytes. The dispersive solvent which is soluble in both water and extractive solvent assist in forming a turbulent or milky solution during the extraction which facilitate the transfer of analytes into the extractive solvent (Mohebi et al., 2020). Low content of organic solvents is used during the extraction steps, and the method does not require any complicated steps. Most DLLME focuses on the extraction of a single class of antibiotics. This is because extraction of multi-class analytes is difficult due to the widespread of physicochemical properties. There are few studies which are based on multi-class extraction of antibiotics using miniaturised liquid-liquid extraction (Liang et al., 2016; Herrera-Herrera et al., 2013) and in most cases, this multi-class extractions are done using SPE, DSPE or QuEChERS (Do et al., 2020; Wang et al., 2019; Santos et al., 2019). If the properties that affect the extraction of these analytes were to be optimised, then their extraction will be possible.

The aim of this work is to develop fast, easy, cheap and environmentally friendly DLLME method for extraction of three-class of antibiotics (fluoroquinolones, tetracyclines and sulfonamides) in macadamia nuts prior to liquid chromatography coupled to quadrupole time-of-flight mass spectrometer. The method was then applied in real samples for extraction of antibiotics. To the best of our knowledge, this is the first time DLLME was applied for extraction of multiclass antibiotics in macadamia nuts.

2. Material and method

2.1 Chemicals

The studied antibiotics such as enrofloxacin, ofloxacin, sulfadiazine, sulfamethoxazole and doxycycline were all > 95% pure from Sigma-Aldrich, (Johannesburg, South Africa). HPLC grade methanol and acetonitrile were purchased from Microsep (Johannesburg, South Africa). NaEDTA, formic acid, acetic acid, dichloromethane, chloroform, tetrachloromethane, sodium hydroxide, trichloromethane, pivalic acid, phosphocholine chloride and hexane were all analytical grade purchase from Sigma-Aldrich (Johannesburg, South Africa). The methanolic stock solution of each analyte (1000 µg L^{-1}) were prepared in a 50 mL polypropylene tube and stored in the dark at 4ºC. Standard solutions were prepared daily by mixing an appropriate amount of stock solution with a new 10 mL polypropylene tube.

2.2 Macadamia nuts samples

The studied macadamia nuts samples were purchased in a supermarket around Thohoyandou (Limpopo province, South Africa). Another sample was obtained in the villages around Thohoyandou which was used as blank during optimisation and validation. The samples were kept at 4ºC in the refrigerator until analysis.

2.3 Instruments and LC-MS/MS conditions

The samples and standard solutions were analysed using Ultra High-Performance Liquid Chromatography – Quadrupole Time-of-Flight Mass spectrometry (UHPLC-QTOF-MS/MS). It is the coupling of ultra-performance liquid chromatography (LCMS-9030), equipped with a column heater and degassing system, and quadrupole time of flight mass spectrometer (The SYNAPT G1 Q-TOF). Orion Star™ A21 pH portable meter from Thermo Fisher Scientific (Waltham, Massachusetts, U.S.A) was used to measure the pH of the aqueous solution during method development. BOECO BAS 31(BOECO Germany, Hamburg, Germany) plus weighing balance was used for weighing of standard analytes and samples. Fisherbrand™ Fixed Speed Vortex Mixer purchased from Fisher Scientific (Waltham, Massachusetts, U.S.A) was used to vortex the sample. NF 1200 Bench-top centrifuge from NÜVE SANAYİ MALZEMELERİ İMALAT VE TİCARET A.Ş (Ankara, Turkey) was used for sample centrifugation. ABH 2 heating block (CDR laboratory services, Brackendowns, South Africa) units with a temperature range from 0 - 200ºC was used to hold the 2 mL Eppendorf tube and regulate their temperature during drying of solvents.

A volume 10 µL injection volume was injected using an auto injector and the analytes were separated using Shim-pack Velox (100 mm x 2.1 mm, with particle size of 2.7 µm, Shimadzu, USA). The binary solvent gradient with a flow rate of 0.4 mL min^{-1} was used for analyte separation for 6 minutes. The mobile phases constitute of A: 0.1% formic acid in Milli-Q water and B: 0.1% formic acid in methanol. The separation conditions were: 5% B for 1 minute, 95% B for 5 min and 5% B for 1 min.

The SYNAPT G1 Q-TOF high-definition mass spectrometer (Shimadzu, Japan) was used as a detector. The analytes were ionised in a positive ionisation mode using electrospray ionisation (ESI). The MS conditions were set as follows: nebulizer gas flow of 3 $L.min^{-1}$, drying gas flow of 10 L min^{-1}, heating gas flow of 10 L min^{-1}, interface voltage of 4 kV, interface current of 8.2 µA, interface temperature of 300ºC, desolvation temperature of 526ºC, DL temperature of 250ºC, heat block temperature of 400ºC and the director voltage of 1.4 kV.

2.4 Sample preparations

The procedure for preparation of macadamia nuts was adopted from Hidalgo-Ruiz et al. (2021) with few modifications. A mass of 2 g of ground macadamia nuts was weighed in a 50 mL polypropylene tube and the working standard and 1% of Na_2EDTA used as a chelating agent was added. A volume of 10 mL of a mixture of ultra-pure water and acetonitrile (50/50, v/v) containing 10% of formic acid was added into the tube. The mixture was sonicated in the ultrasonic bath for 15 min and vortexed for 5 min. The mixture was manually shaken for 60 sec after adding 10% of NaCl. The aqueous and the organic was later separated using a centrifuge at 9000 rpm for 10 min. 5 mL of the supernatant was transferred into a new 15 mL centrifuge tube containing 6 mL of hexane. The mixture was shaken for 60 sec using a vortex and the top hexane layer was discarded. The procedure was repeated two times to ensure all the analytes are extracted. All the organic phases were combined, and the volume was reduced to 1000 µL using nitrogen gas. The same acetonitrile was used as dispersive solvent in the DLLME clean-up procedure below.

2.5 DLLME procedure

A volume of 1000 µL of acetonitrile obtained from the previous procedure was mixed with 600 µL of chloroform used as an extraction solvent. The mixture was vigorously injected into the aqueous phase at pH of 6. The mixture was vortexed for 5 min until the milky solution was formed. The emulsification was broken using a centrifuge at 5000 rpm for 5 min. The top phase

was discarded and the sedimented phase containing the antibiotics was completely dried using pure nitrogen gas. The analytes were reconstituted with 60 µL of methanol and loaded into the HPLC vial for analysis.

2.6 Evaluation of enrichment factor and relative recoveries

The enrichment factor, which was calculated using Equation 1 below, was used to evaluate the extraction efficiency during method development.

$$EF = \frac{C_{extracted}}{C_{added}} \quad (1)$$

where C_o is the initial concentration added into the sample $C_{extracted}$ is the concentration after the extraction.

The relative recovery was calculated using Equation 2 to evaluate the method performance in real samples.

$$RR = \frac{(C_{found} - C_{real})}{C_{added}} \text{ x } 100 \quad (2)$$

where C_{found}, C_{real} and C_{added} is the detected concentration, concentration in unspiked sample and the concentration added, respectively.

3 Results and discussion

3.1 Effect of sample pH

Sample pH of the solution has a very significant impact on the extraction of analytes or the portioning of analytes into the organic phase. Analytes in ionic form tend to have an affinity with aqueous phase due to the electrostatic interaction between the analyte charge and the polar ends of water molecules. The pH of aqueous solution was studied in the range between 4 and 12 by using sodium and acetic acid. The distribution of analytes into the organic layer is very dependent on their dissociation and association or pKa values.

Enrofloxacin and ofloxacin fall in the group of fluoroquinolones with the amino(piperazinyl) and carboxylic functional group. These analytes can be anionic, cationic or zwitterion states depending on the pH state subjected on. They are in their intermediate state between the pKa values of approximately 6 and 10. This was also confirmed experimentally where enrofloxacin and ofloxacin have higher absorption at pH of 8 (Figure 1). Similar results have been found by

Yan et al. (2011) and Yu et al. (2020). Sulfonamides are ampholytes with a basic amine (-NH_2) group and acid (-NH-SO_2). This group exists as a protonated state or neutral state in an acidic state. As can be seen in Figure 1, the analytes have the optimum pH around 4 and the enrichment factor decreases as the pH increases due to the deprotonation and the increase of the degree of ionisation. Similar results have been found by Yao and Du. (2020), Chatzimitakos et al. (2018) and Di et al. (2019). Tetracyclines are amphoteric compounds with three pKa value (i.e., 3.1, 8.0 and 9.3) due to the tricarbonyl system, phenolic diskeleton system and dimethyl ammonium group (Şanli et al., 2009; Ibarra et al., 2011). In most studies, doxycycline is extracted at pH less than its pKa_1 value (i.e., less than 3.1) due to protonation of the analyte (Lorenzetti et al., 2017; Dil et al., 2020). However, the optimum pH value in our case was 6. This was influenced by the neutral state of doxycycline at pH level between 3.09 – 9.0. These results were also consistent with the results obtained by Sereshti et al. (2020).

As can be seen in Figure 1, these analytes have different properties and their optimum pH values are different. However, all analytes have high enrichment factors at pH 6 compared to other pH values. Therefore, the pH of 6 was selected as the optimum value and was used in the subsequent experiments.

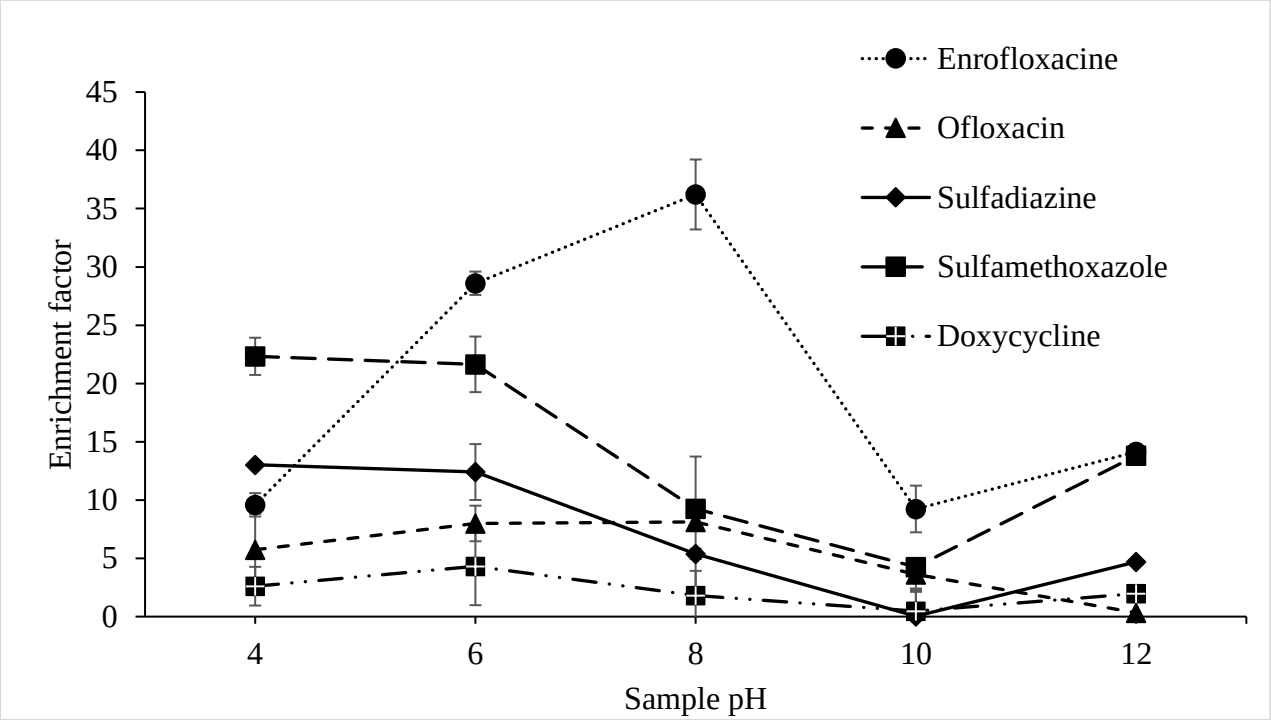

Figure 1: The effect of sample pH. Experimental conditions: Volume of donor solvent = 5mL; extractive solvent = dichloromethane; volume of extraction solvent = 600 µL; dispersive

solvent = methanol; volume of dispenser solvent = 1000 µL; vortex time = 1 min; centrifugation = 5000 rpm for 5 min. (n = 3, RSD)

3.2 Ionic strength

Salting out effect has effectively been used in various LLE techniques to enhance the transfer of analytes in the acceptor phase. However, the ionic strength can also reduce the transfer of analytes due to viscosity of the organic (Sereshti et al., 2020). The type of salt should also have partial solubility with the organic phase. Thus, in this study, sodium chloride has been chosen as the optimum salt based on other studies (Shahi et al., 2020; Sereshti et al., 2020; Ferrone et al., 2018). The concentration of salt in the donor phase was studied in the range between 0% – 12%. Figure 3 shows that increasing the concentration of salt has less impact on improving the enrichment factor in most antibiotics and in some cases the enrichment factor of other antibiotics was being reduced when increasing the ionic strength. This could be contributed by the increase in viscosity of the organic solvent as the ionic strength increases (Sereshti et al., 2020). Thus, further experiments were performed without salt addition.

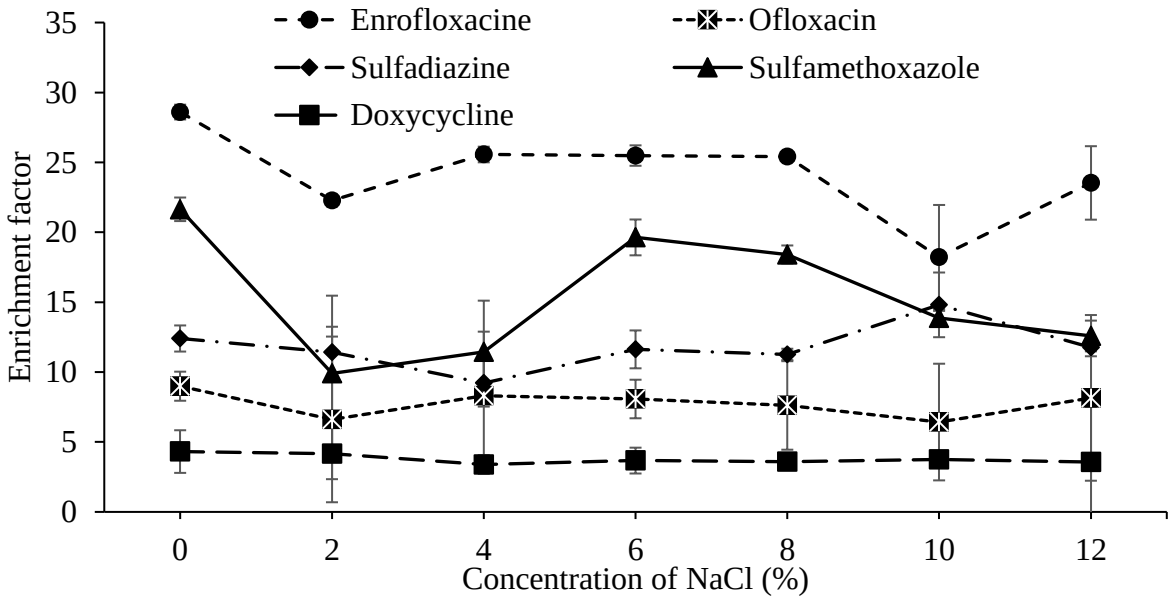

Figure 2: The effect on ionic strength of NaCl. Experimental conditions: Sample pH = 6.04, volume of donor solvent = 5 mL, extractive solvent = dichloromethane; volume of extraction solvent = 600 µL, dispersive solvent = methanol; volume of dispenser solvent = 1000 µL; vortex time = 1 min; centrifugation = 5000 rpm for 5 min. (n = 3, RSD)

3.3 Extraction solvent

Extraction solvents have an impact on the extraction of the desired analytes. The optimum extractive solvent must have higher affinity with the desired analytes and must be immiscible with the aqueous solution and miscible with dispersive solvent. Three extraction solvents, including dichloromethane, chloroform and tetrachloromethane, have been investigated. The quality of the extraction solvent must match the nature of the analytes to enhance their partitioning coefficient (Wang et al., 2019). Figure 3 shows that chloroform and dichloromethane have higher enrichment factors compared to tetrachloromethane. The mechanisms for such phenomenon have never been studied, however it can be speculated that the polarity of the extractant has a major impact. All the studied analytes are polar compounds and due to the principle of "polar-like-polar", it is likely that these analytes will interact with polar solvents. Tetrachloromethane is a non-polar solvent, thus it has a weak interaction with the studied analytes. When comparing the polarity of chloroform with dichloromethane, chloroform has a polarity of 4.1 which is higher than the polarity of dichloromethane with a polarity of 3.1. Similar results have been found by Herrera-Herrera et al. (2013) through the detection of sulfonamides and quinolones in water samples. Therefore, chloroform has been chosen as the optimum extraction solvent.

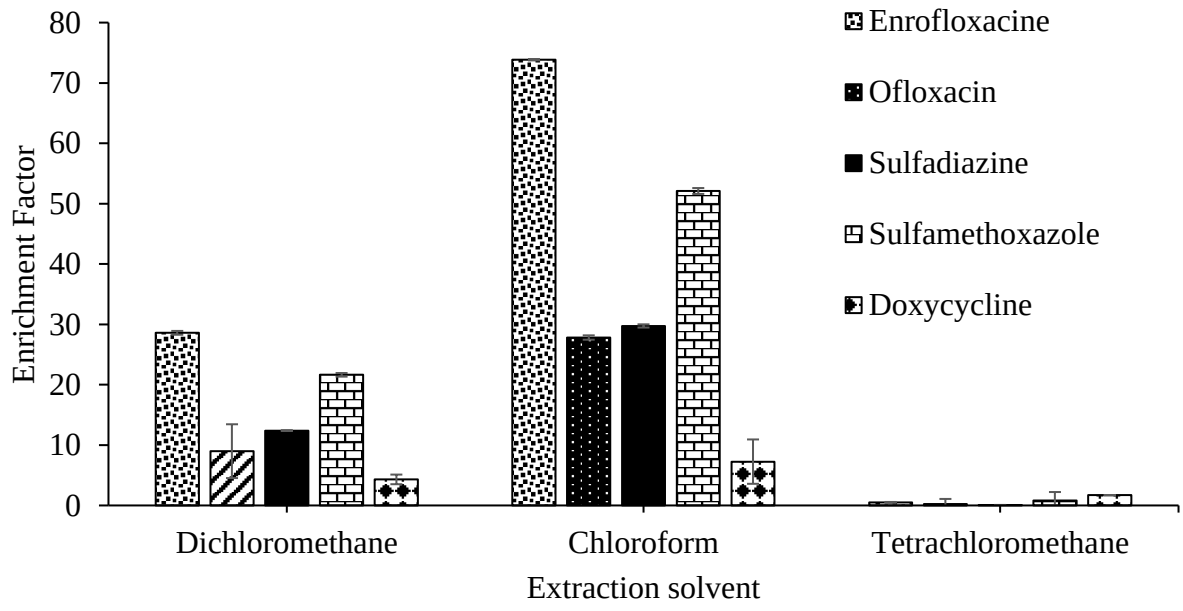

Figure 3: The effect of extraction solvents. Experimental conditions: Sample pH = 6.04; volume of donor solvent = 5mL; volume of extractive solvent = 600 µL; dispersive solvent =

methanol; volume of dispenser solvent = 1000 µL; vortex time = 1 min; centrifugation = 5000 rpm for 5 min (n = 3, RDS).

3.4 Effect of dispersive solvent

Dispersive solvent is very important in DLLME because it facilitates the mobility of analytes into the extractive solvent. The type of a dispersive solvents must be miscible with both the aqueous phase and the extraction solvent. Four dispersive solvents including acetonitrile, methanol, acetone and MeOH/ACN, (50:50, v/v), was investigated to further improve the enrichment factor. Figure 4 shows that acetonitrile is the best dispersive solvent with the highest enrichment factor compared to other solvents. Similar results were found by (Herrera-Herrera et al., 2010; Sereshti et al., 2020). The interaction between the analytes and the dispersive solvent may be influenced by the dipole moments of the acetonitrile, acetone, and methanol which are 3.8 D, 2.85 D and 1.70 D, respectively. As can be seen in Figure 4, there is a trend between the enrichment factor of analytes and the type of dispersive solvent, where acetonitrile > MeOH/ACN > acetone > methanol. Therefore, acetonitrile has been chosen as the optimum dispersive solvent.

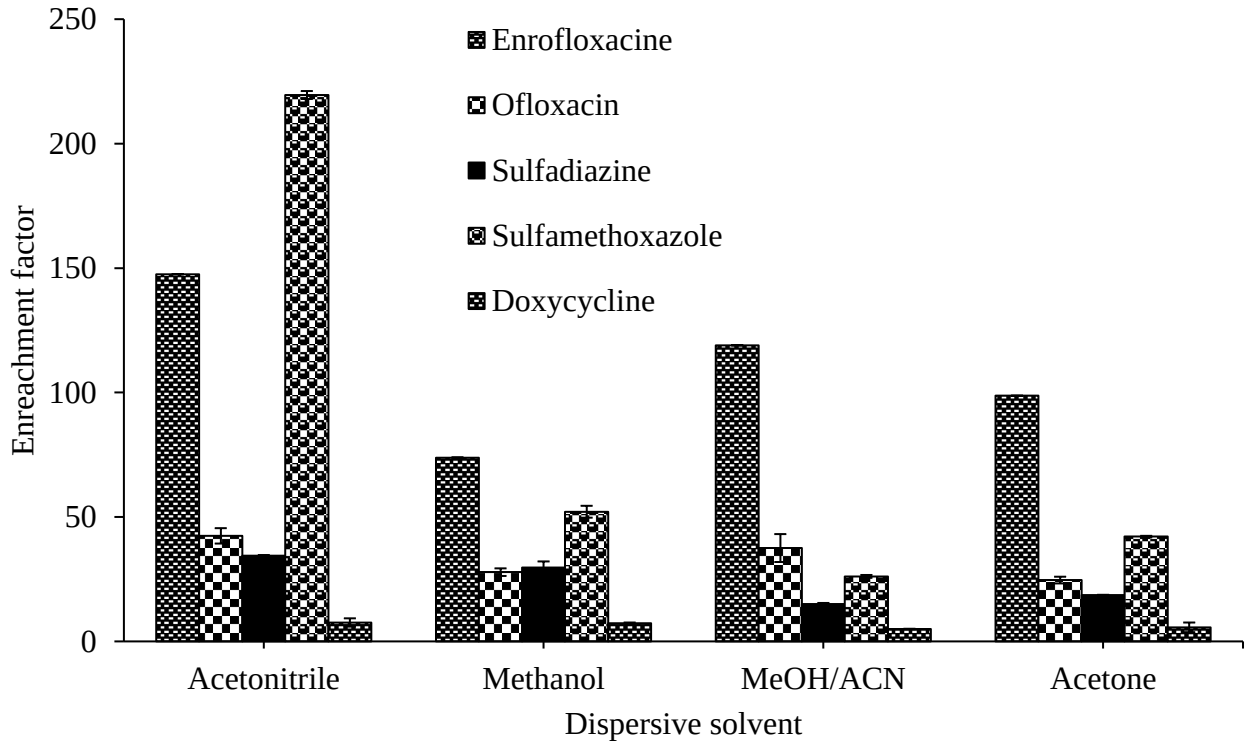

Figure 4: The effect of dispersive solvents. Experimental conditions: sample pH = 6.07; volume of donor solvent = 5 mL; extractive solvent = chloroform; volume of extractive solvent

= 600 µL; volume of dispenser solvent = 1000 µL vortex time = 1 min; centrifugation = 5000 rpm for 5 min. (n = 3, RSD).

3.5 Effect of volume of extraction solvent

The volume of extractive solvent has an impact in increasing the enrichment factor. However, higher extraction volume can also reduce the enrichment factor (Liang et al., 2016). In this experiment, the volume of the extractive solvent has been investigated in the range of 50 µL – 800 µL. As can be seen in Figure 5, there is an increase in enrichment factor in the range between 50 µL – 600 µL and until its reach a maximum at volume of 600 µL. The decrease in enrichment factor was observed at 800 µL. Thus 600 µL have been chosen as the optimum volume of the extraction solvent.

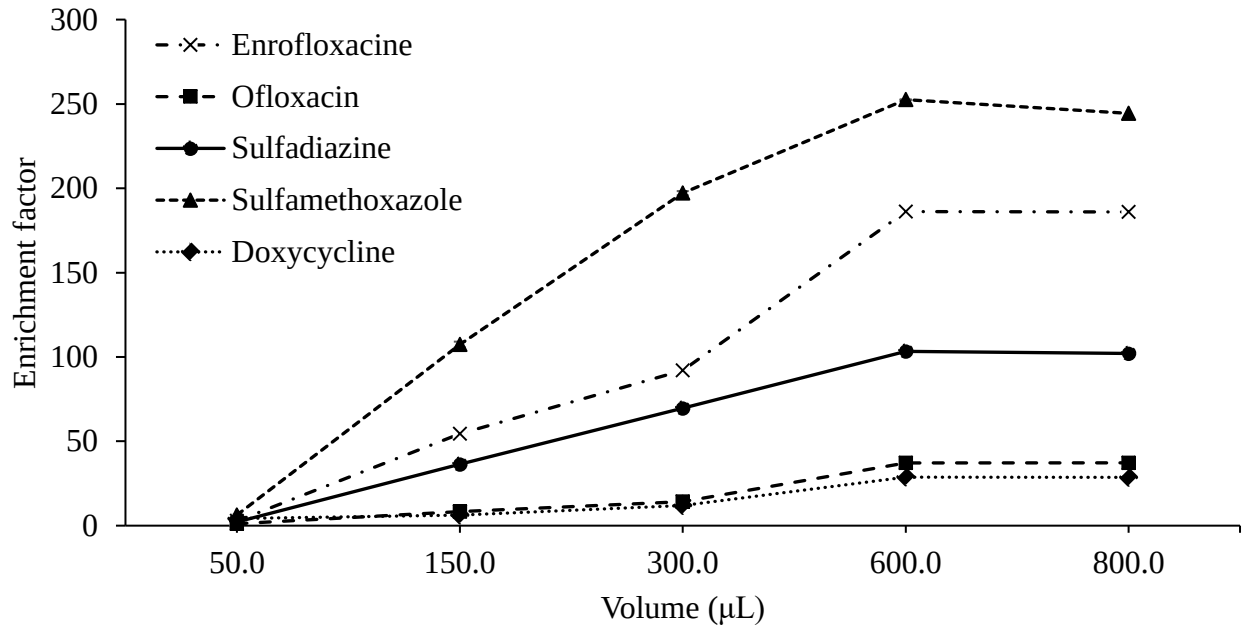

Figure 5: The effect of extraction volume. Experimental conditions: Sample pH = 6.02, volume of donor solvent = 5 mL, extraction solvent = chloroform; dispersive solvent = acetonitrile; volume of dispenser solvent = 1000 µL, vortex time = 1 min; and centrifugation = 5000 rpm for 5 min (n = 3, RDS)

3.6 Effect of vortex time

Agitation helps to increase the contact area between the analytes and the organic phase and facilitates the formation of micro-droplets. In this experiment the time of agitation has been

investigated between the time range of 1 – 20 min to improve the enrichment factor. Figure 6 shows that the increase in vortex time to 5 min increase the distribution of analytes into the organic phase. The enrichment factor remained constant as the shaking time increased from thereafter. Therefore 5 min was chosen as the optimum vortex time.

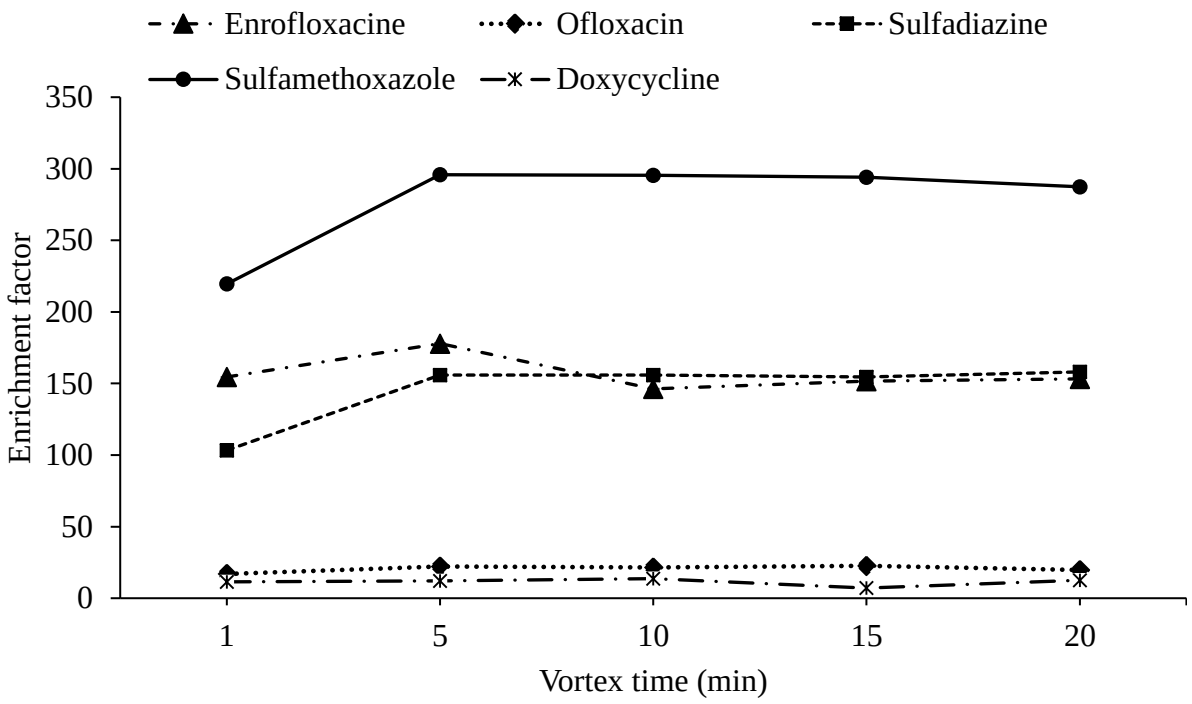

Figure 6: The effect on vortex time. Experimental conditions: Sample pH = 6.04, volume of donor solvent = 5 mL, extractive solvent = 600 µL volume of dispenser solvent = 1000 µL and centrifuged at 5000 rpm for 5 min (n = 3, RDS)

3.7 UHPLC-QTOF-MS/MS

Quantitative analysis was performed using UHPLC-qToF-MS in selective or multiple reaction monitoring mode. This technique is programmed to only detect analytes with the predefined precursor ion and its product ions. To eliminate the false results due to the presence of isomers, the retention time, the precursor ion and the product ions are set in the compound table. To set a compound table the instrument was optimised. First, the analytes of interest are first directly injected into the mass spectrometer to identify their precursor ion. In this project, all antibiotics were ionised in a positive ionisation mode using an electrospray ionisation source. The mass spectrum of each analyte will have different fragments and only $[M+H]^+$ fragment was selected as the precursor ion (Table 1). Each analyte was then run in the MS/MS mode where different

collision energies were set to investigate the optimum collision energy that can fragment analyte to give the product ions that will be used to verify the precursor ion. Lastly, the mixture of all the analytes were run through the column where they were retained, separated and eluted and the retention time for each analyte was determined (Figure 7). This will enable the instrument to separate structural isomers since they have different retention time.

Table 1: Selected transitions and optimisation potentials of different antibiotics

Antibiotic	Retention time (min)	Precursor ion (m/z)	Products ions (m/z)	Collision energy (eV)	Spread (eV)
Enrofloxacin	2.59	390.172	361.182	20	5
Ofloxacin	2.56	360.1723	245.108; 342.161	30	5
Sulfadiazine	2.42	251.0594	108.043; 156.010	20	5
Sulfamethoxazole	2.67	254.059	156.01	20	5
Doxycycline	2.77	445.1610	321.075; 428.133	30	5

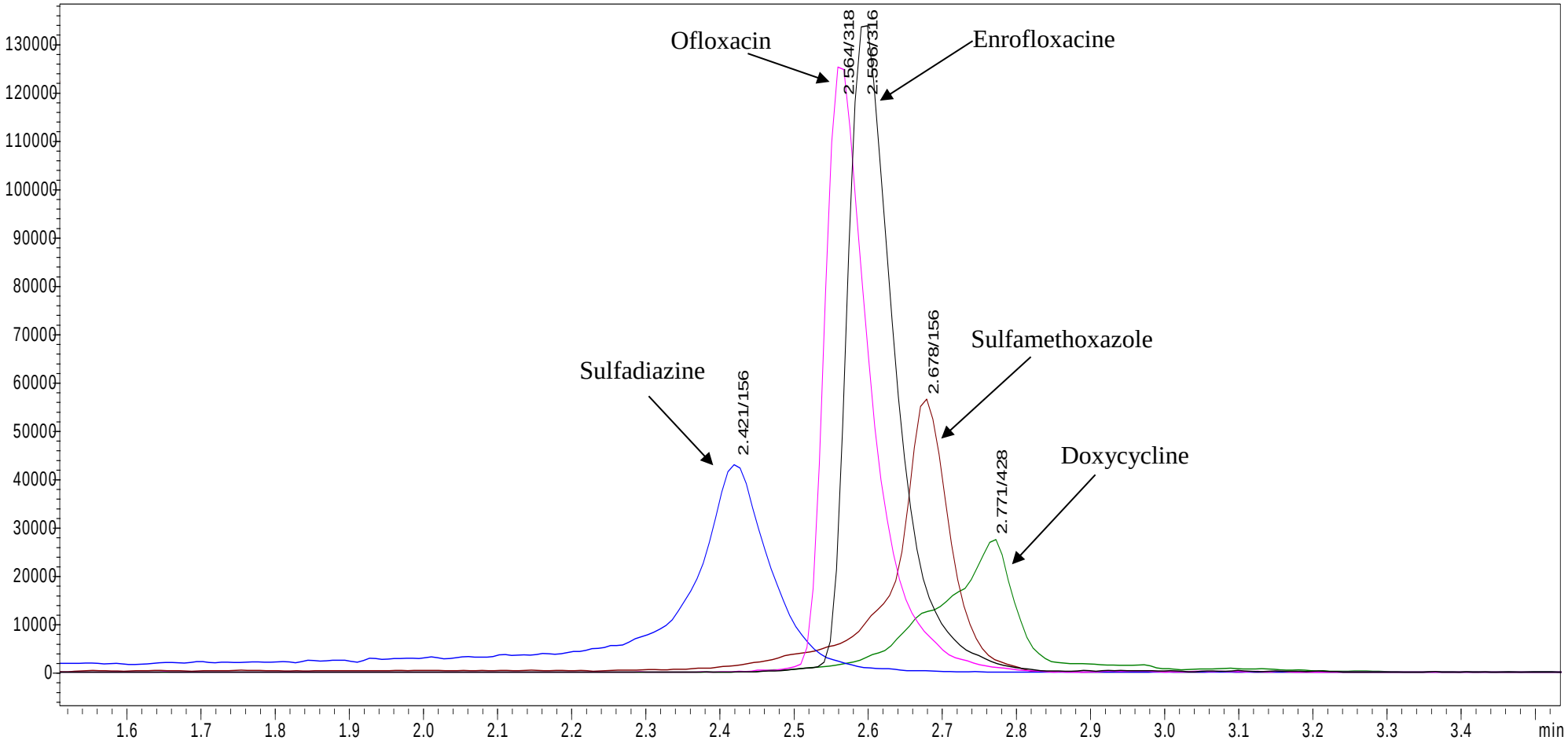

Figure 7: MRM chromatogram of antibiotics with the retention time/ product ion on top of the peak

3.8 Method validation

The developed method was validated based on parameters such as the linear range, limits of detection (LOD), limits of quantification (LOQ), and enrichment factor (EF) to evaluate its analytical performance (Table 2). All experiments were run in triplicate (n = 3) to evaluate its accuracy. The linear range of the developed method was investigated by plotting the spiked concentration in macadamia nuts against the absorbance where an excellent coefficient of determination or $r^2 > 0.998$ found. The LOD and LOQ, which were calculated by multiplying the signal to noise ratio (S/N) by 3 and 10, respectively, were found within the range of 6.91 μg kg^{-1} – 43.50 μg kg^{-1} and 20.75 μg kg^{-1} – 130.73 μg kg^{-1}, respectively. The efficiency of the method, which was evaluated in terms of EF was in the range between 12.01 – 295.93. In this case, doxycycline exhibited the lowest EF while sulfamethoxazole exhibited the highest EF. These results confirm that this method can be applied for a routine monitoring of antibiotics in macadamia nuts.

Table 2: Figures of merit of the introduced method for the studied analytes.

Antibiotics	Equation	Linear range (µg kg^{-1})	r^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	EF
Enrofloxacin	y = 400.27x – 1427.4	12.5 – 800	0.9997	11.35	34.08	177.88
Ofloxacin	y = 435.02x – 3727.4	12.5 – 800	0.9998	8.75	26.26	22.11
Sulfadiazine	y = 301.00x – 1202.5	12.5 – 400	0.9998	6.91	20.75	155.84
Sulfamethoxazole	y = 221.24x – 936.35	12.5 – 800	0.9999	7.66	22.99	295.93
Doxycycline	y = 278.21x – 23484	100 – 800	0.9983	43.50	130,63	12.01

3.9 Real sample analysis

The dispersive liquid-liquid micro-extraction was applied for analysis of antibiotics in macadamia nuts. The concentration of antibiotics was calculated using the standard addition method, and the results are shown in Table 3. Out of five antibiotics, two antibiotics, namely, sulfadiazine and sulfamethoxazole were detected at concentration of 1.28 µg kg^{-1} and 3.8 µg kg^{-1}, respectively. However, to verify the applicability of the method for analysis of antibiotics, blank macadamia nuts were spiked with three concentration levels (10 µg kg^{-1}, 20 µg kg^{-1}, 50 µg kg^{-1}) and the relative recoveries were evaluated. The analyses were all run in triplicate to evaluate the accuracy of the results. The percentage recovery ranges from 59.12% – 131.25% (Table 3).

Table 3: The concentration of analytes in real sample and the percentage recovery of the spiked levels

Antibiotic	Concentration	Recovery (%) 10 µg kg^{-1}	RSD	Recovery (%) 50 µg kg^{-1}	RSD	Recovery (%) 100 µg kg^{-1}	RSD
Enrofloxacin	-	82.07	1.2	59.12	2.63	67.31	1.03
Ofloxacin	-[b]	72.11	4.92	72.00	1.09	68.26	1.45
Sulfadiazine	3.80	64.75	1.27	77.23	1.40	69.25	1.76
Sulfamethoxazole	1.28	93.98	1.36	99.46	2.15	89.11	1.49
Doxycycline	-	131.25	1.92	107.03	2.83	121.75	6.36

a = less than detection limits

3.10 Comparison

The dispersive liquid-liquid micro-extraction developed in this study was compared with the available techniques used for pre-concentration of multi-class antibiotics. The method was compared based on the LOD, LOQ, percentage recoveries and RSD. Table 4 shows that these parameters are comparable with those in the literature. However, the relative standard deviation was much better, which confirms the reputability of the method. Therefore, instead of using a long, tedious and non-environmentally friendly classical techniques, a cheap, quick and environmentally friendly technique developed in this study can be used for multi-class analysis of antibiotics in complex matrix such as macadamia nuts.

Table 4: Comparison of multiclass extraction techniques used for pre-concentration of antibiotics in food samples

Matrix	Analytes	Method	LOD and LOQ	RSD (%)	% Recovery	Reference
Lettuce, tomato, cauliflower, and broad beans	Fluoroquinolones, sulfonamides, lincosamides and metoxybenzylpyrimidines	SPE-LC-MS/MS	0.1 – 5.8 ng g^{-1}	< 18	40 – 118	Tadić et al. (2019)
Chicken Eggs	Sulfonamides, quinolones, tetracyclines, macrolides, lincosamide, nitrofurans, β-lactams, nitromidazoles, and cloramphenicols	SLE – LC-MS/MS	0.005 – 0.72 and 0.015 – 6.66 µg kg^{-1}	< 14	70 – 116	Wang et al. (2017)
Chicken manure, swine manure, poultry feed and soil	Cephalosporin, fluoroquinolone, lincosamide, macrolide, nitroimidazole, quinolone, sulfonamide and tetracycline	QuEChERS – LC-MS/MS	0.01 – 1.86 and 0.05 – 5.91 µg kg^{-1}	< 20	24 – 162	Rashid et al. (2020)
Fatty food	Sulfadimidine, sulfadiazine, enrofloxacin, erythromycin, roxithromycin, and flumequine	Pressurised liquid extracyion - LC-MS/MS	0.4 – 25.0 µg kg^{-1} and 1.3 – 75 µg kg^{-1}	< 28.5	82.2 – 115.0	Wang et al. (2019)
Macadamia nuts	Fluoroquinolones, sulfonamides and tetracycline	LC-MS/MS	6.91 – 43.50 µg kg^{-1} and 20.75 –	< 10.92	59.12 – 111.25	This study

	130.73 µg kg^{-1}

4. Conclusion

In this study, for the first time, a DLLME technique was developed and applied for pre-concentration of two fluoroquinolones, two sulfonamides and one tetracycline in macadamia nuts using chloroform and acetonitrile as an extraction solvent and a dispersive solvent, respectively. The performance of this technique was comparable with those available in literature. However, compared with available study, this technique is easy, cheap and environmentally friendly. During the method development, factors which affect the enrichment of analytes such as the effect of sample pH, type of extraction and dispersive solvent, volume of extraction solvent, concentration of NaCl and agitation time were optimised. The sensitivity of the method was evaluated in terms of LOD, and LOQ which range from 6.91 – 43.50 µg kg^{-1} and 20.75 – 130.73 µg kg^{-1}. The efficiency and accuracy were evaluated in terms of relative recovery and relative standard deviations which range between 59.12 – 111.25 and 1.03 – 6.36, respectively. The method has an enrichment factor of 12.01 – 295.93. In real samples, three antibiotics were detected in the range between 1.28 µg kg^{-1} to 3.8 µg kg^{-1}. These results show that this technique can be applied in a complex matrix such as macadamia nuts for analysis of multiclass antibiotics.

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4.4 paper IV

This paper “Liquid-liquid micro-extraction of multiclass antibiotics using deep eutectic in green beans prior to liquid chromatography coupled quadrupole mass spectrometer analysis” is a manuscript in preparation. It describes the application of a novel DES in development of LLME for extraction of multiclass antibiotics in green beans and its application in real sample. Factors affecting the extraction of analytes were optimised, validated and applied in real analysis.

Liquid-liquid micro-extraction of multiclass antibiotics using deep eutectic in green beans prior to liquid chromatography coupled quadrupole mass spectrometer analysis

Gershom Mpela Ntshani, Nikita Tawanda Tavengwa*

Department of Chemistry, School of Mathematics and Natural Science, University of Venda, Private Bag X5050, Thohoyandou, South Africa, 0950

* Corresponding author: nikita.tavengwa@univen.ac.za

Abstract

The presence of antibiotics in the environment has gained a lot of attention due to the severe effects they pose on human health and the ecosystem. The presence of antibiotics in food is inevitable and it is important to monitor their presence in food before ingestion. In this paper, a simple LLME has been developed and applied for pre-concentration of multiclass antibiotics in the green beans using a novel DES which was prepared from the combination of aliquat-336 and butyric acid. The solvent was characterised using a FTIR spectroscopy. The analysis was carried out using the ultra-high performance liquid chromatography coupled to quadrupole time of flight mass spectrometer. The factors that affect the enrichment of analytes such as the type of DES, ratio of DES, concentration of NaCl, volume of DES and agitation time were optimised. The method sensitivity in terms of LOD and LOQ was ranging between 6.91 μg kg^{-1} – 43.50 μg kg^{-1} and 20.75 μg kg^{-1} – 130.73 μg kg^{-1}, respectively. The linear range was found between 12.5 μg kg^{-1} – 800 μg kg^{-1} with an excellent determination coefficient or $r^2 > 0.99$. The applicability and accuracy of the method was evaluated in terms of relative recovery and relative standard deviation which range from 64, 65% – 101.09% and 0.01 – 6.60, respectively.

Keyword: Antibiotics, liquid-liquid microextraction, deep eutectic solvents, green extraction techniques, multi-class analysis, green beans, LC-qToF-MS

1. Introduction

Antibiotics are broadly used for treatment of various infectious diseases (Straub, 2016). They are also used in agriculture for treatment and to improve animal production (Giang et al., 2015). Due to their excessive usage and misuse, high quantities of antibiotic residues have been detected in water bodies, living organisms and food. The presence of antibiotic residues is

linked with severe danger to living organisms especially those that live in aqueous environment (Hernández-Pérez et al., 2020; Cheng et al., 2020). Ingestion of food and water and inhalation of air which is contaminated with antibiotic residues is associated with severe effects on the body ranging from carcinogenic effect to death. The selection of antibiotic resistance bacteria is a serious concern since the available treatments are no longer effective for treatment.

Recently, analysis of antibiotic has been carried out using chromatographic techniques, enzyme linked immunosorbent assay, fluorescence spectroscopy and capillary electrophoresis (Park et al., 2020; Krall et al., 2018; Li et al., 2020; Liu et al., 2017). The above-mentioned instruments are very effective in analysis of antibiotics, but to analyse substances that are present in trace level in the environment and food samples might require extraction techniques such as liquid phase extraction (LPE) and solid phase extraction (SPE). This is because food samples are complex matrices which are loaded with a wide variety of biological, organic and inorganic compounds. The use of miniaturised LLE known as liquid-liquid microextraction (LLME) has gained a lot of attention in analytical chemistry because they comply with the principles of green analytical chemistry. The use of LLME has many advantages over the classical LLE and SPE. This is because the use of SPE is tedious, consumes high quantities of solvents and it takes time for the synthesis of adsorption particles (Chen et al., 2019). Ionic liquids have been recommended for extraction of antibiotics and other contaminants due to their environmental friendliness (Liang et al., 2016; Feng et al., 2020). They are also applied in a variety of industries such as electrochemical, food processing and petrochemical industries (Gomes et al., 2019). Their wide range of application arises from their physico-chemical properties such as being liquid below 100˚C, low vapour pressure and thermal and chemical stability (Vafaeezadeh et al., 2016).

The aim of this project was to use LLME for extraction and preconcentration multiclass antibiotics using ionic liquid as extractive solvent in green vegetables prior to spectrophotometry analysis in the spiked solvents. After optimisation, the technique will be used for extraction of two fluoroquinolones, two sulfonamides and one tetracycline in the green vegetables and analysis using LC-QToF-MS.

2. Materials and methods

2.1. Chemicals and reagent:

The chemicals used for this experiment were of high quality and purity (> 95 % purity). The investigated analytes such as ofloxacin, sulfamethoxazole, sulfadiazine and doxycycline were purchased from Sigma-Aldrich (Johannesburg, South Africa). 1-octanol, butyric acid, 1-decyl-3-methylimmidazilium chloride and tricaprylmethylammonium chloride (aliquat-336) used for preparation of DES were purchased from Sigma-Aldridge (Johannesburg, South Africa). HPLC grade methanol and acetonitrile was purchased from Microsep (Johannesburg, South Africa). Distilled water was obtained from milli-Q water purifier. The stock solution of all the analytes (1000 µg L^{-1}) were in methanol in a 10 mL polypropylene tube and stored in the dark at -3ºC until analysis. The standard solutions were prepared by dissolving each standard solution in a new 10 mL centrifuge tube. The standard solutions were prepared on the day of the experiment.

2.2 Green beans sample

The studied green beans samples were purchased in a supermarket around Thohoyandou (Limpopo province, South Africa). Another sample was obtained in a local farm around Thohoyandou which was used as blank during optimisation and validation. The samples were blended using a blender and kept at 4ºC in the refrigerator until analysis.

2.3 Instruments and LC-MS/MS conditions

The samples and standard solutions were analysed using Ultra High-Performance Liquid Chromatography – Quadrupole Time-of-Flight Mass spectrometry (UHPLC-QTOF-MS/MS). it is the coupling of ultra-performance liquid chromatography (LCMS-9030), equipped with a column heater and degassing system, and quadrupole time of flight mass spectrometer (The SYNAPT G1 Q-TOF). Orion Star™ A21 pH portable meter from Thermo Fisher Scientific (Waltham, Massachusetts, U.S.A) was used to measure the pH of the aqueous solution during method development. BOECO BAS 31(BOECO Germany, Hamburg, Germany) plus weighing balance was used for weighing of standard analytes and samples. Fisherbrand™ Fixed Speed Vortex Mixer purchased from Fisher Scientific (Waltham, Massachusetts, U.S.A) was used to vortex the sample. NF 1200 Bench-top centrifuge from NÜVE SANAYİ MALZEMELERİ İMALAT VE TİCARET A.Ş (Ankara, Turkey) was used for sample centrifugation. ABH 2 heating block (CDR laboratory services, Brackendowns, South Africa) units with a temperature range from 0 - 200ºC was used to hold the 2 mL Eppendorf tube and regulate their temperature during drying of solvents. Alpha FTIR spectrometer Bruker (Johannesburg, South Africa) was used for characterisation of deep eutectic solvent.

A volume 10 µL injection volume was injected using an auto injector and the analytes were separated using Shim-pack Velox (100 mm x 2.1 mm, with particle size of 2.7 µm, Shimadzu, USA). The binary solvent gradient with a flow rate of 0.4 mL min^{-1} was used for analyte separation for 6 minutes. The mobile phases constitute of A: 0.1% formic acid in Mili-Q water and B: 0.1% formic acid in methanol. The separation conditions were: 5% B for 1 minute, 95% B for 5 min and 5% B for 1 min.

The SYNAPT G1 Q-TOF high-definition mass spectrometer (Shimadzu, Japan) was used as a detector. The analytes were ionised in a positive ionisation mode using electrospray ionisation (ESI). The MS conditions were set as follows: nebulizer gas flow of 3 $L.min^{-1}$, drying gas flow of 10 L min^{-1}, heating gas flow of 10 L min^{-1}, interface voltage of 4 kV, interface current of 8.2 µA, interface temperature of 300ºC, desolvation temperature of 526ºC, DL temperature of 250ºC, heat block temperature of 400ºC and the director voltage of 1.4 kV.

2.4 Preparation of deep eutectic solvents

The method used for DES preparation have been described by Pirsaheb et al. (2019) with few modifications. Methyltrioctylammonium chloride as hydrogen bond acceptor was mixed with butyric acid as hydrogen bond acceptor in a mole ratio of (1:1) in a 15 mL polypropylene centrifuge tube. The tube was immersed in a water bath for 5 min at 85ºC. After, the mixture was vortexed for 5 and returned into the water bath for another 5 min. The heating and vortex cycle was repeated three times until the yellow homogeneous liquid (DES) was formed. The formed DES was characterised using FTIR.

2.5 Sample preparation

The procedure for sample preparation was adopted from the procedure described by Tadić et al. (2019) with few modifications. A mass of 2 g of blended green beans sample was weighed in a 50 mL polypropylene tube. A mixture of water and acetonitrile (3:7, v/v) was added, and the mixture was vortexed for 5 min, sonicated for 15 min and centrifuged for 10 min at 9000 rpm. The supernatant was taken into a new 15 mL centrifuge tube and the extraction was repeated for times to ensure all analytes were extracted. The supernatant was combined and dried under a nitrogen stream.

2.6 LLME procedure

The analytes in previous procedure were reconstituted with 5 mL of pure water at a pH of 6 and vortexed for 6o sec. 60 µL of DES was added into the aqueous solution and sonicated for 5 min in the ultrasonic bath. 3% of NaCl was added into the solution, vortexed for 60 sec and centrifuged for 5 min at 5000 rpm. The bottom aqueous phase was carefully removed and discarded using a syringe until only the top DES remains in the polypropylene tube. A 140 µL of methanol was used to rinse the wall of the tube, while also diluting the viscosity of DES. The resultant was lorded into the autosampler vial for analysis.

3. Results and discussion

3.1 Characterisation of DES

The FTIR spectroscopy was used for identification of functional groups present in the synthesised DES. The spectrum of the synthesised DES was compared with the spectrum of butyric acid and aliquat-336 used for its synthesis. The interaction between the butyric acid (hydrogen bond donor) and aliquat-336 (hydrogen bond donor) is expected to be on the carboxylic acid functional group of butyric acid and the ammonium chloride functional of aliquat-336 (Figure 1). The FTIR spectrum of butyric acid has the sharp peak around 1702 cm^{-1} for C=O, a sharp peak at 2969 cm^{-1} for C-H and broad peak which stretches around 3091 cm^{-1} for OH. Aliquat-336 has a strong sharp peak around 2859 cm^{-1} - 2937 cm^{-1} for C-H and a very weak sharp peak around 1165 cm^{-1} for C-N transition. The spectrum for the synthesised DES still shows a strong sharp C-H around 2859 cm^{-1} - 2937 cm^{-1} for C-H. There is a shift in wavenumber and a decrease in intensity for C=O which was absorbing around 1702 cm^{-1} in the butyric acid spectrum to 1712 cm^{-1} for the new DES. The OH bond which was absorbing around 3091 cm^{-1} was not detected or maybe, the intensity was very low. The sharp and weak absorbance of C-N peak which was absorbing around 1165 cm^{-1} in the aliquat-336 spectrum was found to have higher intensity in the DES spectrum. This change in vibrational frequencies and change in intensity of the absorbance confirms the interaction hydrogen bond accepting and hydrogen bond donation between the aliquat-336 and butyric acid, respectively.

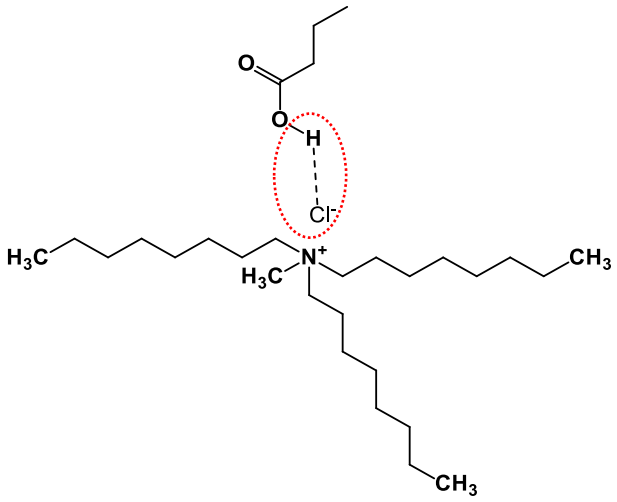

Figure 1: The structure of deep eutectic solvent showing the interaction between the hydrogen bend accepter and the hydrogen bond doner.

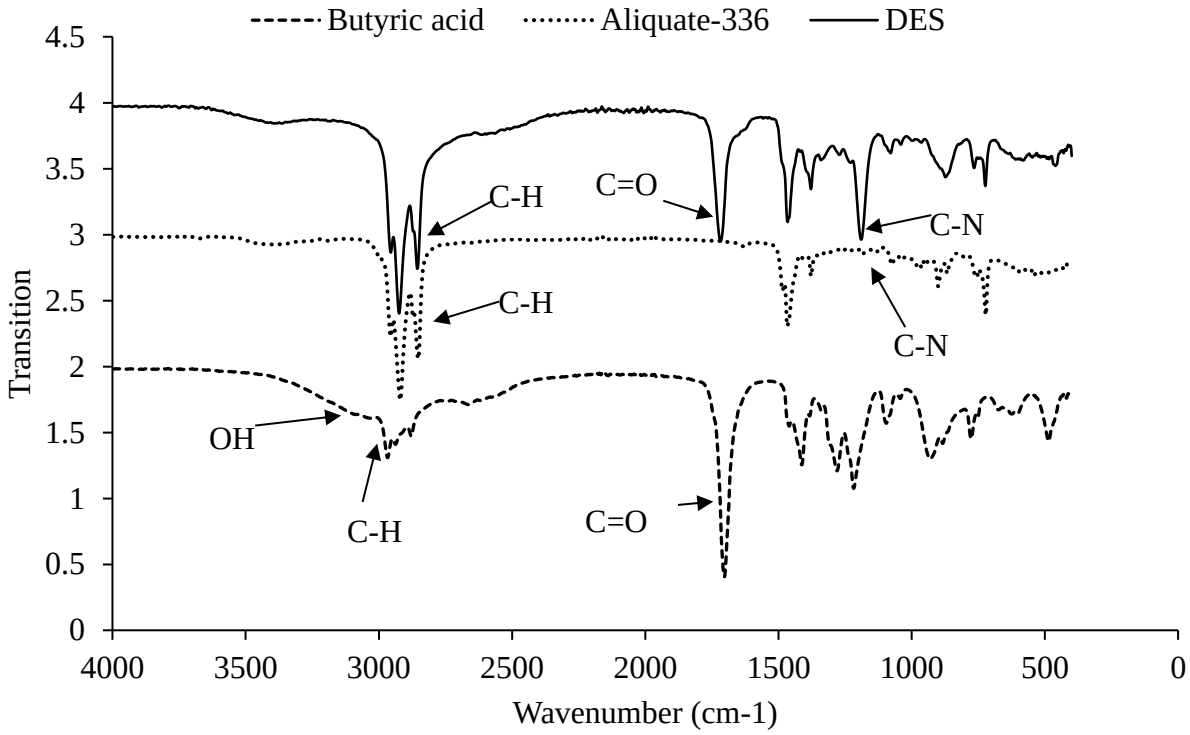

Figure 2: FTIR spectrum of DES, aliquate-336 and butyric acid

3.2 Optimisation of sample pre-treatment

3.2.1 The type of DES

The previous study has demonstrated that these studied antibiotics have different physico-chemical properties, and they have different optimum pH absorbance. However, it was apparent that at pH of 6 they all have the highest enrichment factors and the pH of 6 was chosen as the optimum pH. In this study the type of DES was investigated while the pH of the donor solution was kept at 6. Selection of DES is very important in this study. The desired solvents were chosen based on the characteristics such as low water solubility, low toxicity, high enrichment factor, density lower than water and melting point close to room temperature. In this experiment four DES, including [DMIM]Cl : butyric acid, [DMIM]Cl : octanol, aliquat-336 : butyric acid and aliquat-336 : octanol, were investigated. In other studies, Pirsaheb et al. (2019) have developed a VALPME extraction technique for extraction of amoxicillin and ceftriaxone in hospital sewage water where [DMIM]Cl : butyric acid was found to be an optimum DES. Ji et al. (2020) have developed an USALLME for extraction of sulfonamides in fruit juice where the DES prepared using aliquat-336 and 2-octanol was found to be optimum. In this study, Figure 3 shows that the DES formed using aliquat-336 : butyric acid resulted in higher enrichment factor compared to other DES. To the best of our knowledge, this is the first time aliquat-336 : butyric acid DES was prepared for extraction of fluoroquinolones, sulfonamides and tetracyclines. Therefore, this solvent has been chosen as the optimum.

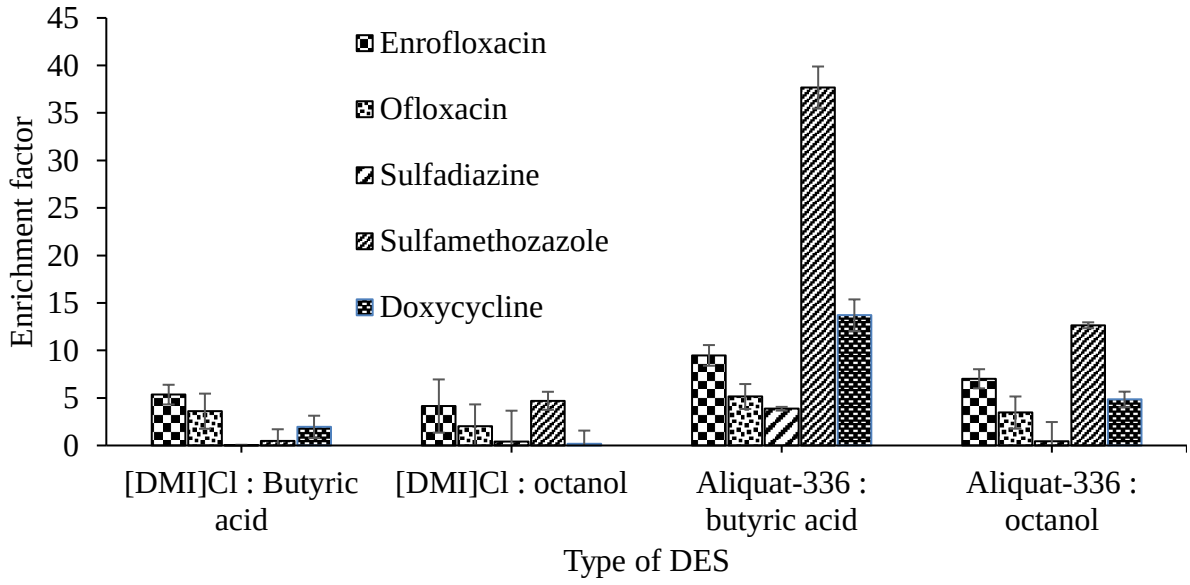

Figure 3: The effect of extractive solution. Experimental conditions: pH of donor solution = 6.2; volume of donor solvent = 5 mL; volume of extractive solvent = 60 µL, sonication = 5 min; vortex time = 5 min and centrifugation = 5000 rpm for 10 min (n = 3, RDS)

3.2.2. Ionic strength

The ionic strength is increased by addition of salt into the sample solution. The ionic strength in solution is associated with facilitating phase separation and strengthening the transfer of analytes into the organic phase (Saei et al., 2020). Too much ionic strength can also increase the viscosity of the organic phase and therefore reduce the transfer of analytes (Sereshti et al., 2021). In this step the percentage of ionic strength has been varied from 3% to 15%. It was observed that when the extraction was performed without addition of salt it was difficult to break the emulsion formed between the two layers which resulted in a longer extraction process and loss of analytes. Figure 4 shows that the increase in ionic strength has a negative impact in this technique as the enrichment factor decreased as the percentage salinity was increased. Similar results have been observed by Di et al. (2020), through the extraction of tetracyclines in wastewater using aliquat-336 : nonanoic acid DES, Saei et al. (2020) through the extraction of three antibiotics (penicillin G, oxytetracycline and tilmicosin) in hamburger sing choline chloride : pivalic acid as DES and Sereshti et al. (2020) through the extraction of tetracyclines using thymol : octanoic acid as DES. Therefore, 3% of salt concentration have been chosen as the optimum through the experiments.

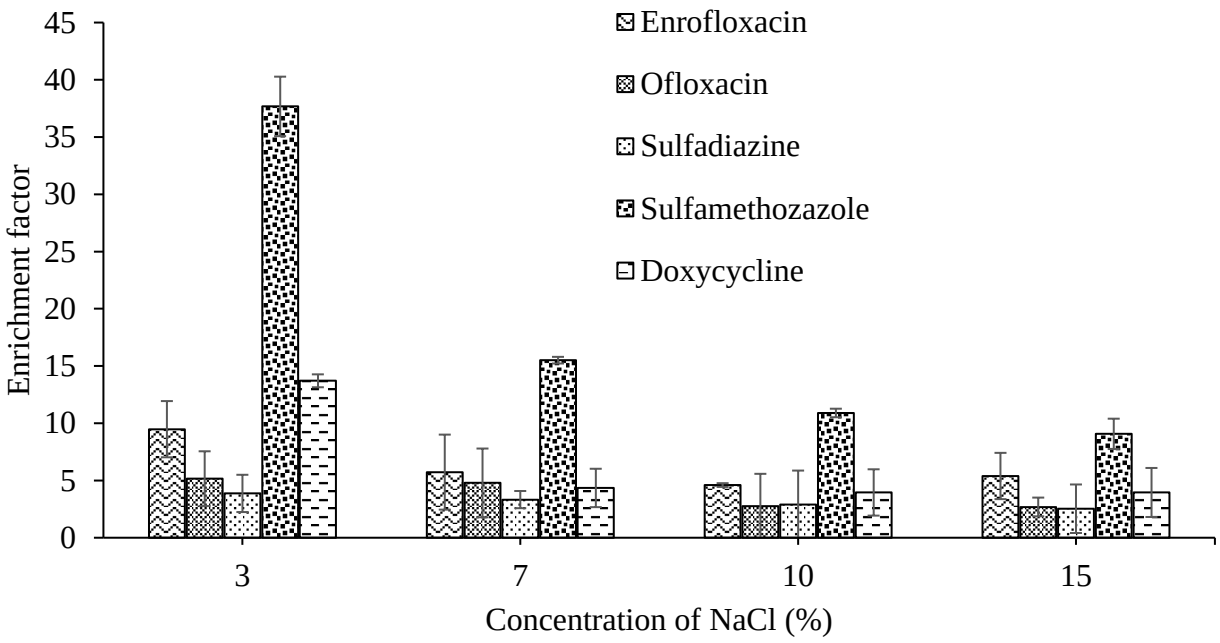

Figure 4: The effect of ionic strength. Experimental conditions: pH of done solution = 6.2, volume of donor solution = 5 mL, extractive solvent = Aliquat-336 : butyric acid at (1:1 molar ratio), volume of extractive solvent = 60 µL, molar ratio of extraction solvent (1:1) sonication for 5 min, vortex time = 5 min and centrifuged at 5000 rpm for 10 min (n = 3, RDS)

3.2.3. Molar ratio of extraction solvent

Mole ratio of Aliquat-336 : butyric acid DES was adjustment to investigate its effect on the enrichment of antibiotics. The mole ratio of DES is very important in this extraction since it can directly affect the physico-chemical properties of the solvent and this also affects the enrichment factor. In this study three different mole ratios, including 1:1, 1:2, 1:5 and 1:7, have been investigated. Figure 5 shows that the mole ratio of 1:1 gives higher enrichment factors, and the enrichment factors decrease as the increase. This means that the increase in proportion of butyric acid decreases the enrichment factor of analytes. Therefore, the mole ratio of 1:1 has been chosen as optimum.

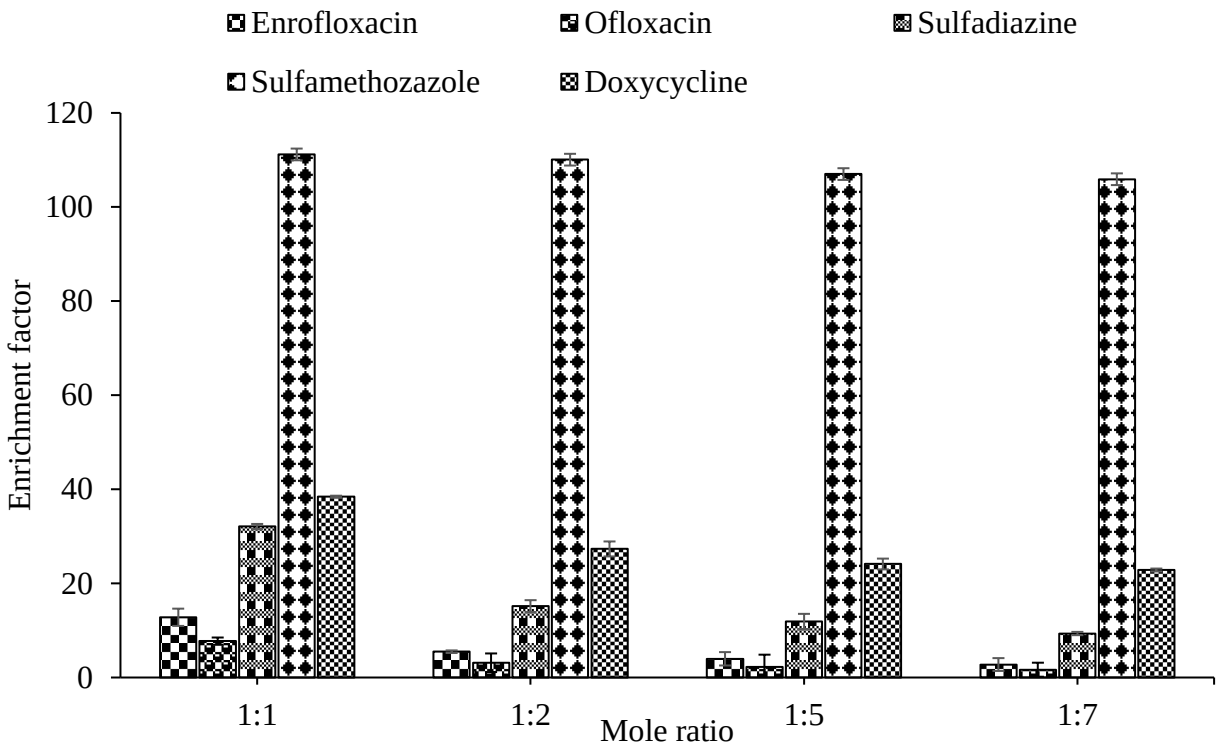

Figure 5: The effect of mole ratios. Experimental conditions: pH of done solution = 6.2; volume of donor solution = 5 mL; extractive solvent = Aliquat-336 : butyric acid; volume of extractive solvent = 60 µL; sonication = 5 min; vortex time = 5 min and centrifugation = 5000 rpm for 10 min (n = 3, RDS)

3.2.4. Effect of extraction volume

The volume of extraction solvent has an important role during the extraction process since an increase in volume can increase the interaction of analytes with DES. In contrast higher extraction volume can also decrease the enrichment factor through dilution effect (Ma and Row., 2021). In this study, the volume of extraction solvent has been varied from 60 µL – 120 µL to investigate the effect into the enrichment factor. Figure 6 shows that an increase in extraction volume effectively decreased the enrichment factors. Therefore, 60 µL have been chosen as an optimum extraction volume.

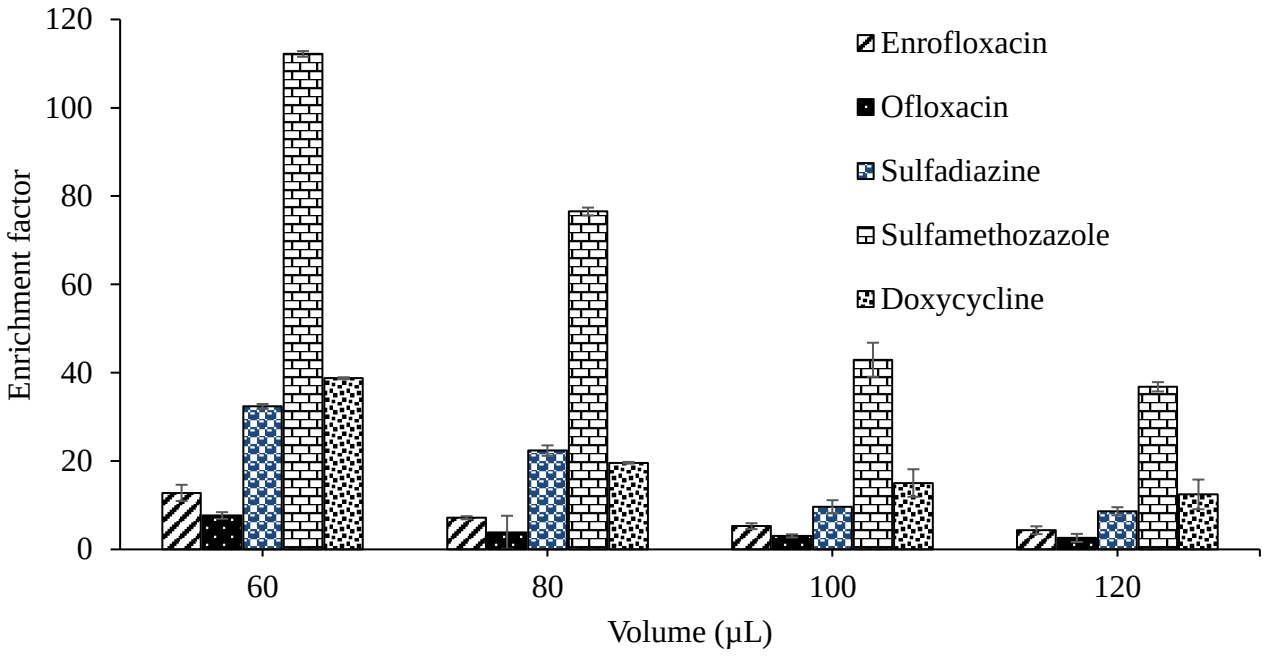

Figure 6: The effect of volume of extractive solvent. Experimental conditions: pH of done solution = 6.2; extraction solvent volume of donor solution = 5 mL, extractive solvent = Aliquat-336 : butyric acid at (1:1 molar ratio); ionic strength = 3% NaCl; volume of extractive solvent = 60 µL; sonication = 5 min; vortex time = 5 min and centrifugation = 5000 rpm for 10 min (n = 3, RDS)

3.3 UHPLC-qToF-MS

Detection of analytes were performed in the UHPLC-qToF-MS using the multiple reaction monitoring (MRM) mode. This was done by first determining the precursor ion of each analyte by direct injection of a volume of 0.5 µL into the MS. The product ions of each analyte were determined by running the MS/MS spectrum. In this case different collision energies ranging between 20 eV to 50 eV in the spread of 5 eV were investigated to identify best product ions that will be used for quantification. The optimum collision energies for each analyte and their

precursor ions are presented in Table 1. The optimum precursor ion and product ion were used to create a compound table that was used for quantitative analysis. The chromatogram of a standard solution containing a mixture of all analytes was run and only the analytes with the pre-defined precursor ion and its product ion was detected (Figure 7). The chromatogram was used to create a compound table where the retention time, product ion and precursor of each analyte was entered. This will allow the instrument to only detect the analyte with pre-defined precursor ion, product ion and retention time and eliminate other contaminants such as structural isomers.

Table 1: Selected transitions and optimisation potentials

Analytes	Retention time (min)	Precursor ion (m/z)	Products ions (m/z)	Collision energy (eV)*	Spread (eV)
Enrofloxacin	2.62	390.172	361.182	20	5
Ofloxacin	2.56	360.1723	245.108; 342.161	30	5
Sulfadiazine	2.42	251.0594	108.043; 156.010	20	5
Sulfamethoxazole	2.67	254.059	156.01	20	5
Doxycycline	2.76	445.1610	321.075; 428.133	30	5

* = electro volts

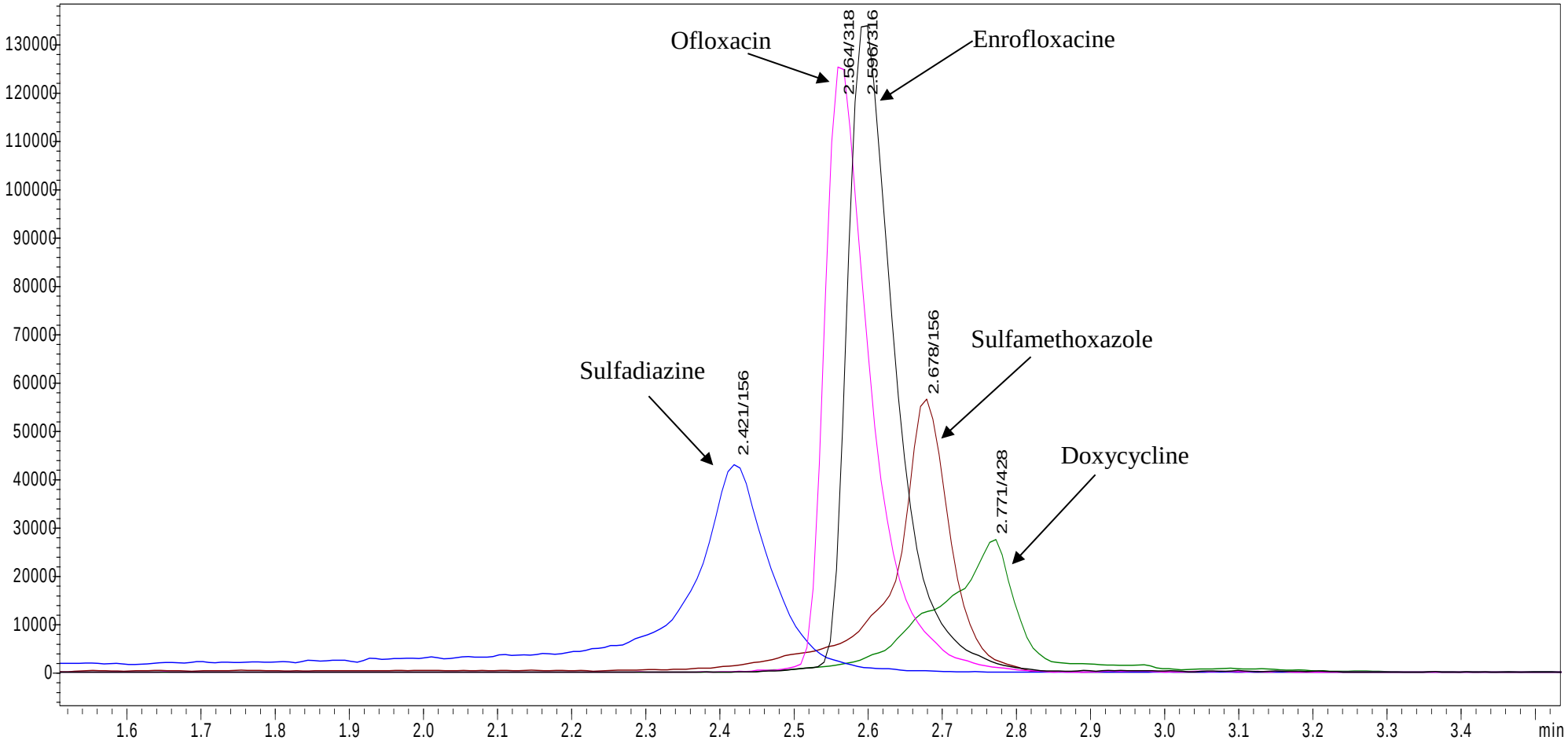

Figure 7: MRM chromatogram of antibiotics with the retention time/ product ion on top of the peak

3.3 Method validation

The liquid-liquid micro-extraction technique developed in this study was validated using parameters such as LOD, LOQ, linear range, intercept, relative standard deviation (RSD) and enrichment factor to evaluate its analytical performance. All the parameters were performed in triplicate (n = 3) to evaluate the accuracy of the method of which the range was from < 1.83 – 3.86. The linear range was investigated by plotting the spiked concentration against the absorbance. This was done by first investigating the lowest concentration which can be detected by the instrument. As can be seen in Table 2 the linear range for each analyte is broad. The intercept was evaluated as the minimum absorbance at zero concentration. The LOD and LOQ was calculated by multiplying the signal to noise ratio (S/N) by 3 and 10, respectively, which range between 6.91 µg kg^{-1} – 43.50 µg kg^{-1} and 20.75 – 130.73 µg kg^{-1}. The efficiency of the method was evaluated by the enrichment factor which range from 7.67 – 112.19, where ofloxacin exhibited the lowest enrichment and sulfamethoxazole exhibited the highest enrichment factor.

Table 2: Figures of merit of the introduced method for the studied analytes.

Antibiotics	LOD[a] (µg kg^{-1})	LOQ[b] (µg kg^{-1})	Linear range (µg kg^{-1})	Intercepts	r^2	EF[c]	RSD[d] (%)
Enrofloxacin	11.35	34.08	12.5 – 800	1427.4	0.9997	12.8	1.83
Ofloxacin	8.75	26.26	12.5 – 800	3727.4	0.9998	7.67	3.74
Sulfadiazine	6.91	20.75	12.5 – 400	1202.5	0.9998	32.40	1.46
Sulfamethoxazole	7.66	22.99	12.5 – 800	936.35	0.9999	112.19	3.86
Doxycycline	43.50	130.63	100 – 800	23484	0.9983	38.76	3.36

a = Limits of detection

b = Limits of quantification

c = Enrichment factor

d = Relative standard deviation

3.4 Applications

The Liquid-liquid microextraction technique developed in this study was applied for extraction of the studied antibiotics in green beans purchased in the supermarkets around Thohoyandou (Limpopo, South Africa). However, none of the studied antibiotics were detected. To evaluate its applicability in detection of antibiotics, the samples were spiked at three different concentration levels (10 µg kg^{-1}, 20 µg kg^{-1} and 50 µg kg^{-1}) (Table 3). All the experiments were performed in triplicate to evaluate its precision. Table 2 indicates very satisfactory results where the percentage recovery was ranging between 64, 65% – 101.09%. The repeatability of the method which was expressed as the relative standard deviation range around 0.01 – 6.60.

Table 3: The percentage recovery of analytes in three spiked levels

Antibiotic	10 µg kg^{-1}		20 µg kg^{-1}		50 µg kg^{-1}	
	Recovery (%)	RSD	Recovery (%)	RSD	Recovery (%)	RSD
Enrofloxacin	76.32	0.45	85.84	2.26	76.21	0.91
Ofloxacin	89.51	2.28	98.51	4.89	78.18	1.80
Sulfadiazine	64.65	6.60	94.45	3.71	64.77	4.35
Sulfamethoxazole	79.59	0.70	101.09	5.23	78.51	1.71
Doxycycline	79.84	0.01	81.23	5.75	69.65	3.25

a = non-detection limits

b = not detected

c = relative standard deviation

3.4. Comparison of miniaturised liquid phase extraction technique

The method was compared with other extraction techniques based on miniaturised liquid phase extraction using DES (Table 4). However, the available publications are based on a single class of antibiotics. In this case, the LOD, EF, and the percentage recovery were compared. Though in this paper, three classes of antibiotics were considered, the performance of the method was comparable with those developed for a single class analysis. The LOD found in this study was much better compared to the available data. The enrichment factor found in this study was comparable or better that the one found by Shirley et al., (2020). These results verify that LLME is applicable for analysis of multiclass antibiotics.

Table 4: Comparison of miniaturised liquid phase extraction of antibiotics using deep eutectic solvents

Food matrices	Antibiotics	Method	LOD	Enrichment factor	% Recovery	References
Fruit juice	SAs	UALLME-HPLC-UV	0.02 – 0.04 µg mL^{-1}	-*	88.09 – 97.84	Yinghe et al., (2020)
Wastewater	FQs	VADLLME-HPLC-DAD	0.63 – 1.2 ng. L^{-1}	48 – 83	-*	Shirley et al., (2020)
Environmental water	TCs	LLME HPLC-UV	0.5 – 2.0 ng mL^{-1}	-*	77.5 – 89.6	Di et al., (2020)
Milk	TCs	HF-dynamic LLME	0.95 – 3.6 µg mL^{-1}		93.38 – 107.3	Xu et al., (2017)
Green beans	FQs, SAs and TC	VALLME LC/QTOF-MS	6.91 – 43.50 µg kg^{-1}	7.67 – 112.19	64.65 – 101.09	This study

* = no data

4. Conclusion

In this study, a novel deep eutectic solvent was synthesised using aliquat-336 as a hydrogen bond acceptor and butyric acid as a hydrogen bond donor. The solvent was characterised using FTIR. The interaction between aliquat-336 and butyric acid was through the carboxylic acid functional group of butyric acid and ammonium chloride functional group of aliquat-336. These interactions were observed by the change in vibrational frequencies and a decrease or increase in peak intensities in the FTIR spectra. The solvent was applied for the first time in pre-concentration of multi-class antibiotics in green beans. The factors which affect the enrichment of analytes were carefully optimised. Though this technique was developed for multiclass class analyses, the analytical performance, which was evaluated by the LOD, LOQ and EF, were comparable or better than other techniques developed for single class analyses.

Therefore, this technique can be used for routine analyses of antibiotics in complex matrices such as green beans.

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4.5 Paper V

This paper “Dispersive liquid-liquid micro-extraction of PFAS in food contact materials prior to liquid chromatography coupled to quadrupole time-of-flight mass spectroscopy” is a manuscript in preparation. It describes the development of DLLME for extraction of multiclass antibiotics in food contact material. Factors affecting the extraction of analytes were optimised validated and applied in real analysis.

Dispersive liquid-liquid micro-extraction of PFAS in food packaging materials prior to liquid chromatography couples to quadrupole time-of-flight mass spectroscopy analysis

Gershom Mpela Ntshani, Nikita Tawanda Tavengwa*

Department of Chemistry, School of Mathematics and Natural Science, University of Venda, Private Bag X5050, Thohoyandou, South Africa, 0950

* Corresponding author: nikita.tavengwa@univen.ac.za

Abstract

Ingestion of food that is contaminated with PFAS is associated with adverse effects on human health even when they are in low concentrations. Detection of analytes that are present in trace level requires an extraction technique with the capability to isolate the desired analytes from impurities. In this study, a dispersive liquid-liquid microextraction (DLLME) has been developed for extraction of perfluorooctanoic acid, perfluorooctane sulfonic acid and perfluorohexane sulfonic acid in food contact materials prior to liquid chromatography coupled to quadrupole time of flight mass spectrometry. The sample was pre-treated using an ion-pair extraction and cleaned-up using DLLME. Chloroform was used as an extraction solvent and acetonitrile was used as a dispersive solvent. During the extraction, the solvents were rapidly injected and agitated until the mixture turned milky. The phase was separated in the centrifuge. The bottom phase containing the analytes was transferred in a new polypropylene tube, completely dried using nitrogen gas and reconstituted for LC-MS analyses. The factors such as the effect of sample pH, type of extraction and dispersive solvent, volume of extraction and dispersive solvent, concentration of NaCl, and agitation time were optimised. The linearity of the method was evaluated between 1.25 to 160 µg kg^{-1}. The sensitivity was evaluated by looking at the LOD and LOQ which range from 0.64 – 2.75 µg kg^{-1} and 1.82 µg kg^{-1} – 8.26 µg kg^{-1}, respectively. The efficiency and accuracy of the method were evaluated in terms of relative recovery and RSD which range from 67.80% – 96.11% and < 2.86 to 5.32, respectively.

Keywords: perfluoroalkyl substances, DLLME, miniaturised-LPE, green analytical chemistry, food packaging materials

1. Introduction

PFAS are synthetic industrial chemicals that have a wide range of applications in metal plating industries, paper industries, adhesives and surface coatings (Kim et al., 2021; Sim et al., 2021). The structure of PFAS has a hydrophobic C-F chain and a hydrophilic functional group. The bond between the carbon and fluorine atom is considered one of the strongest Van der Waal bonds. They are found to be resistant to most degradation processes. These properties have rendered PFAS to be applied in a wide range of products such as aqueous film forming forms, non-stick cookware, detergents, surface coating, cosmetics and food packaging (Li et al., 2020). For example, PFAS can be applied in food contact materials which are used to contain hot and greasy food such as chips. The presence of PFAS in the environment have recently gained a lot of attention globally due to the adverse effect they pose in human health and the ecosystem (Abercrombie et al., 2021; Szilagyi et al., 2020). Other concerns arise from their toxicity, persistency and high accumulation potential in the environment and human body (Di Nisio et al., 2020).

Ever since their discovery around the 1940s, they have been used in many industries and household applications (Chen et al., 2020). However, starting from the year 2000, many countries and regulatory agencies have placed regulation and the maximum residue limits (MRLs) in food and drinking water to control the environment and human exposure from PFAS (McGlinchey et al., 2020; Herkert et al., 2020). These compounds have been replaced by novel PFAS which, unlike legacy PFAS, are less toxic, biodegradable and volatile. However, PFAS are still detected in most environmental samples (Kim et al., 2021; Choi et al., 2021; Van der Schyff et al., 2020). This might be due to the persistent PFAS that were remaining in the environment or the degradation product of novel PFAS (Nguyen et al., 2019)

The recent development of analytical instruments such as LC-MS/MS and GC-MS/MS revolutionised everything. These instruments have the capability to detect analytes in parts per trillion. Since environmental matrices are complex, it is crucial to develop a pre-concentration technique to isolate the analytes from the contaminants to improve matrix effect. Liquid-liquid extraction (Meng et al., 2019; Pan et al., 2017) and solid phase extraction (Jin et al., 2021; Zhang et al., 2020) techniques are traditional extraction techniques that are routinely used for PFAS extraction. However, these techniques are associated with shortfalls because they are expensive, not environmentally friendly and they require long tedious steps before one can get the extracted analytes (Gutiérrez-Serpa et al., 2021). Dispersive liquid-liquid microextraction

(DLLME) is one of miniaturised LLE that have been used for extraction of a wide range of analytes. In contrast to conventional LLE techniques, DLLME is considered an environmentally friendly technique since it uses less solvents and does not require any complicated steps (Lu et al., 2020). There are few reports based on DLLME of PFAS, however the already used methods have proven effective. Vela-Soria have recently published two articles using the combination chloroform as extraction solvent and acetonitrile as dispersive solvent for extraction of PFCA (C_6– C_{13}) and PFSA (C_6 and C_8) in placenta (Vela-Soria et al., 2021) and milk (Vela-Soria et al., 2020). Backe et al. (2013) have added 10% 2,2,2-trifluoroethanol in extraction solvent ethyl acetate for extraction of wide range of PFAS in aqueous through LLME and the method had a percentage recovery of 96 – 106% and lower detection and quantification.

The aim of this project is to develop a DLLME technique for pre-concentration of PFAS analytes in food contact material. The technique will be applied in real samples to evaluate the prevalence of PFAS. Analysis of PFAS in food contact material was motivated by the application of heat and moisture resistance substances in fiber based food contact materials used in fast food stores. To the best of our knowledge, this is the first time a DLLME was applied for preconcentration of PFAS in food contact materials.

2. Material and methods

2.1 Chemicals and reagents

Perflurohexanoic acid, perfluorooctanoic acid, perfluoroheptanoic acid, perfluorobutane sulfonic acid, perfluorooctane sulfonic acid, perfluorohexane sulfonic acid, methyl-tert-butyl ether, tetrabutylammonium hydrogen sulphate and formic acid were all > 95% pure and purchased from Sigma-Aldrich (Johannesburg, South Africa). HPLC grade Methanol and acetonitrile were purchased from Microsep (Johannesburg, South Africa). Distilled water was obtained from Mili-Q water purifier (University of Johannesburg, South Africa). Ammonium acetate, sodium chloride, hydrochloric acid, sodium carbonate, sodium hydroxide were all of analytical grade and was purchased from Sigma-Aldrich (Johannesburg, South Africa). The stock solution (1000 µg L^{-1}) of each analyte were dissolved in a mixture of water and methanol (1:9, v/v) in a 50 mL polypropylene tube and stored in the dark at – 3ºC. The working standard solution was prepared daily during the day of experiment for sample spiking.

2.2 Food contact materials

The fibber-based food contact material that was used for analysis of PFAS were found in the fast-food stores around Thohoyandou (Limpopo, South Africa). For optimisation and method validation, a food contact material was first analysed to check if there are no residues of PFAS present and then used as a blank. The paper was cut into a very small using a stainless-steel scissor and stored in a polypropylene tube in a refrigerator at -3ºC.

2.3 Instruments and LC-MS/MS operations

The samples and standard solutions were analysed using Ultra High-Performance Liquid Chromatography – Quadrupole Time-of-Flight Mass spectrometry (UHPLC-QTOF-MS/MS). it is the coupling of ultra-performance liquid chromatography (LCMS-9030), equipped with a column heater and degassing system, and quadrupole time of flight mass spectrometer (The SYNAPT G1 Q-TOF). Orion Star^{TM} A21 pH portable meter from Thermo Fisher Scientific (Waltham, Massachusetts, U.S.A) was used to measure the pH of the aqueous solution during method development. BOECO BAS 31 from BOECO Germany (Hamburg, Germany) plus weighing balance was used for weighing of standard analytes and samples. Fisherbrand™ Fixed Speed Vortex Mixer purchased from Fisher Scientific (Waltham, Massachusetts, U.S.A) was used to vortex the sample. NF 1200 Bench-top centrifuge from NÜVE SANAYİ MALZEMELERİ İMALAT VE TİCARET A.Ş (Ankara, Turkey) was used to centrifuge the sample. ABH 2 heating block (CDR laboratory services, Brackendowns, South Africa) units with a temperature range from 0 - 200ºC was used to hold the 2 mL Eppendorf tube and regulate their temperature during drying of solvents. SSM1 miniorbital shaker, Stuart (Staffordshire, ST15 OSA, UK) with the speed range up to 300 round per minute, was used to shake the sample during sample preparation of PFAS.

A volume of 10 µL was injected using an autoinjector. The analytes were separated using Shim-pack Velox (100 mm x 2.1 mm, with particle size of 2.7 µm, Shimadzu, USA). The binary solvent gradient with a flow rate of 0.4 mL.min^{-1} was used for analyte separation for 6 minutes. The mobile phases constitute of A: 20 mM ammonium acetate in Milli-Q water and B: acetonitrile. The separation conditions were: 5% B for 1 minute, 95% B for 5 minutes and 5% B for 1 minute.

The analytes were analysed in a negative ionisation mode using electrospray ionisation (ESI) source. The MS conditions were set as follows: nebulizer gas flow of 3 L.min^{-1}, drying gas flow of 10 L.min^{-1}, heating gas flow of 10 L.min^{-1}, interface voltage of 4 kV, interface current

of 8.2 µA, interface temperature of 300 ºC, desolvation temperature of 526 ºC, DL temperature of 250 ºC, heat block temperature of 400 ºC and the director voltage of 1.4 kV.

2.4 Sample preparation

A mass of 1 g of pieces of food contact material sample was weighed in a 50 mL polypropylene tube and spiked with 10 µg kg^{-1} of internal standard solution. A volume of 1 mL 0.5 M tetrabutylammonium hydrogen sulfonate solution and 2 mL of 0.25 M sodium carbonate solution at pH of 10 was added into the polypropylene tube. A volume of 5 mL MTBE were added, and the mixture was shaken in a shaker for 20 min and centrifuged for 20 min at 3000 rpm. The organic layer was transferred into a new 15 mL polypropylene tube. The process was repeated twice. The resulted organic later was mixed in a 15 mL polypropylene tube and was completely dried under streams of nitrogen gas. The tube was taken for further clean up using DLLME.

2.5 DLLME

A volume of 5 mL of aqueous sample was added into 15 mL polypropylene containing PFAS and vortex for 60 sec. A volume of 800 µL of chloroform was mixed with 1000 µL of acetonitrile and the mixture was rapidly injected into 15 mL polypropylene containing the analytes. The tube was shaken for 60 sec, vortexed 2 min and centrifuged for 10 min at 5000 rpm. The extractive solvent (bottom phase) with the analytes was transferred into a new 2 mL Eppendorf tube and the solvent was completely dried under nitrogen streams. The analytes were reconstituted with the mixture of 20 mM of ammonium acetate solution and acetonitrile (3:7, v/v) and transferred into an autosampler vial for LC-MS/MS analysis.

3. Results and discussion

3.1 Effect of sample pH

Perfluoroalkyl carboxylic acid and perfluoroalkyl sulfonic acid are groups of acidic compounds composed of C-F hydrophobic alkyl chain and carboxylic acid and sulfonic acid functional groups, respectively. These compounds have a lower pK_a value of which PFOA have pK_a range from 0.5 – 4.2, PFHxSA have a pK_a value range from -5.8 – 0.14 and PFSA have a pK_a value range from -3.27 – 0.9. They dissociate into anionic compounds at a pH above its pKa values which makes them have higher affinity with water. In contrast, their C-F alkyl chain is hydrophobic, and given the fact that these compounds have higher octanol to water partitioning

coefficient (log K_{ow}) that ranges around 5.9 – 6.4, it is more likely that the C-F alkyl chain will have higher affinity with the organic phase. Therefore, in an organic/aqueous layer the pH should be adjusted to more acid condition for the analytes to be neutral and be partitioned to the organic layer as demonstrated by (Vela-Soria et al., 2021). However, the formation of an emulsion may not be possible at lower pH as was demonstrated by Yao and Du., (2020). Similar effect was observed in this study when the effect of pH was studied in the range from 2 – 12. It was observed that at the lowest pH values there was less formation of emulsion which poor partitioning of analytes into the organic phase. Better emulsification was observed starting from the pH of 4, and it was richer at pH of 12 hence the higher enrichment factor of analytes (Figure 1). Therefore, the pH of 12 has been used throughout the study.

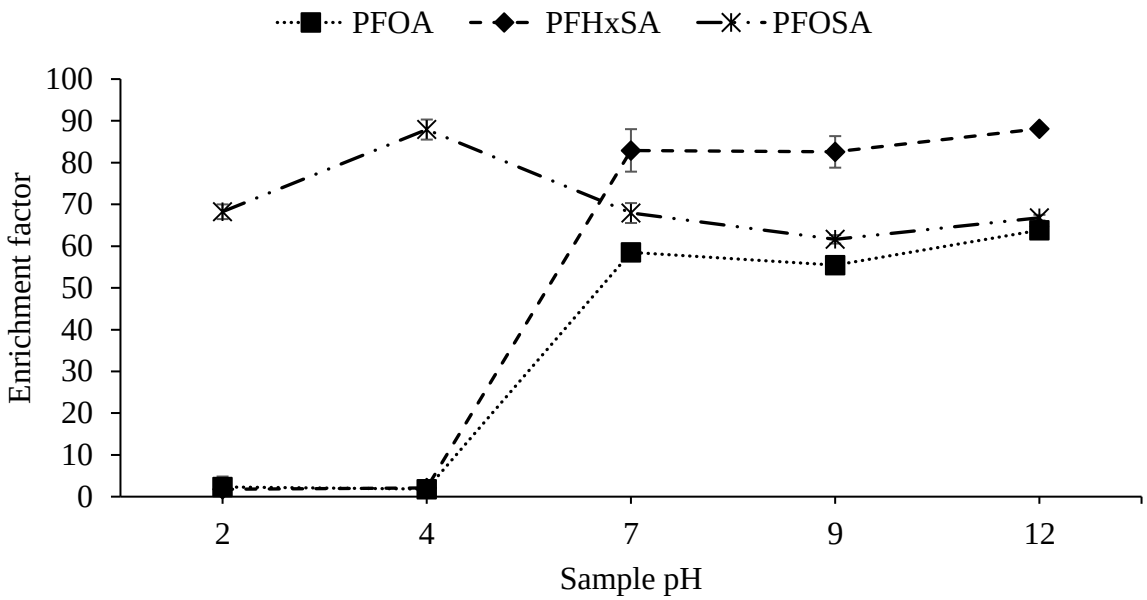

Figure 1: The effect of sample pH. Experimental conditions: volume of donor solution = 5 mL; extraction solvent = chloroform: volume of extraction solvent = 800 µL; dispersive solvent = acetonitrile; volume of dispersive solvent = 1000 µL; manual shaking time = 1 min; vortex time = 2 min; centrifugation = 5000 rpm for 10 min. (n = 3, RSD)

3.2 Effect of extraction solvents and volume of extraction solvent

Selection of extraction solvent is very important in this DLLME. The solvent must be immiscible with aqueous later and must have higher affinity with the target analytes. In this

study, dichloromethane, chloroform and tetrachloromethane have been selected to investigate the optimum extraction solvent. The results from Figure 2 shows all the solvents can enrich the target analytes better, with chloroform having the highest enrichment capacity. The interaction between the analytes and the solvents can be through electrostatic interaction and lipophilic interaction. Chloroform was selected as the optimum extraction solvent and it was used in subsequent study to investigate the effect of extraction volume. It was apparent from Figure 3 that enrichment factor increased with an increase in extraction volume and reaches a maximum from 800 µL. Therefore, 800 µL was used as the volume of extraction solvent.

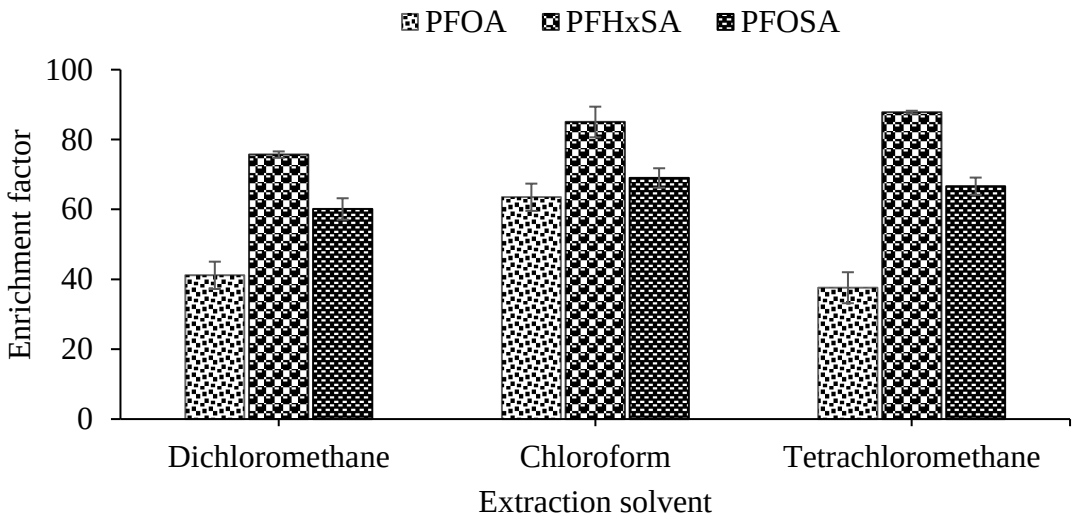

Figure 2: The effect of extraction solvent. Experimental conditions: volume of donor solution = 5 mL; pH of donor solution 12.0; volume of extraction solvent = 800 µL; dispersive solvent = acetonitrile; volume of dispersive solvent = 1000 µL; manual shaking time = 1 min; vortex time = 2 min; centrifugation = 5000 rpm for 10 min. (n = 3, RSD)

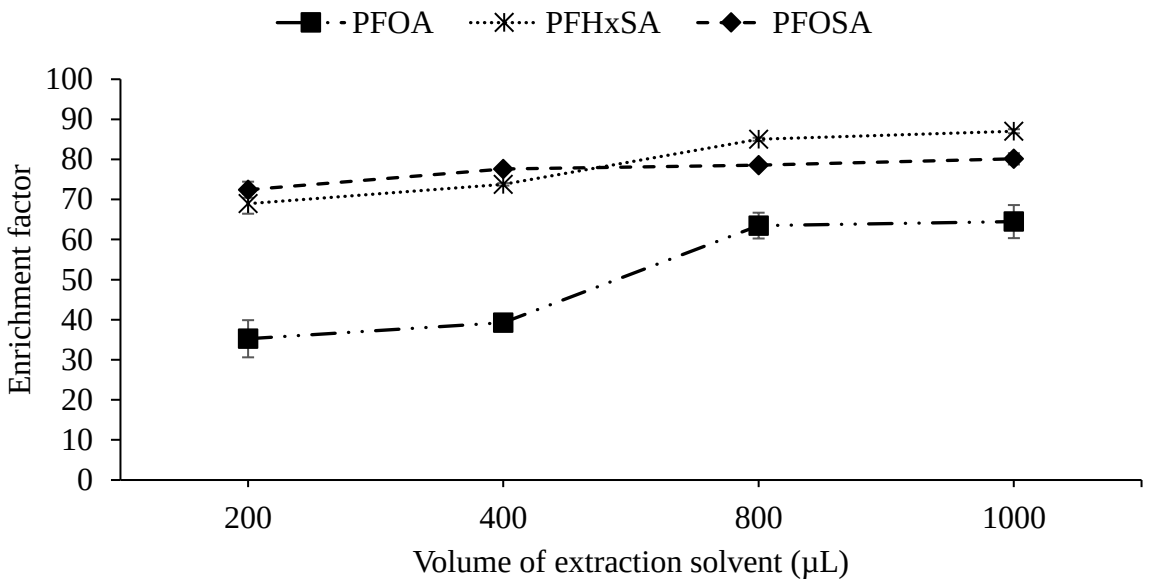

Figure 3: The effect of volume of extraction solvent. Experimental conditions: volume of donor solution = 5 mL; pH of donor solution 12.02; extraction solvent = chloroform; dispersive solvent = acetonitrile; volume of dispersive solvent = 1000 µL; manual shaking time = 1 min; vortex time = 2 min; centrifugation = 5000 rpm for 10 min. (n = 3, RSD)

3.3 Effect of dispersive solvent and volume of dispersive solvent

The use of dispersive solvent during DLLME is very important because it facilitates the movement of analytes into the extraction solvent by inducing the formation of emulsion. The principles are the dispersive solvent must be miscible with both the aqueous solution and the extraction solvent. In this paper, methanol, acetonitrile, MeOH/ACN, which is methanol/acetonitrile mixture (50:50, v/v), and acetone have been selected based on the principle mentioned above to identify the best dispersive solvents. The results from Figure 4 shows that acetonitrile contributes to higher enrichment of analytes. This result is consistent with the results found by (Wang et al., (2018) and Vela-Soria et al. (2021) where acetonitrile has been found to be an optimum dispersive solvent during PFAS extraction. The interaction between acetonitrile and PFAS could be through hydrogen bond interaction since acetonitrile is a polar aprotic, compared to methanol and acetone, and the PFAS investigated in this study are hydrogen bond donors. Therefore, acetonitrile was chosen as the optimum dispersive solvent. The next experiment was to investigate the volume of dispersive solvent in the range between 400 µL to 1200 µL. The results are presented in Figure 5 where it was found that an increase in dispersive volume has a positive effect on the enrichment factor. The enrichment

factor increased until it reached a maximum at a volume of 1000 µL which was chosen as the optimum volume of dispersive solvent.

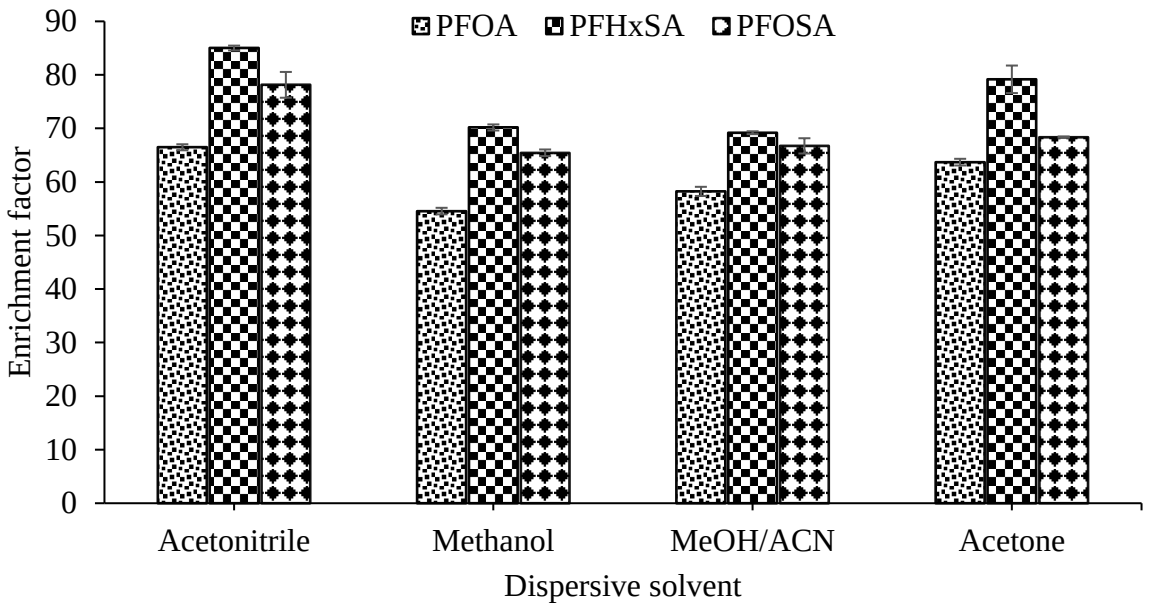

Figure 4: The effect of dispersive solvent. Experimental conditions: volume of donor solution = 5 mL; pH of donor solution 12.01; extraction solvent = chloroform; volume of extraction solvent = 800 µL; volume of dispersive solvent = 1000 µL; manual shaking time = 1 min; vortex time = 2 min; centrifugation = 5000 rpm for 10 min. (n = 3, RSD)

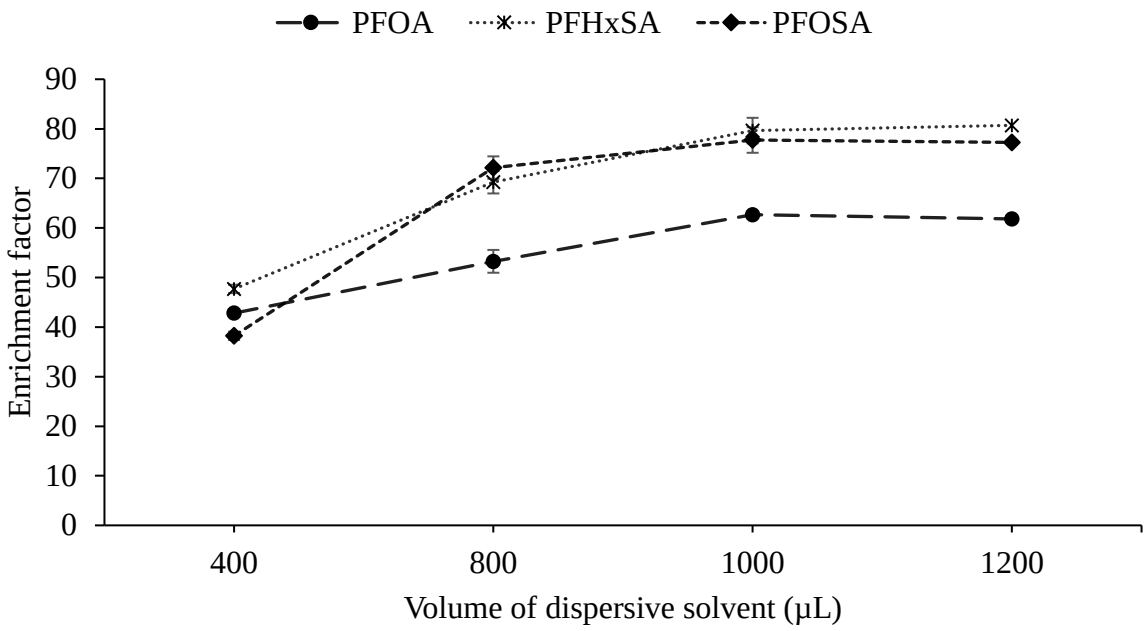

Figure 5: The effect of dispersive solvent. Experimental conditions: volume of donor solution = 5 mL; pH of donor solution 12.0; extraction solvent = chloroform; volume of extraction solvent = 800 µL; dispersive solvent = acetonitrile; manual shaking time = 1 min; vortex time = 2 min; centrifugation = 5000 rpm for 10 min. (n = 3, RSD)

3.4 The effect of ionic strength

Adding salt into the aqueous solution increases its ionic strength which decreases the solubility of analytes from aqueous solution and facilitates their migration into the organic layer through salting out effect (Shi et al., 2020). In contrast, addition of salts can also have a negative impact in analyte enrichment because an increase in ionic strength can increase the viscosity of the organic solvent. The type of salt used must be insoluble or partially soluble in the organic phase. In this study sodium chloride has been chosen as the optimum salt based on the positive effect of analyte enrichment from the literature (Yao and Du., 2020). The concentration of salt has been investigated from the range of 0 to 15%. Figure 6 shows that addition of salt has a little impact on the enrichment of analytes, and it was also found that the enrichment of other analytes decreased as the concentration of salt increases. Therefore, further experiments were performed without salt addition.

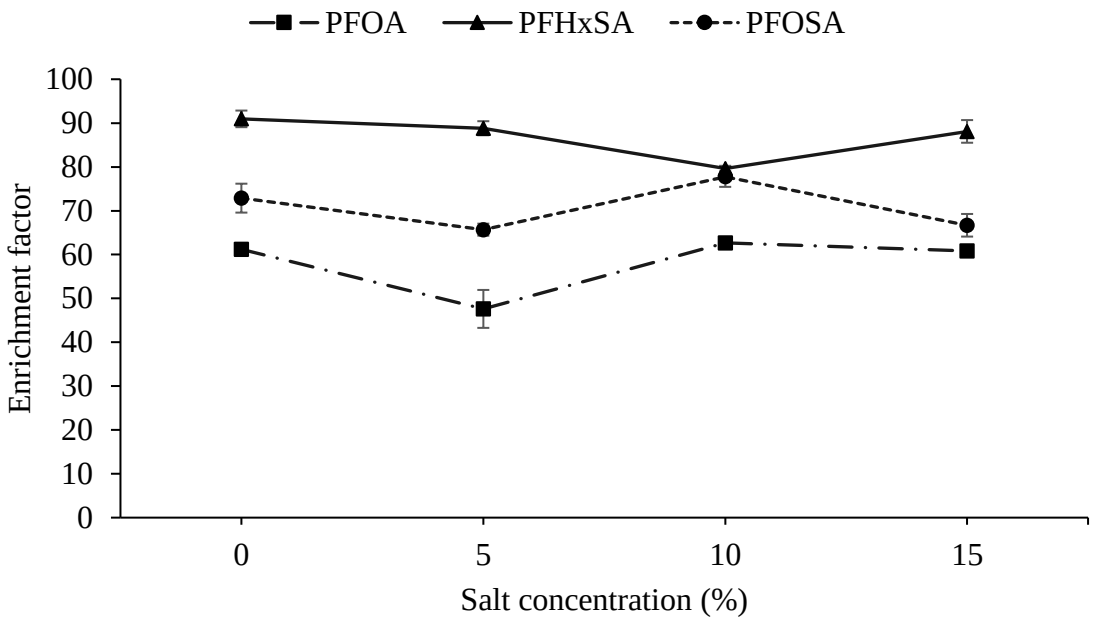

Figure 6: The effect of ionic strength. Experimental conditions: volume of donor solution = 5 mL; pH of donor solution 12.01; extraction solvent = chloroform; volume of extraction solvent = 800 µL; dispersive solvent = acetonitrile; volume of dispersive solvent = 100 µL; manual shaking time = 1 min; vortex time = 2 min; centrifugation = 5000 rpm for 10 min. (n = 3, RSD)

3.5 The effect of agitation time

Agitation is very important in DLLME because it assists in the formation of cloudy solutions and therefore, the movement of analytes into organic layer. In this study, the effect of manual shaking time was investigated in a time range of 1 – 10 min. However, the results from Figure 7 shows that the increase in agitation time has no influence on the increase of enrichment factor. Therefore, the mixture was shaken for 1 min because it was important for the formation of emulsification.

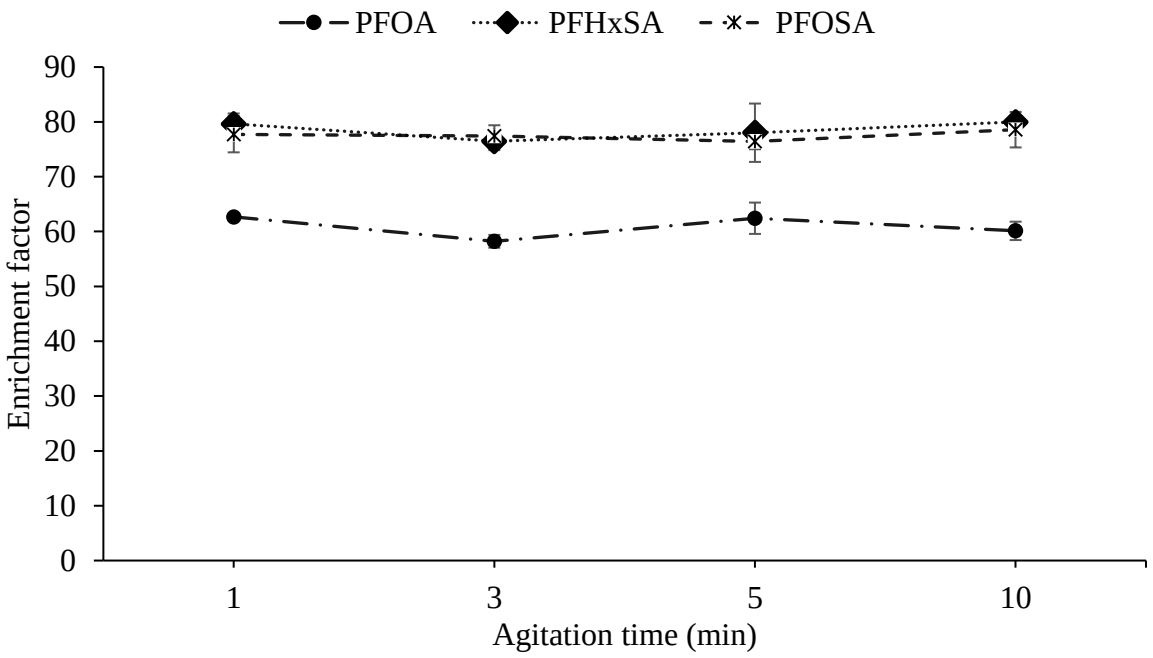

Figure 7: The effect of ionic strength. Experimental conditions: volume of donor solution = 5 mL; pH of donor solution 12.01; extraction solvent = chloroform; volume of extraction solvent = 800 µL; dispersive solvent = acetonitrile; volume of dispersive solvent = 100 µL; vortex time = 2 min; centrifugation = 5000 rpm for 10 min. (n = 3, RSD)

3.2 UHPLC-qToF-MS

Quantitative analyses were performed using multiple reaction monitoring mode on the UHPLC-qToF-MS instrument. The compound table was set using the values in Table 1. These values enabled the instrument to detect and quantify only the analytes with pre-defined retention time, precursor ion and its product ion. First, the precursor ion was determined by ionising the analytes in the MS in negative ionisation mode using electrospray ionisation source. The analytes were then run in MS/MS mode where the optimum collision energy, which can effectively fragment the precursor ion, was chosen together with the product ion. Lastly, the full chromatogram of the mixture of all PFAS were run where the retention time of each analyte was determined (Figure 8).

Table 1: Selected transitions and optimised potentials

Analyte	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)	Spread (eV)
PFOA	3.66	612.962	368.973	10	5
PFOSA	3.70	398.934	79.955	40	5
PFHxSA	3.79	498.926	79.955	50	5

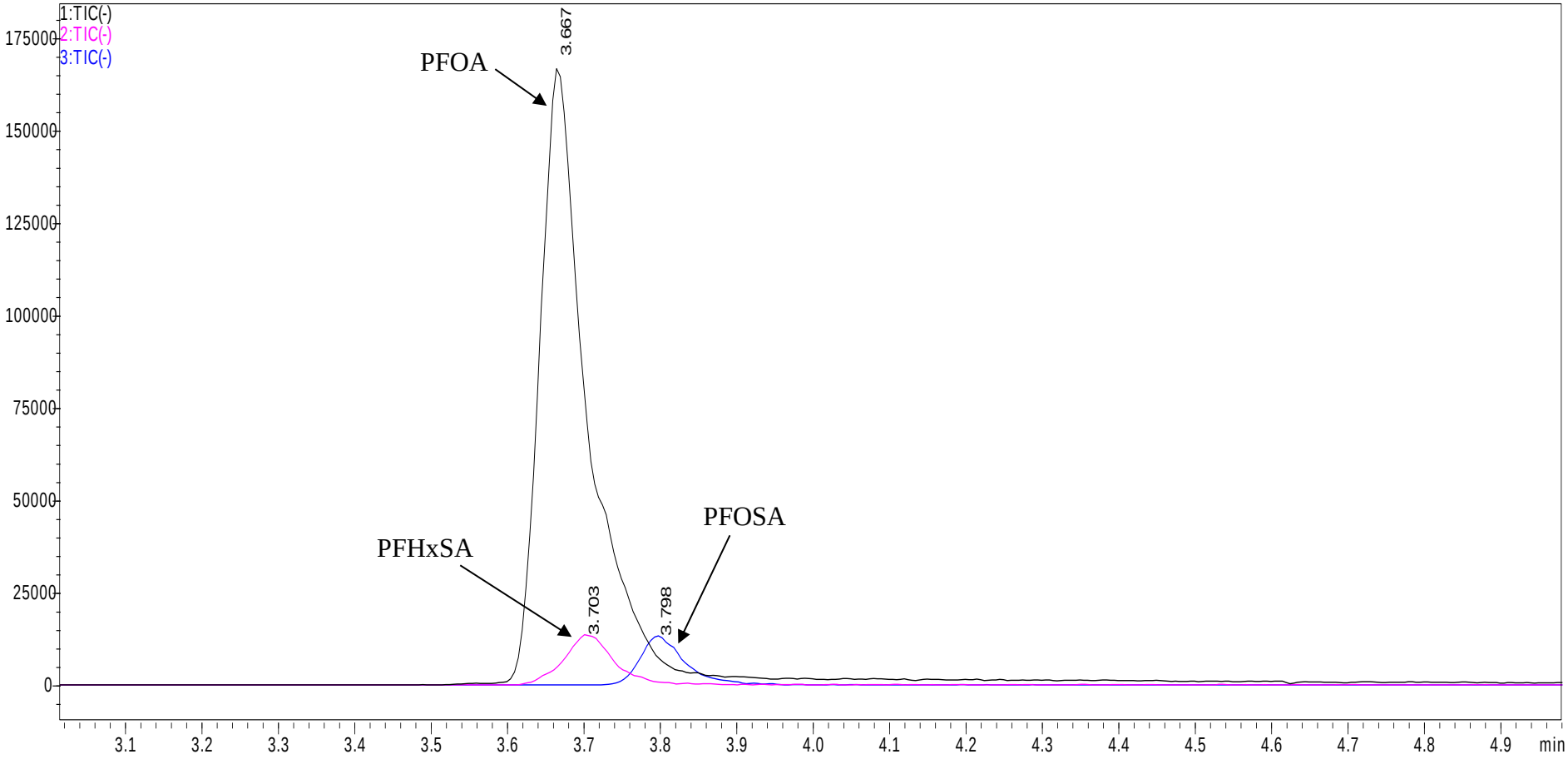

Figure 8: The MRM chromatogram of PFAS with different the retention time

3.6 Method validation

The developed method was validated by determining the linear range, limit of detection (LOD), limit of quantification (LOQ), relative standard deviation (RSD), percentage recovery and enrichment factor to evaluate the analytical performance. The analysis was run in triplicate (n = 3) to evaluate its precision. The minimum detectable concentration was investigated by diluting the concentration of each analyte. The linear range of the developed method was investigated by plotting the spiked concentration against the absorbance. The equations for linear regression lines for each antibiotic are represented in Table 2. The standard curve exhibited an excellent coefficient of or $r^2 > 0.99$ with a linear range of 1.25 µg kg^{-1} - 160 µg kg^{-1}. The LOD and LOQ were calculated by multiplying the signal to noise ratio (S/N) by 3 and 10, respectively. The range of LOD and LOQ was found between 0.64 µg kg^{-1} – 2.75 µg kg^{-1} and 1.82 – 8.26 µg kg^{-1}, respectively. The accuracy and precision of the method was evaluated based on the RSD which was ranging between range 2.86 to 5.32. The method validity and accuracy show that this technique can be applied for routine monitoring of PFAS in food contact materials.

Table 2: Validation results of the developed method based of DLLME

PFAS	Equation	Linear range (µg kg^{-1})	r^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	EF	RSD
PFOA	y = 2947.1x – 329114	1.25 – 160	0.9999	0.64	1.82	62.68	2.86
PFHxSA	y = 205.56x – 24373	1.25 – 160	0.9995	1.86	5.60	79.64	5.32
PFOSA	y = 333.74x – 85.342	1.25 – 160	0.9990	2.75	8.26	77.47	3.30

3.7 Application

The performance of DLLME developed in this study was applied for analysis of PFAS in fiber-based food contact material purchased in fast-food stores around Thohoyandou (Limpopo, South Africa). None of the PFAS were detected in this matrix (Table 3). However, to evaluate the performance and efficiency of the developed method for extraction of PFAS in food contact material, three concentration levels were spiked into blank fiber-based materials. The concentration that was detected was compared with the concentration added and used to calculate the relative recovery. Table 3 shows that the method has an excellent relative recovery which ranges between 67.80% to 96.11% with low RSD which range from 0.26 – 9.47.

Table 3: The concentration of analytes in real sample and the percentage recovery of the spiked levels

PFAS	Real sample	Recovery (%) 10 µg kg^{-1}	RSD[c]	Recovery (%) 50 µg kg^{-1}	RSD[c]	Recovery (%) 100 µg kg^{-1}	RSD[c]
PFOA	-[a]	94.93	4.28	96.11	7.51	82.92	2.10
PFHxSA	-	91.77	8.96	88.15	9.47	76.76	0.28
PFOSA	-	67.80	6.48	70.80	3.23	90.90	0.87

a = less than limit of quantification

3.7 Comparison

The method performance was compared with the miniaturised liquid phase extraction techniques available in the literature. The parameters that were compared are the LOD, LOQ and relative recoveries, and the values are summarised in Table 4. The method developed in this study was found to be more effective or comparable with the other methods. The LOD were in the similar range with the other studies, but the LOQ was much better. The extraction efficiency which was evaluated in terms of percentage recovery of the method was also comparable with those in the literature. These results prove that miniaturised extraction techniques, like the one developed in this study, can be applied for routine analyses of PFAS in food contact materials or other complex matrices. This is because, compared to the classical extraction technique, these methods are simple, cheap and environmentally friendly.

Table 4: Comparison of miniaturised liquid phase extraction techniques used for pre-concentration of antibiotics

Matrix	Analytes	Methods	LOD	LOQ	% Recovery	Reference
Breast milk	PFCA (C_6 – C_{13}) and PFSA (C_6 and C_8)	SALLE – DLLME-LC-MS/MS	-	20 pg mL^{-1}	85.9–110.8	Vela-Soria et al. (2020)
Placenta	PFCA (C_6 – C_{13}) and PFSA (C_6 and C_8)	SALLE – DLLME-LC-MS/MS		0.02 ng g^{-1}	88.2 – 113.9	Vela-Soria et al. (2021)
Aqueous film forming forms (AFFF) and groundwater	26 newly identified and 21 legacy PFAS	LLME-LC-MS/MS	0.71 – 67 ng L^{-1}		69 – 106	Backe et al. (2013)
Tape and river water and urine	PFCA (C_3 – C_{17}) and	DLLME-LC-MS/MS	0.6 – 8.7 ng L^{-1}	-	80.6 – 121.4	Wang et al. (2018)

	PFSA (C_4 and C_{10})					
Food contact material	PFOA, PFHxSA and PFOSA	DLLME – LC-MS/MS	0.64 – 2.75 µg kg^{-1}	1.82 – 8.26 µg kg^{-1}	67.80 – 96.11	This study

4. Conclusion

In this study, for the first time, a DLLME has been developed and applied for pre-concentration of PFAS in fiber-based food contact materials prior to UHPLC-qToF-MS analysis. The method was optimised by looking on the factors like sample pH, extraction and dispersive solvent, volume of extraction and volume of dispersive solvent, the concentration of NaCl and agitation time which affect the enrichment factors of analytes. The linearity was found within the range of 1.25 µg kg^{-1} – 160 µg kg^{-1} with a good coefficient of determination $r^2 > 0.999$. The method exhibited excellent enrichment factors which range from 62.68 to 79.64. The sensitivity of the method was evaluated by the LOD which ranged from 0.64 – 2.75 µg kg^{-1}, LOQ which ranged from 1.82 µg kg^{-1} – 8.26 µg kg^{-1}. The efficiency was evaluated by the percentage recoveries which range from 67.80% – 96.11%.

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Chapter 5: General conclusion and future work

This chapter give the general conclusion of the project and the future recommendations.

5.1 General conclusion

Antibiotics and PFAS are ubiquitous because they are readily used. However, the misuse of these substances has resulted in their residues present in the environment. Higher quantities have been detected in wastewater, sludge and manure, and eventually in food samples like vegetables, milk and meat. They are distributed in the environment through agricultural runoffs, air particulates and leaching. Another source of antibiotics and PFAS distribution is the insufficient treatment of wastewater treatment plants. Therefore, it is important to monitor the presence of these substances in the environment. In this project, two extraction techniques, namely, DLLME and LLME have been developed for the pre-concentration of antibiotics and PFAS before liquid chromatography quadrupole time of flight mass spectrometric analysis in complex food samples such as macadamia nuts and green beans, and food contact materials. The techniques were developed based on the principles of green analytical chemistry.

Dispersive liquid-liquid microextraction has been applied for multi-class extraction of antibiotics in **Paper III**, and extraction of perfluoro carboxylic acid and perfluoro sulfonic acids in **Paper V**. The developed methods proved to be sensitive with lower detection limits, higher accuracy with lower relative standard deviation, effective with higher percentage recovery and enrichment factor. During the method development, different factors that affect the enrichment of analytes were optimised. Analyte's physico-chemical properties such as pK_a values and polarity determine their partitioning of analytes into the organic phase. The use of a dispersive solvent such as acetonitrile, used in **Paper III** and **Paper V**, enhanced the partitioning of analytes into chloroform (used as an extractive solvent in both **Paper III** and **Paper V**) by forming a dispersion or small droplets that facilitated the movement of analytes.

Extraction of antibiotics has further improved by the development of novel deep eutectic solvent using the combination of aliquat-336 as hydrogen bond acceptor and butyric acid as hydrogen bond donor in **Paper IV**. The solvent was effective with higher enrichment factors and higher recoveries. Multiclass analytes of antibiotics were extracted in a very low extraction solvent in a short extraction time.

5.2 Future work

The use of deep eutectic solvent was very effective in the analysis of three classes of antibiotics without any solvent. In the future, the solvent can also be applied in DLLME in a combination with a dispersive solvent to improve the enrichment of analytes. Many published papers have focused on the use of DES for the extraction of a single class of antibiotics. In **Paper IV**, it

was demonstrated that DES can be applied in multiclass extraction. It is advisable to apply these kinds of solvents in other classes of antibiotics.

Extraction of PFAS using miniaturised liquid phase extraction is scarce. In **Paper V**, DLLME has been successfully developed for the extraction of PFAS with higher enrichment factors. In the future, green extraction solvents, such as DES, SUPRAS and ionic liquids, and extractive sorbents, such as magnetic nanoparticles, molecularly imprinted polymer, molecular organic frameworks and natural sorbents, needs to be investigated to further improve the analysis of PFAS in the environment. Different miniaturised extraction techniques also need to be developed to improve the extraction of PFAS without the introduction of high quantities of organic waste in the environment and expensive experimental procedures.

In this project, the developed extraction techniques were applied in food samples. However, they are not limited to food samples. They can be applicable to other matrices such as environmental samples (e.g., wastewater, surface water, drinking water, soil and sediments) and biological samples (e.g., blood plasma, whole blood and body organs).

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Appendix

Supplementary data

This section gives the supplementary data for the papers in this study

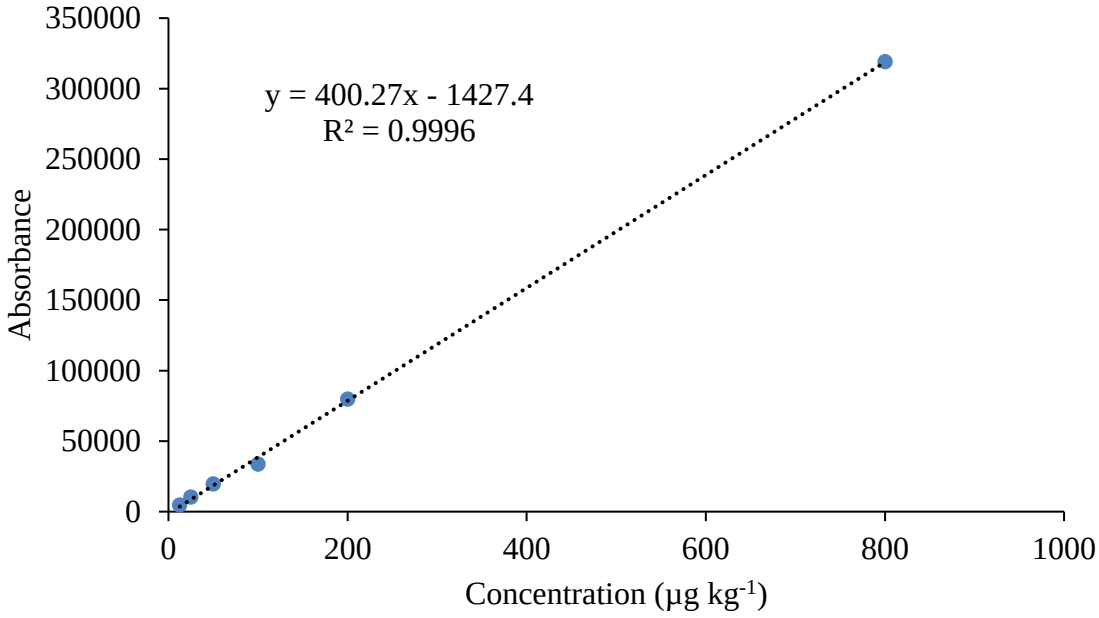

Figure A 1: Calibration curve for enrofloxacin

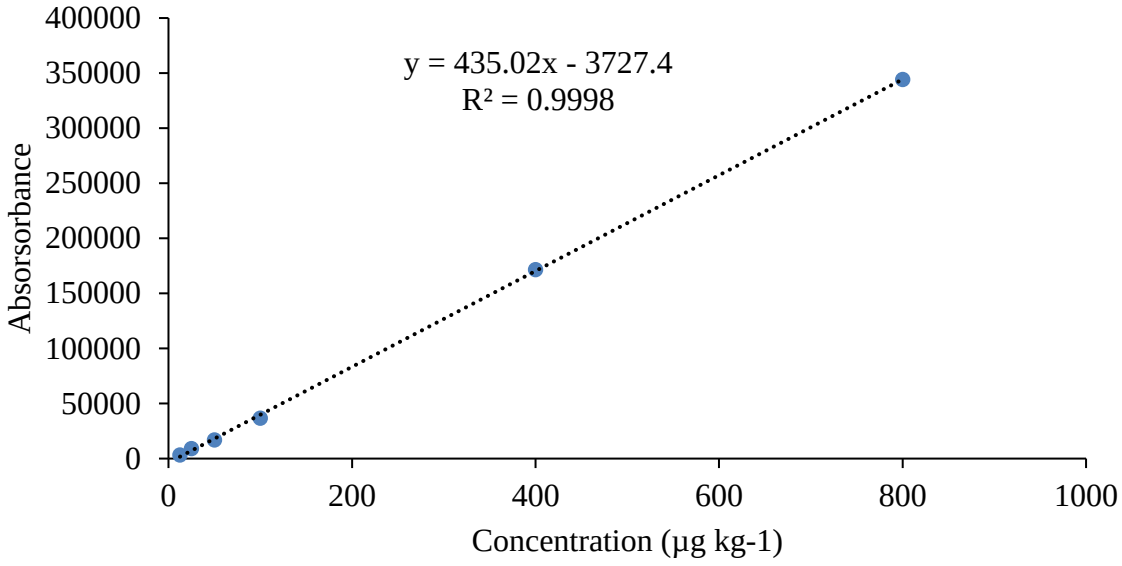

Figure A 2: Calibration curve for ofloxacin

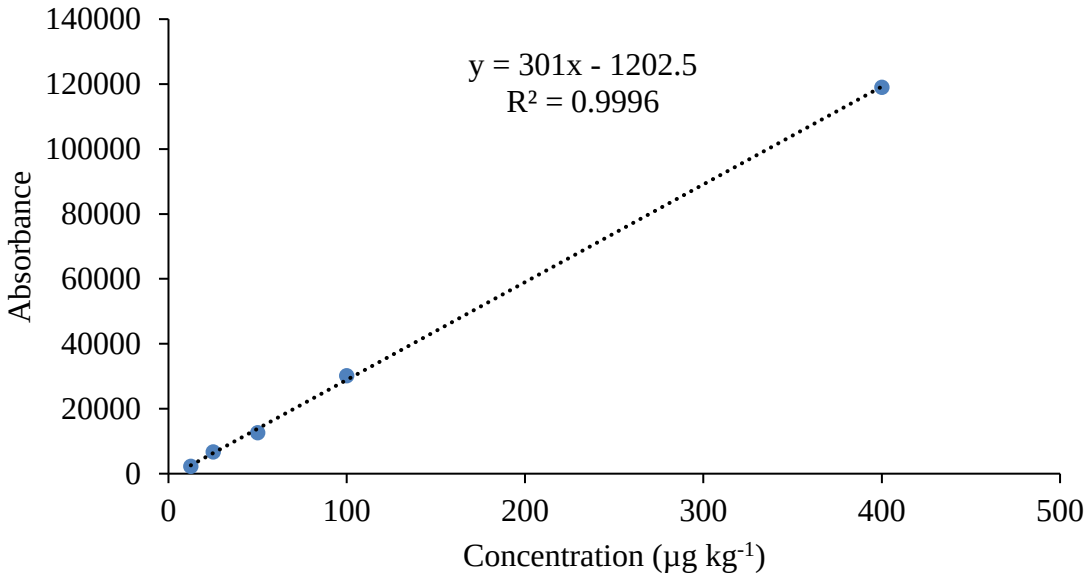

Figure A 3**:** Calibration curve for sulfadiazine

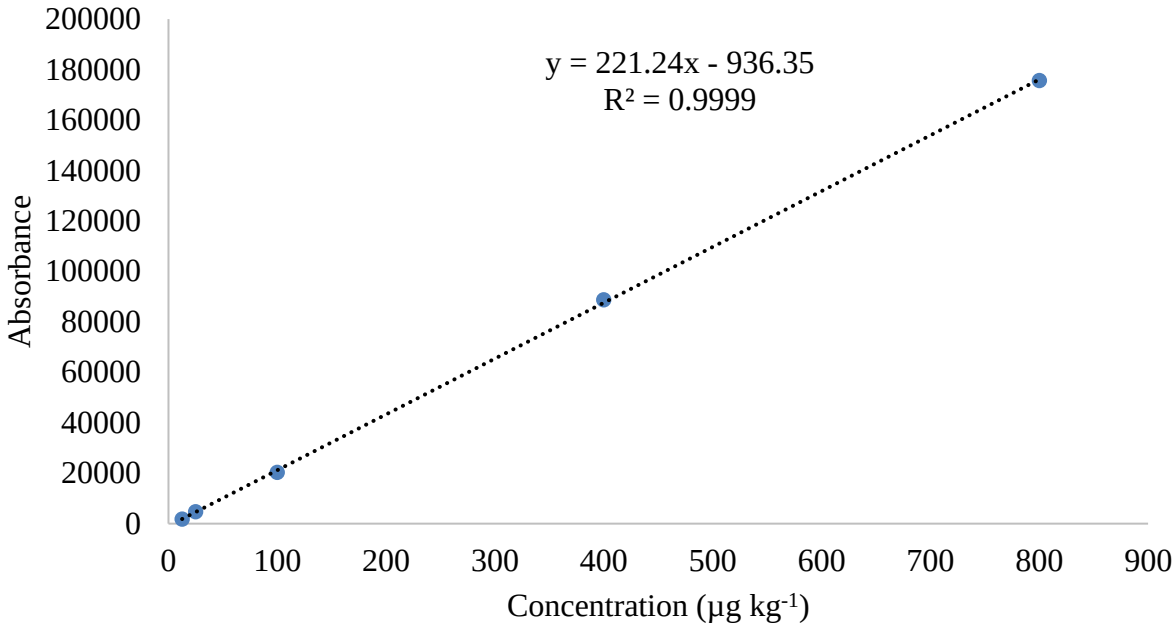

Figure A 4: Calibration curve for sulfamethoxazole

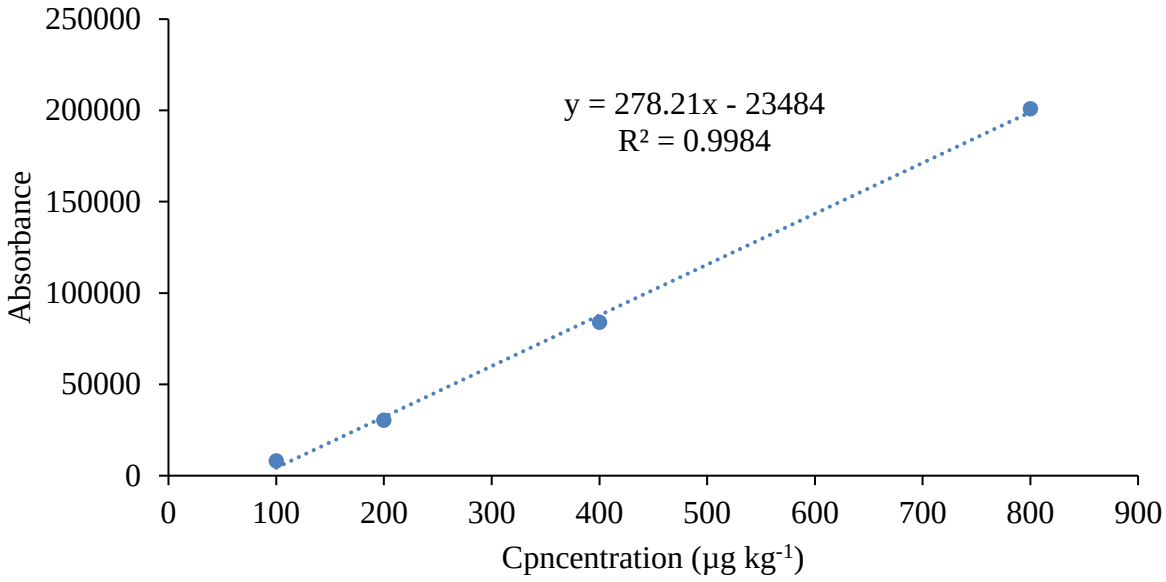

Figure A 5**:** Calibration curve for doxycycline

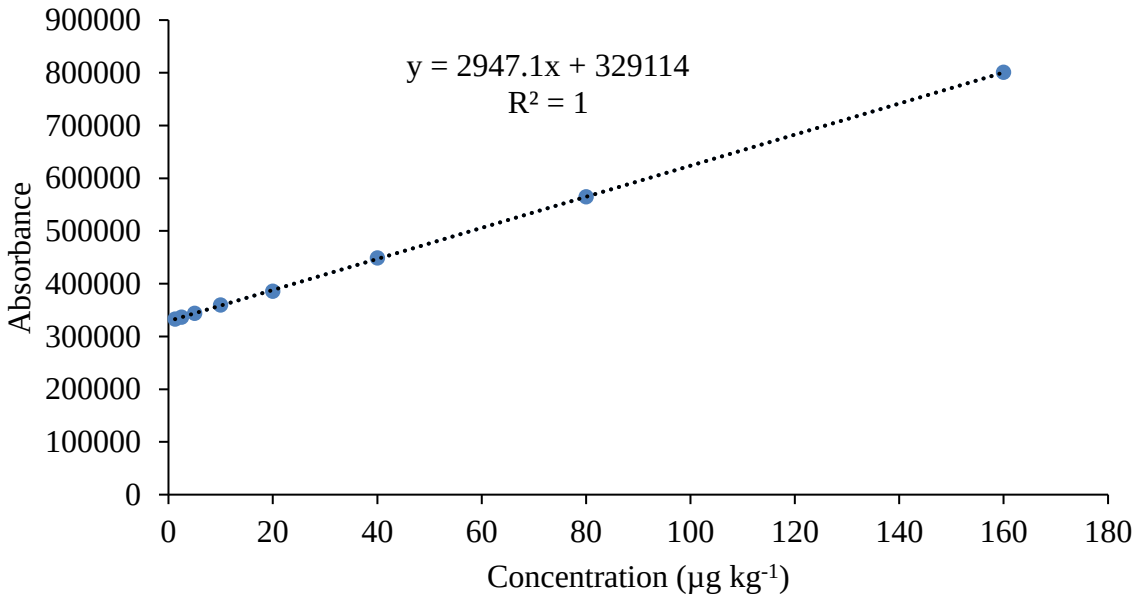

Figure A 6 : Calibration curve for PFOA

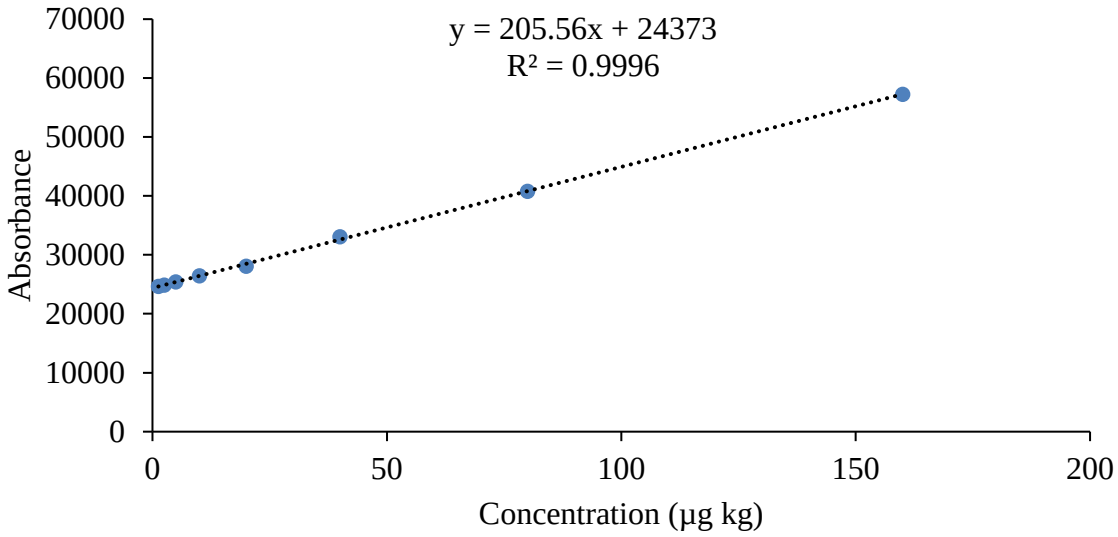

Figure A 7: Calibration curve for PFHxSA

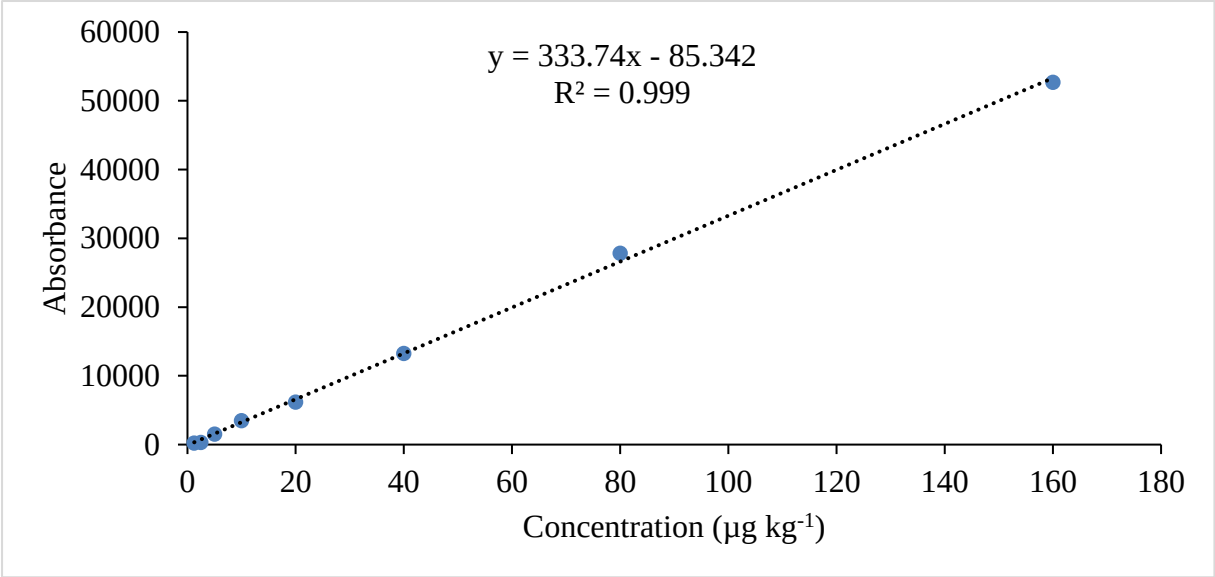

Figure A 8: Calibration curve for PFOSA

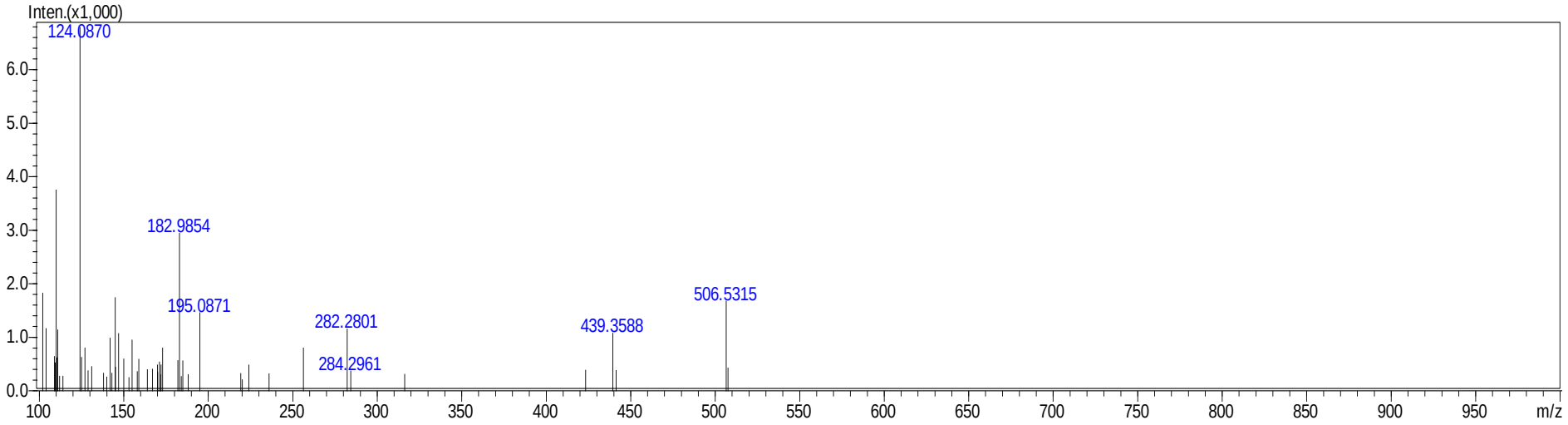

Figure A 9: MS spectrum of enrofloxacin

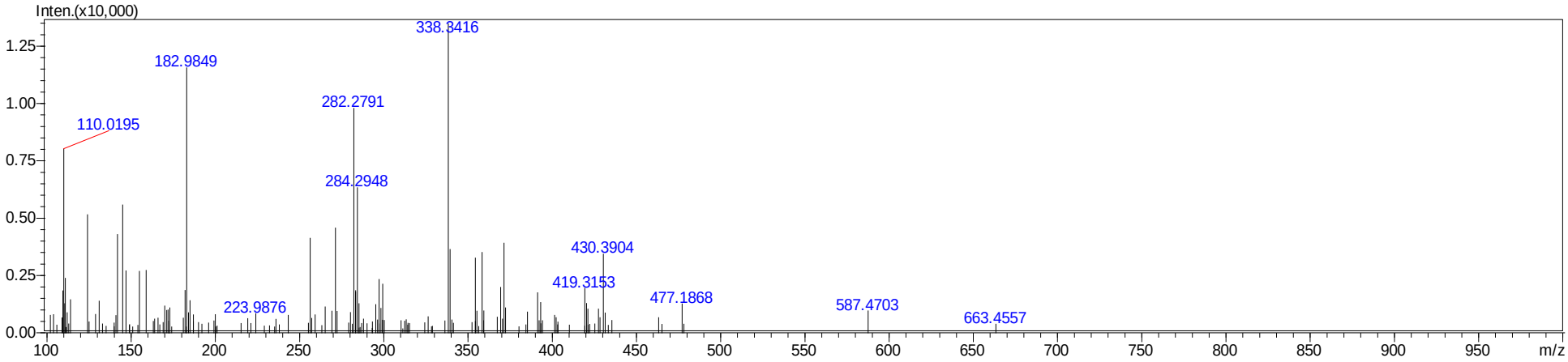

Figure A 10: MS spectrum of ofloxacine

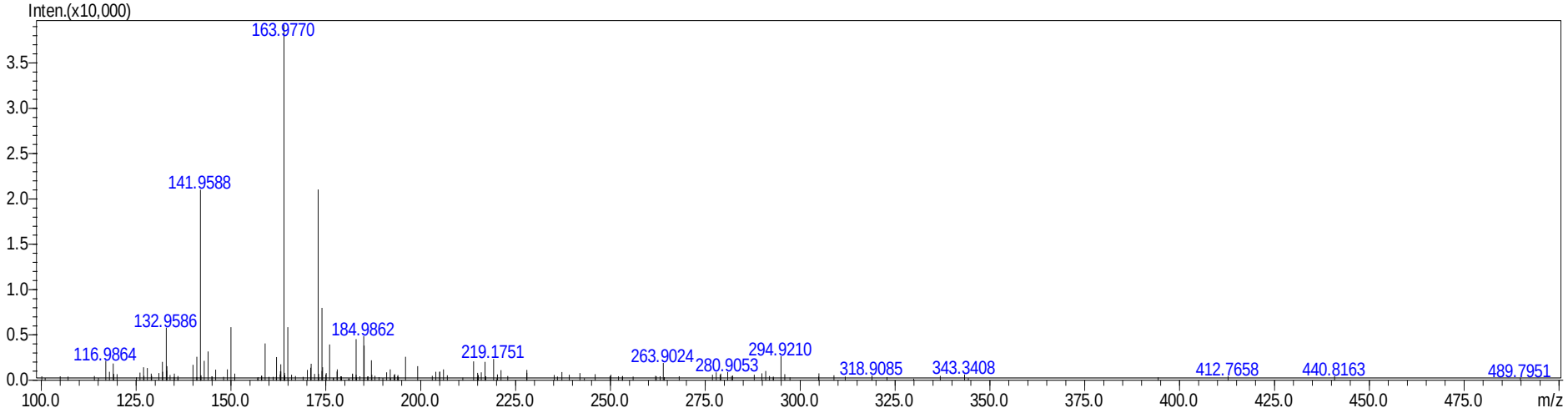

Figure A 11: MS spectrum of sulfadiazine

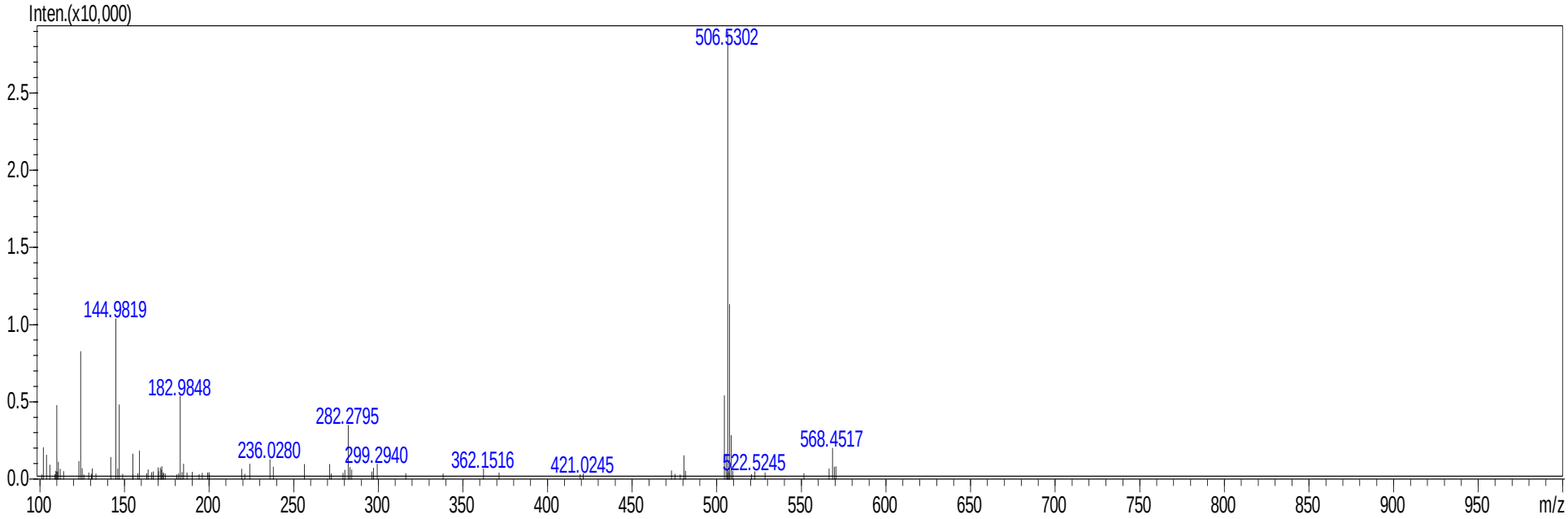

Figure A 12: MS spectrum of sulfamethoxazole

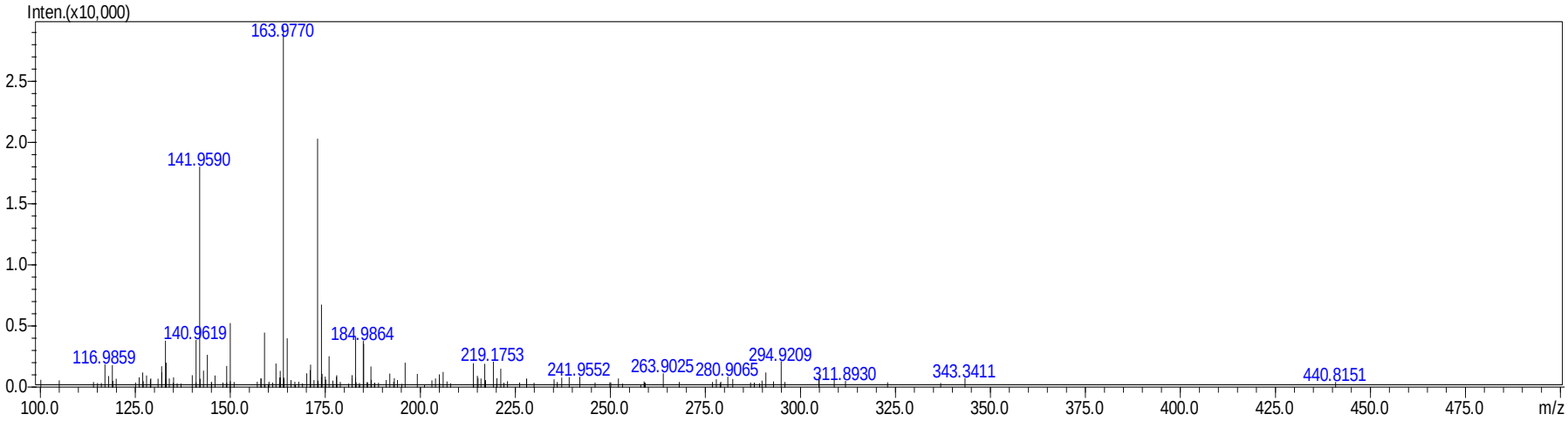

Figure A 13: MS spectrum of doxycycline

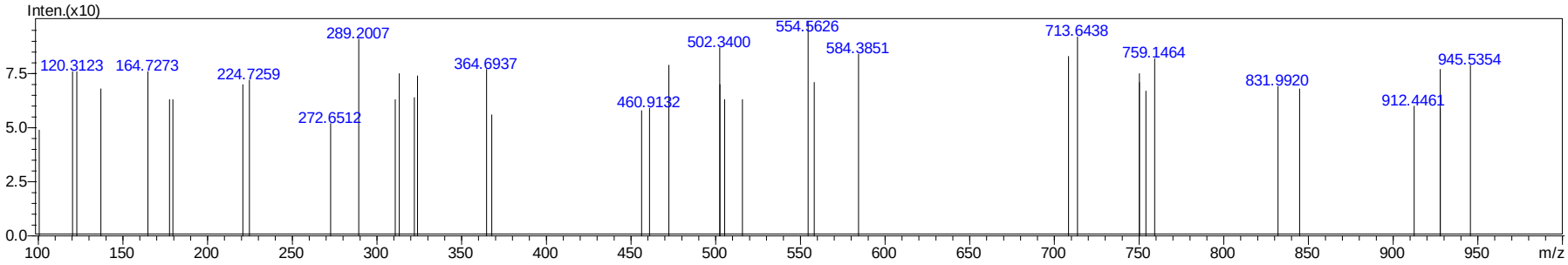

Figure A 14: MS/MS spectrum of enrofloxacin at collision energy of 20 eV with the spread of 5 eV

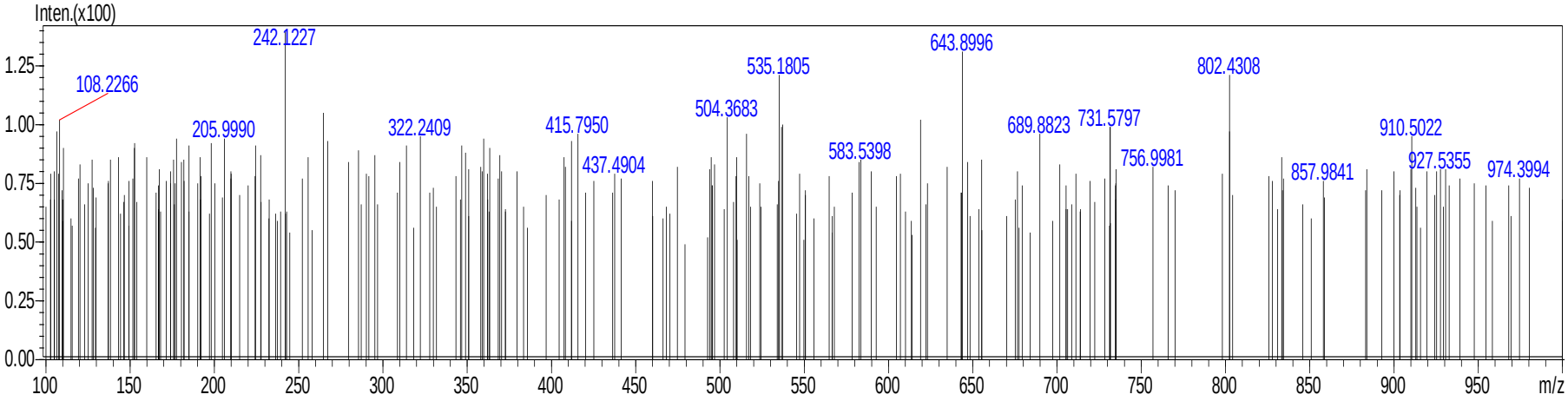

Figure A 15: MS/MS spectrum of ofloxacin at collision energy of 30 eV with the spread of 5 eV

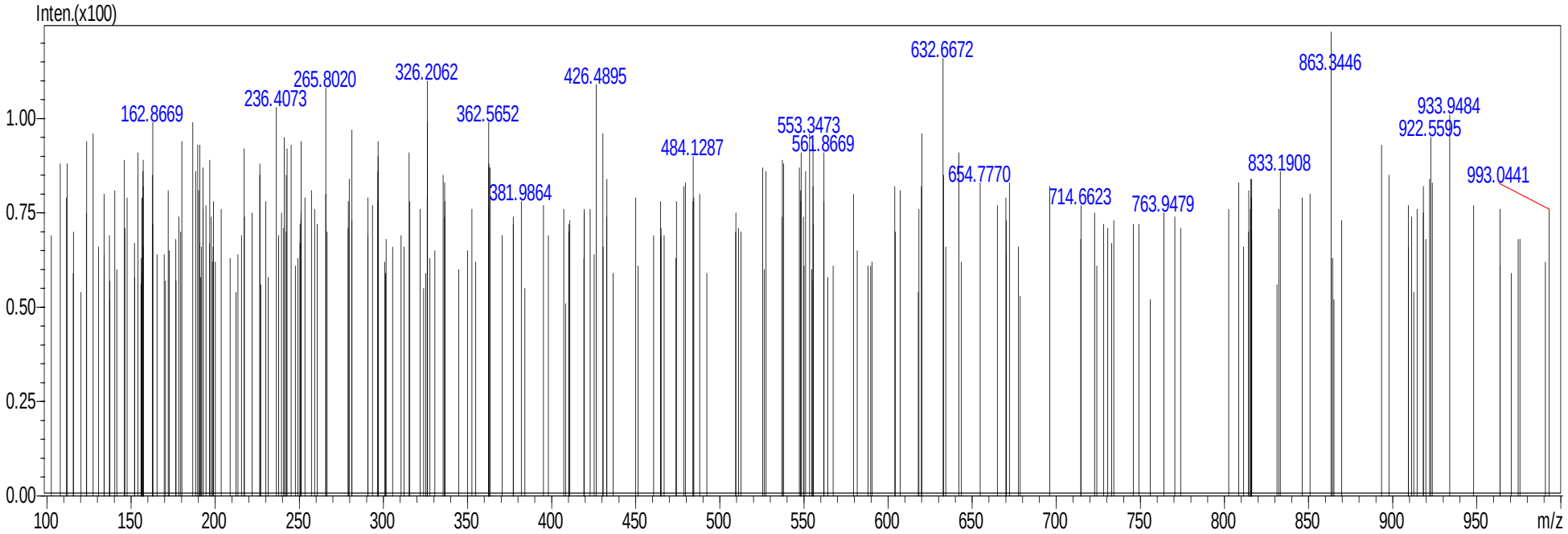

Figure A 16: MS/MS spectrum of sulfadiazine at collision energy of 20 eV with the spread of 5 eV

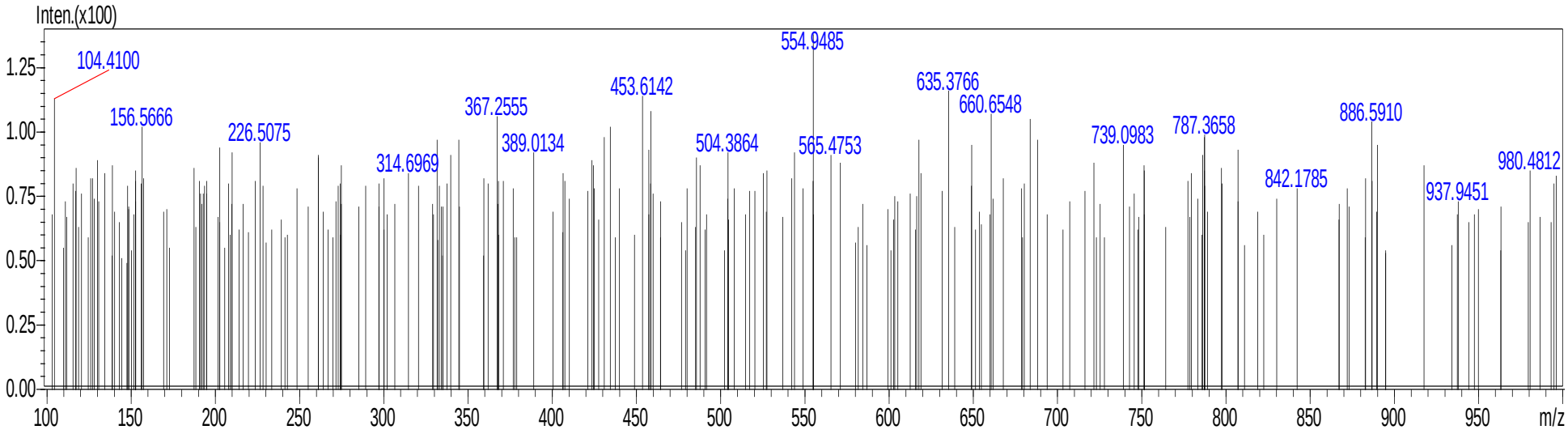

Figure A 17: MS/MS spectrum of sulfamethoxazole at collision energy of 20eV with the spread of 5 eV

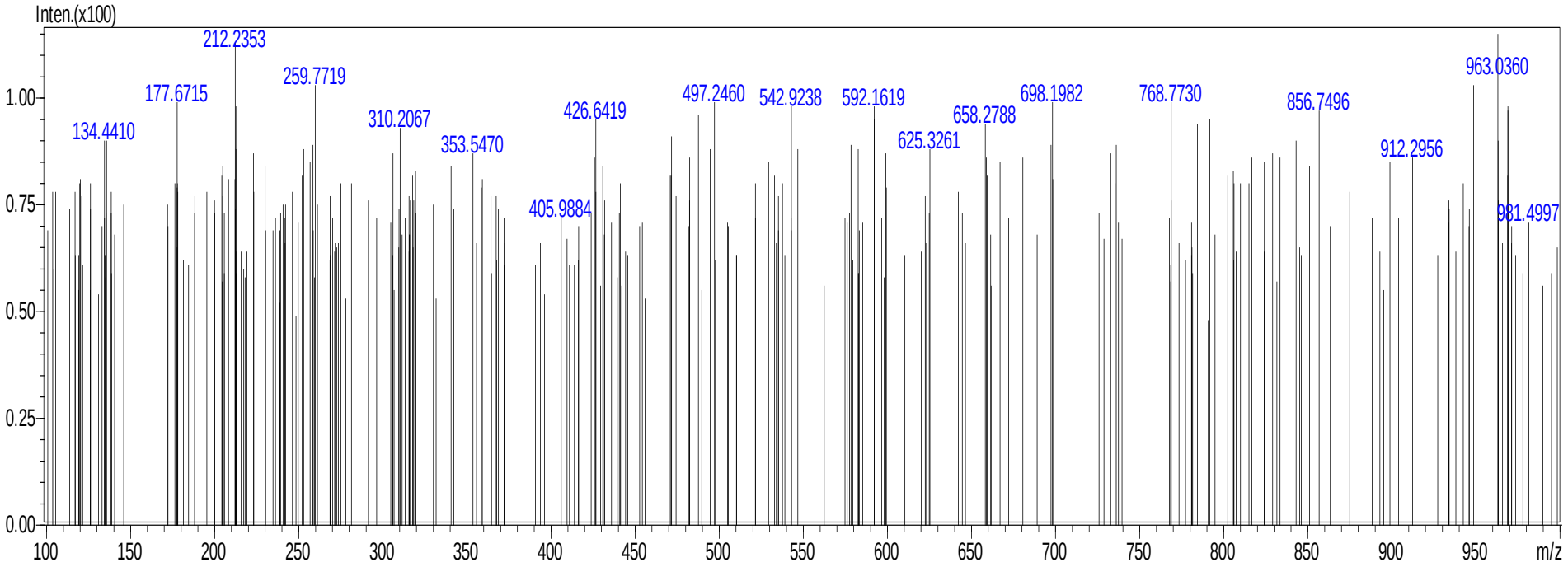

Figure A 18: MS/MS spectrum of doxycycline at collision energy of 20eV with the spread of 5 eV

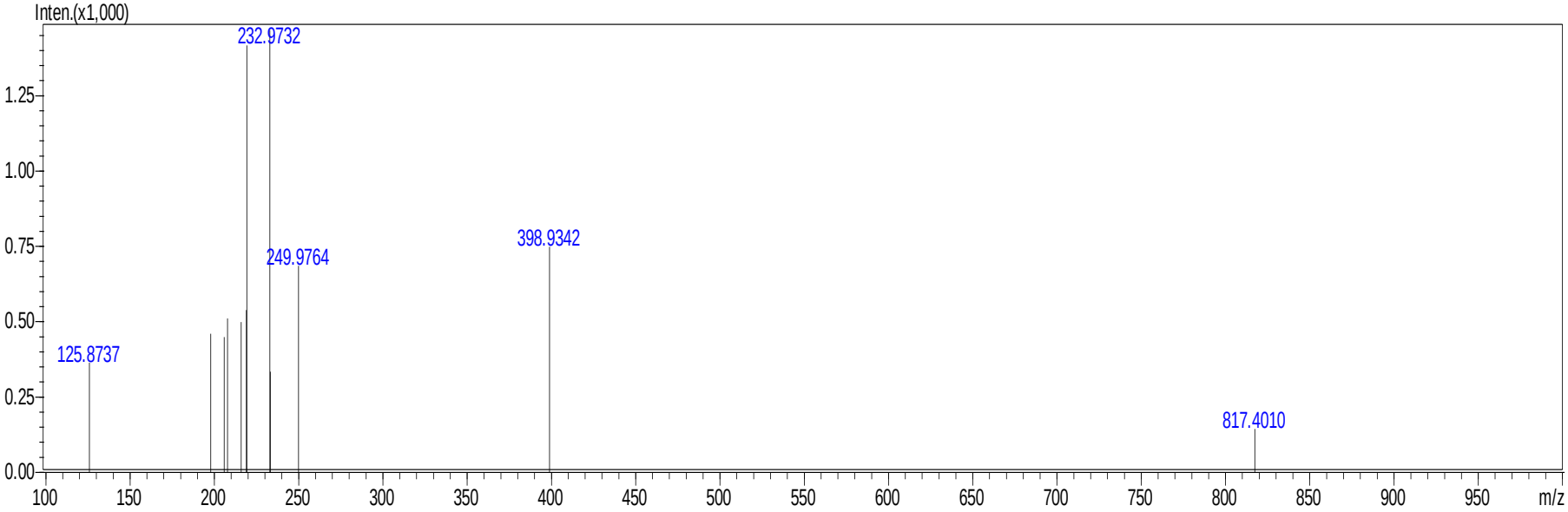

Figure A 19: MS spectrum of PFOA

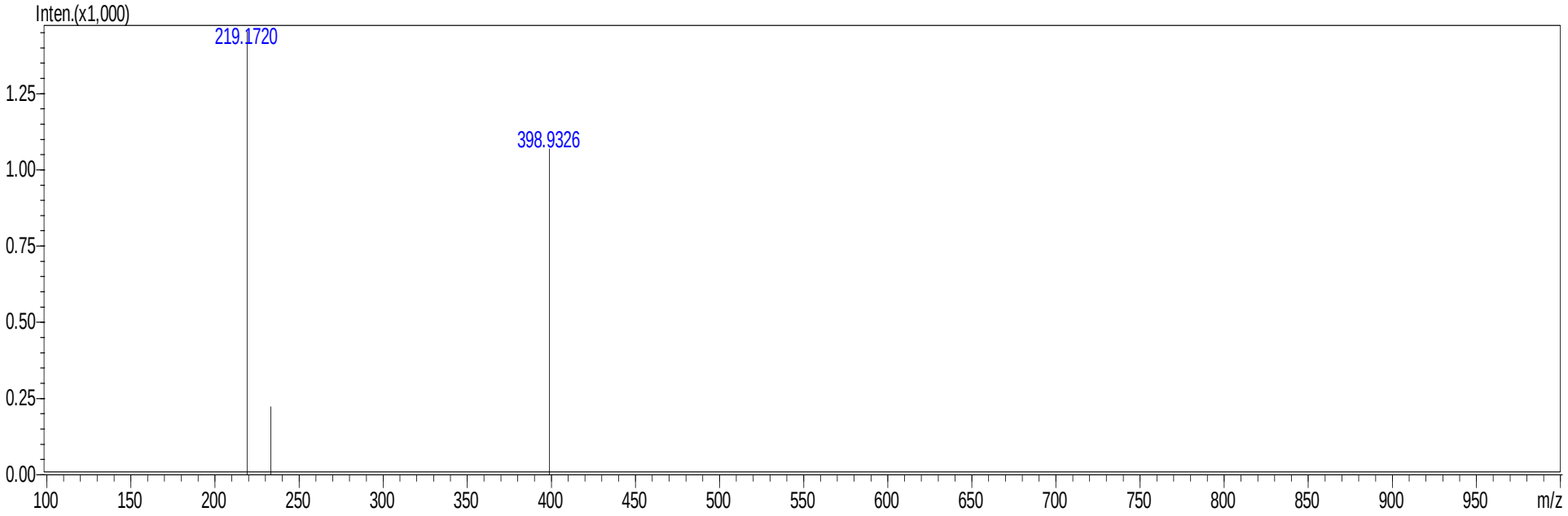

Figure A 20: MS spectrum of PFHxSA

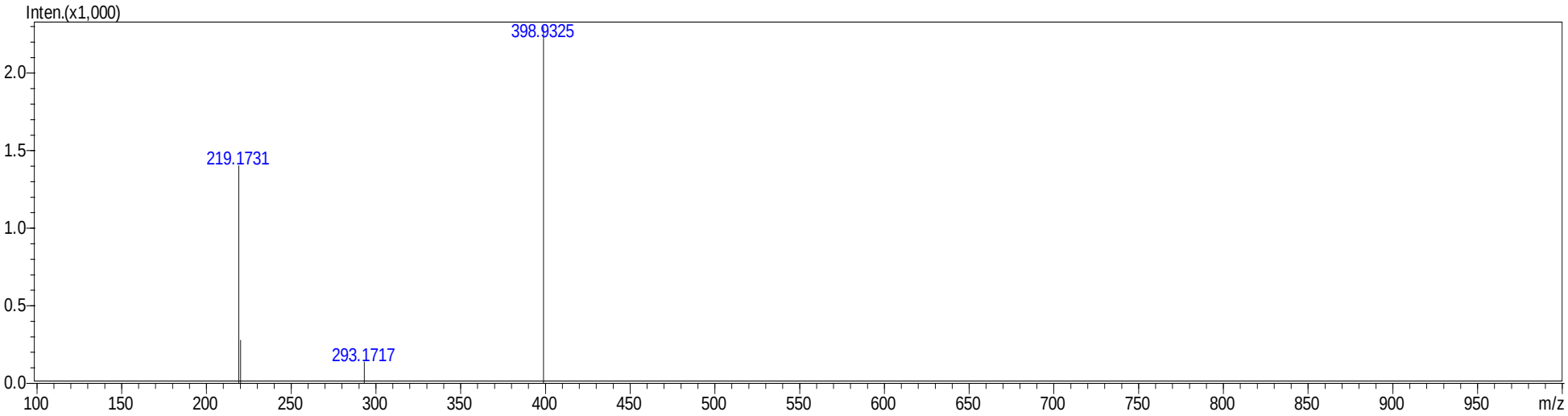

Figure A 21: MS spectrum of sulfadiazine

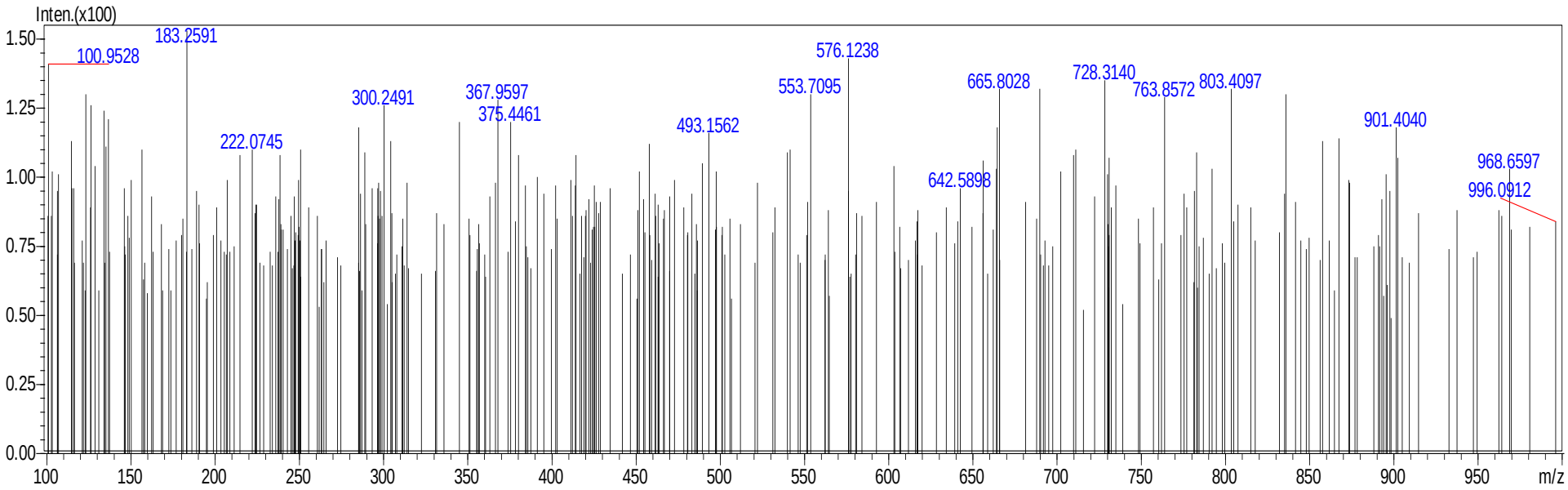

Figure A 22: MS/MS spectrum of PFOA at collision energy of 10 eV with the spread of 5 eV

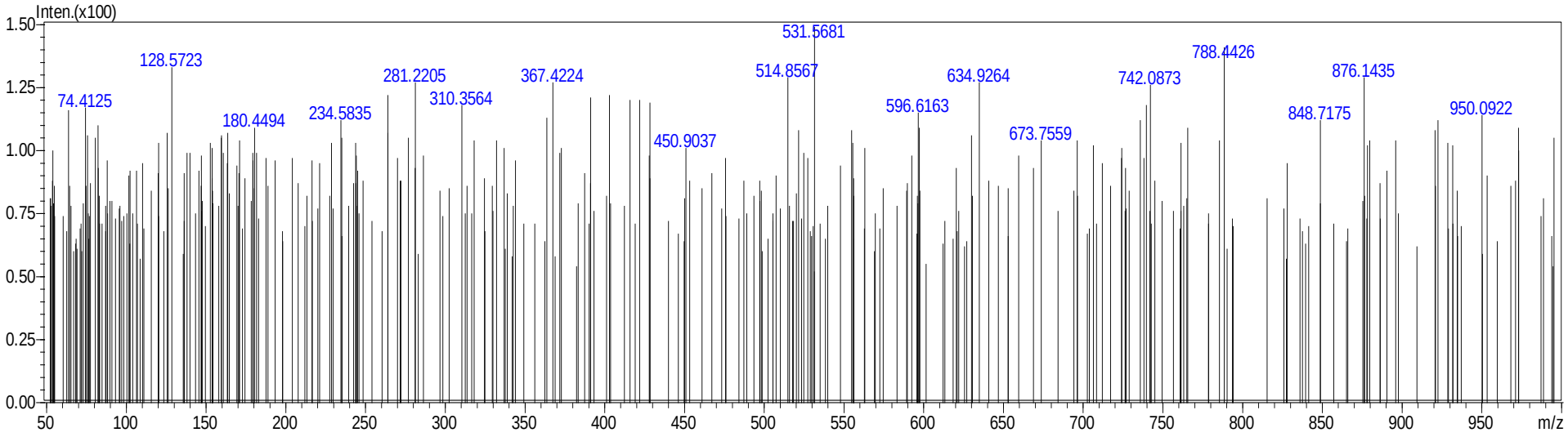

Figure A 23: MS/MS spectrum of PFHxSA at collision energy of 40eV with the spread of 5 eV

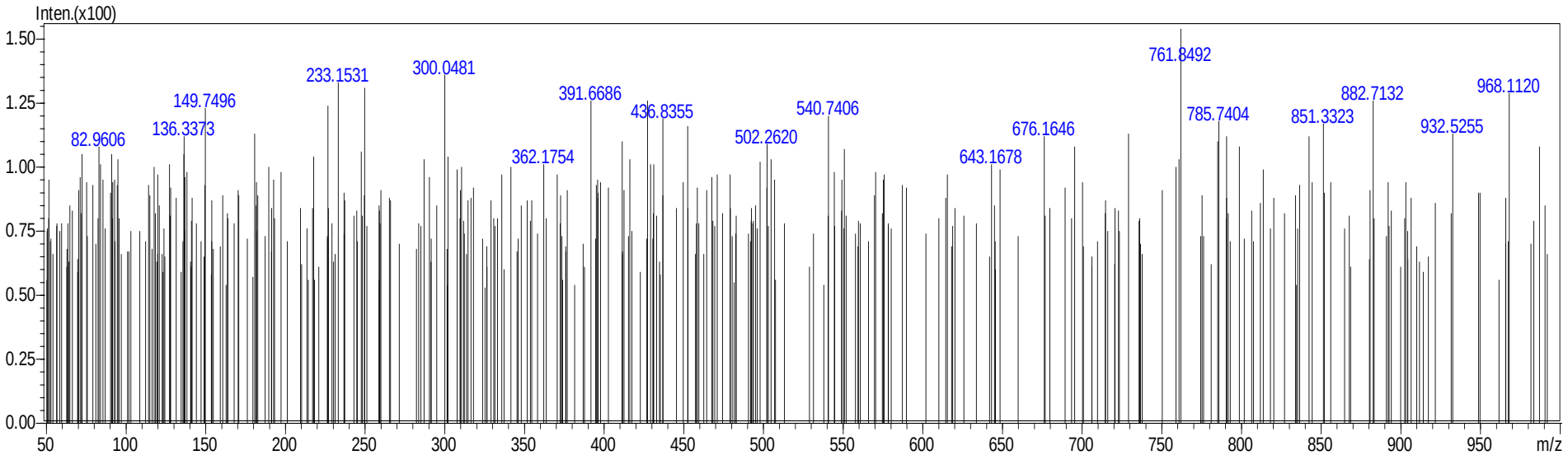

Figure A 24: MS/MS spectrum of PFOSA at collision energy of 50 eV with the spread of 5 eV