UNIVERSITY OF TARTU

FACULTY OF SCIENCE AND TECHNOLOGY

Institute of Chemistry

Joshua Onyeka Osagu

SIMULTANEOUS DETERMINATION OF SELECTED ANTIMICROBIAL AGENTS IN SEWAGE SLUDGE BY PRESSURISED LIQUID EXTRACTION AND LC-MS/MS

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Supervisor:

Koit Herodes (Associate Prof.)

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List of Abbreviations

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ABBREVIATIONS

AMR	antimicrobial resistance
ASE	accelerated solvent extraction
CIP	ciprofloxacin
ENR	enrofloxacin
ESI	electrospray ionization
GC	Gas chromatography
FA	formic acid
FF	florfenicol
FQs	fluoroquinolones
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol (hexafluoroisopropanol)
HLB	hydrophilic lipophilic balance
HPLC	high performance liquid chromatography
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MAE	microwave-assisted extraction
MAR	marbofloxacin
ME	matrix effect
MS	mass spectrometry
NOR	norfloxacin
OFL	ofloxacin
PLE	pressurized Liquid extraction
PVDF	polyvinylidene fluoride

SAs sulfonamides

SCX strong cation exchange

SD standard deviation

SDM sulfadimethoxine

SMX sulfamethoxazole

SPE solid phase extraction

SRM single reaction monitoring

RP reverse phase

RSD relative standard deviation

USE ultrasonic-assisted extraction

WWTP wastewater treatment plant

1. INTRODUCTION

Sewage sludge is a distasteful but unavoidable semi-solid residue produced during industrial or municipal wastewater treatment. It is considered a host to many pharmaceuticals; hence, it has drawn an increasing attention in recent years. Although, some of these pharmaceuticals especially antibiotics usually exist in sludge at trace concentration levels, but their persistent nature has raised concern in the scientific community. This has led to a growing need for determining and monitoring their concentrations for a healthier environment. Hence, screening of these substances at trace levels have become very essential in order to ameliorate or limit their impact and further adopt innovative strategies for their complete elimination if possible. However, for their concentration to be determined with good level of certainty there must be one or more reliable means of extracting these substances from such a complex matrix.

Several techniques have been employed in extracting antibiotics including ultrasound-assisted extraction (USE), microwave assisted extraction (MAE) and pressurized liquid extraction (PLE) also referred to as accelerated solvent extraction (ASE). The efficiency and other advantages of one technique over another must therefore be brought into perspective before the development of an analytical procedure. Considerations of the matrix effect that comes with any extraction technique of choice must also be made to enable the detection of target compounds even at very low concentration.

Achieving trace screening of pharmaceuticals in sewage sludge requires an analytical technique that can provide reliable information at that level. It therefore begs for a reliable method that could also be employed in subsequent time if required to be done at defined intervals.

2. LITERATURE REVIEW

2.1 Sewage sludge as waste and useful material

Sewage sludge as an industrial waste can be considered a repository of pathogens, heavy metals and a host of other micro-pollutants and substances that could threaten both human and ecological health. Monitoring of sewage sludge has proved the presence of many polar anthropogenic pollutants since LC/MS techniques came into routine use (Sena et al., 2010). As tonnes of sludge produced has rapidly increased over the years, (Schowanek et al., 2004) and (Carbonell et al., 2009) reported that the policy of the European Commission is to encourage beneficial use of sewage sludge for agricultural purposes (soil application and land filling) provided that its content and quality is compatible with public health and environmental requirement because it serves as a sustainable solution to sludge disposal. Disposal alternatives often included incineration and dumping at the sea but the latter was banned in the United states as at December 1991 and in the European community seven (7) years later (Hill et al., 1996; T. Xie & Wang, 2013). Thus, the incentive of using sewage sludge for soil-related purposes is great since it could serve to improve soil fertility by adding several nutrients and organic materials which helps to recovers soil structure. However, several researches have indicated the presence of potentially harmful substances including pharmaceuticals, microplastics, detergents, hormone disruptors, pesticides, flame retardants contained in treated sewage sludge (Carbonell et al., 2009; Li et al., 2018; Lillenberg et al., 2009). These constituents make sewage sludge usage a major environmental challenge and further underscore the need for a proper analytical evaluation to ensure compliance to existing regulations and directives before being put in use.

Studies (Carballa et al., 2008; Chen et al., 2013) have shown that the polar functional groups of pharmaceuticals, including antibiotics could interact with dispersed organic matters leading to their high concentration in the sludge. The increase in concentration is a consequence of their high removal efficiency by biological waste water treatment plants which brings about the sedimentation of these compounds in sludge (Jia et al., 2012). According to a report (Chen et al., 2013), the use of highly concentrated pharmaceutical-containing sludge in agriculture may contaminate the food chain and water supply. Even very small amount of antibiotics in crops and vegetables may generate resistant strains of bacteria in both humans and animals (Lillenberg et al., 2009). The presence of pharmaceuticals including antibiotics in sewage sludge (Lillenberg et al., 2009) therefore becomes an important issue in Estonia which produces a considerable amount on a yearly basis.

2.2 A major problem with sludge: antimicrobials and antimicrobial resistance

Antimicrobials are a group of chemical or physical compounds (agents) that are used to inhibit the growth of microorganisms or to kill them permanently. Different groups of antimicrobials exist; those that act against viruses (antiviral agents), those that are specific towards fungi (antifungal agents) as well as those antimicrobials used against bacteria known as antibiotics.

Antibiotics being introduced into clinical practice in the 1940s has been rated one of the most successful forms of therapies against infectious diseases specifically known for its efficiency against pathogenic bacteria (Aminov, 2009). However, the extensive use of antibiotics in recent decades has led to the emergence and rapid dissemination of antibiotic-resistant pathogens and antibiotic resistance genes (ARGs) in the environment, especially multi-drug-resistant bacteria, further revealing our lack of knowledge about the ecological and evolutionary processes taking place in microbial ecosystems (Aminov, 2009; Xu et al., 2015).

According to World health organisation, 2018, many infections such as tuberculosis, salmonellosis and gonorrhoea are already becoming more difficult to handle as the antibiotics used to treat them become less effective due to antibiotic resistance. Hence, antibiotic resistance has become a threat to global health. The major mechanism of antibiotic resistance has been reported to be as a result of the evolution of resistant strains which provides the working material for natural selection of such bacteria due to the emergence of mutations in their nucleic acids (Woodford & Ellington, 2007). The surge of new resistances and of multi-drug resistances therefore begs for a proper elucidation of the factors and hot spots involved in its diffusion and development. All the known antibiotic resistance mechanisms, acquired by pathogenic and opportunistic bacteria, evolve by means of mutations occurring in pre-existing genes of the bacterial DNA that are naturally selected (Gullberg et al., 2011; Q. Zhang et al., 2011). Mutations within the DNA can be responsible for the decreased affinity of antibiotics to their targets. Also, other resistance mechanisms such as efflux pumps are finely regulated in their expression and at a basal level confer a naturally reduced susceptibility to the drugs. These mutations in the architecture of the bacterial genome regulate such mechanisms resulting in their over-expression that leads to a high level of resistance (Lupo et al., 2012).

A case study is that of the continuous rise in the prevalence of quinolone-resistant isolates which can be traced to extensive use and misuse of these antibacterial agents in clinical and veterinary medicine (Ruiz, 2003). This resistance is mainly due to the presence of mutations in specific sites where quinolones elicits their functions, or the presence of decreased uptake. Horizontal transfer of quinolone resistance (Aminov, 2009) would facilitate the rapid

dissemination of the quinolone resistance genes, even between animal and human pathogens, further compromising the use of these antimicrobial agents (Ruiz, 2003) and also validating the concern of the World health organisation.

2.3 The routes of antibiotics to nature

Many antibiotics have been reported in the environment especially those belonging to the family of fluoroquinolones, tetracyclines and sulfonamides (Jia et al., 2012). Their relatively high concentrations in wastewater, surface water, ground water as well as drinking water begs the question of their routes to these natural media (Dorival-García et al., 2013; Jia et al., 2012; Rossmann et al., 2014). According to (Rossmann et al., 2014), a high dose of administered pharmaceuticals especially antibiotics passes the human body (by excretion) unmodified and end up in wastewater which is treated in the wastewater treatment plants (WWTPs) but their incomplete removal during treatment allows them into the immediate environment.

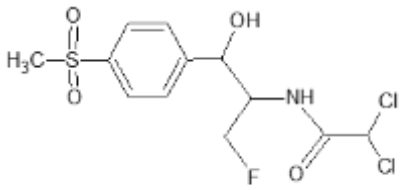
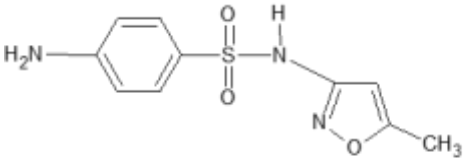
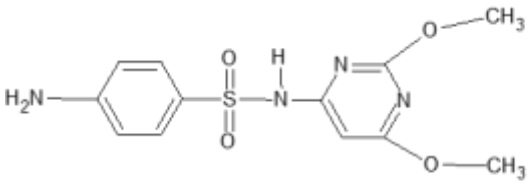
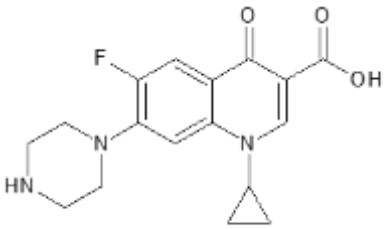
These antibiotics may be taken up by plants in the agricultural ecosystem through wastewater reuse, causing potential exposure to human and animals which in effect poses health risk to both. Most antibiotics in the environment has been reported to be sewage-derived, which are partly eliminated in wastewater treatment processes and are therefore present in the effluents that finally re-enters the immediate environment (Xu et al., 2015). Therefore, it has become more than necessary to elucidate the occurrence and distribution of antibiotics in sludge in order to monitor and control the continued spread and proliferation of antibiotic resistance genes in the ecosystem.

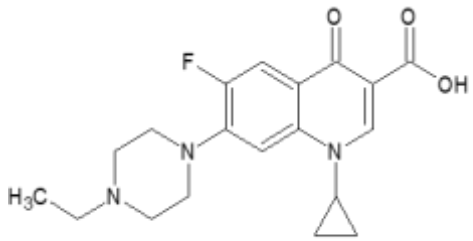
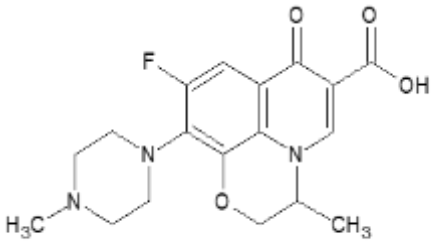
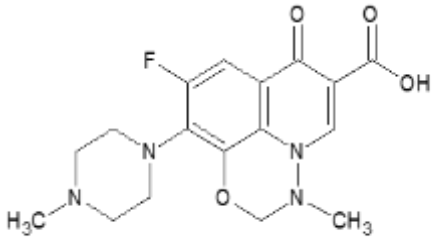
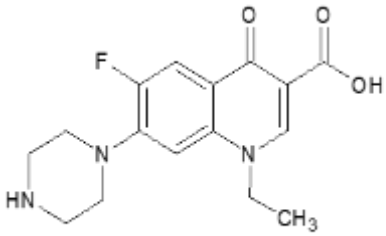
2.4 Selected antibiotics for monitoring

Considering the vast range of pharmaceuticals that are widely present in the natural media, special consideration has been given to specific groups due to high dosage administration both in human and animals as well as their persistence properties. Examples of those groups include the amphenicols (APs), sulfonamides (SAs) and fluoroquinolones (FQs). Specific focus would therefore be directed towards these groups with some few examples (Table 1) to elucidate some of their characteristic properties.

Table 1.

Chemical structures of selected antibiotics and some of their properties. The pKa values and molecular weights are adapted from *drugbank.ca* as well as from the work of (Kipper et al., 2011).

Compound structure	pK_{a1}	pK_{a2}
 Florfenicol (358.21 g/mol)	-3.4	8.49
 Sulfamethoxazole (253.28 g/mol)	1.97	6.61
 Sulfadimethoxine (310.33 g/mol)	2.11	6.17
 Ciprofloxacin (331.34 g/mol)	5.76	8.62

 Enrofloxacin (359.39 g/mol)	5.69	6.68
 Ofloxacin (361.37 g/mol)	5.45	6.2
 Marbofloxacin (362.36 g/mol)	5.38	6.16
 Norfloxacin (319.33 g/mol)	5.66	8.68

2.4.1 Amphenicols

Amphenicol, shortly referred to as phenicol is a wide-spectrum antibiotics drug family that has been applied in the treatment of various bacterial infections and have been considered specially active against both Gram positive and Gram negative bacteria (X. Xie et al., 2018; S. Zhang et al., 2008). It is the antibiotic family to which chloramphenicol, thiamphenicol and

florfenicol belongs. Thiamphenicol and florfenicol are two newer, veterinary, synthetic analogues that were proposed to replace chloramphenicol which was discovered to be associated with several health side effects that led to its ban in both the European Union and the United State of America in 1994 (Alechaga et al., 2012). The administration of these veterinary drugs in modern livestock farming is usually achieved by adding certain dosage to drinking water or as feed additives to serve for both prophylactic and therapeutic essence. However, these newer analogues have been reported to have some health hazards; some have shown inhibition to the formation of red blood cells, white blood cells and platelets, florfenicol have specifically been identified with embryonic toxicity in animal and has therefore received some restrictions in clinical applications even in the European Union, China, as well as the United States (X. Xie et al., 2018; S. Zhang et al., 2008). Notwithstanding, illegal use of these phenicols especially in developing countries still exist which leads to the gradual accumulation of these compounds in sewage sludge. Although no trigger values of these drugs have been reported in sewage sludge (Lillenberg et al., 2009), the European community have established the maximum residue limit (MRL) of florfenicol and its metabolite from 100-2500 ug/kg according to the type of food matrix (Alechaga et al., 2012).

2.4.2 Sulfonamides

Sulfonamides represent a crucial class of antibiotics whose bacteriostatic actions essentially inhibit the biochemical synthesis of folate needed for bacterial metabolism, growth and development. They are a group of antimicrobial agent widely used for veterinary applications and are very active against gram positive and gram-negative bacteria (Dorival-García et al., 2013; Pamreddy et al., 2013). Although, sulfonamides were frequently used as human antibiotics for the treatment of several kinds of infection, recently higher quantities are being used for the treatment and prevention of infectious diseases in different forms of animal husbandry especially in ranching practices (García-Galán et al., 2010). These recent practices, which is often accompanied by inadequate waste management procedures have been associated with grave environmental issues as it constitutes one of the major release sources of this group of antibiotics in the environment (García-Galán et al., 2010). Medicated livestock excrete waste products that eventually serve as manure for agricultural use and this could in turn be a major route by which residual sulfonamides find their way to natural media. Research studies of (Haller et al., 2002; Schmitt et al., 2005) have shown that residues of sulfonamides found in manure goes up to 12.4 mg/kg in concentration. These antibiotics are weak acids, slightly water-soluble and are also polar in nature, as such, they are loosely retained in soil and

have the tendency of draining away into both surface and ground water after being released into the immediate environment (Batt et al., 2006; Blackwell et al., 2004; García-Galán et al., 2010; Sacher et al., 2001).

2.4.3 Fluoroquinolone

Fluoroquinolones are a large group of antibiotics which includes ciprofloxacin, norfloxacin, marbofloxacin, ofloxacin. They are anti-bacterial group which are effective against known pathogenic gram-positive and gram-negative bacteria. Generally, they act by interfering with the unwinding activity of the bacterial topoisomerase enzyme which eventually inhibits the entire deoxyribonucleic acid (DNA) replication process (Ballesteros et al., 2002). Fluoroquinolones are synthetic broad-spectrum antibiotics that have been applied in both veterinary medicine and human medicine, e.g. to treat infections caused by *pseudomonas aeruginosa* and they are characterized by good tissue penetration and distribution in biological fluids with very few side effects (Janusch et al., 2014). Enrofloxacin for example has been used in the treatment of respiratory and gastrointestinal tract infections in different species of cattle, poultry, horses and pigs. The antibiotic is then metabolized in the liver by de-ethylation to ciprofloxacin which is its main metabolite that is solely applied in human medicine (Janusch et al., 2014). Fluoroquinolones are being increasingly used in recent time especially as growth promoters in animal husbandry. According to (He & Blaney, 2015), Over twenty-five (25) of these compounds have been developed for human health purposes and eight (8) for veterinary applications. The increased use of these antibiotics has received both public and environmental concerns and as such, analytical methods capable of detecting and monitoring trace concentrations of fluoroquinolones in environmental samples have become necessary (He & Blaney, 2015). In fact, these concerns have necessitated the visit of the European Centre for Disease Prevention and Control (ECDC) and the European Commission's Directorate General for Health and Food Safety to Estonia from 25 to 29 March 2019 in the wake of having to develop strategies for tackling antimicrobial resistance (AMR) from antibiotic consumption including fluoroquinolones. Correspondingly, the veterinary competent authorities were also concerned about the increasing use of critically important antimicrobials (CIAs) and emerging high levels of AMR in the country.

2.5 Sample Preparation (Pressurized Liquid Extraction)

Whilst most studies have focus on the determination of antibiotics in aqueous matrices (He & Blaney, 2015; Rossmann et al., 2014), a handful have also directed their focus towards

biosolids and sewage sludge ((Ezzariai et al., 2018; García-Galán et al., 2010). However, the issue of which techniques to employ for the extraction of antibiotics from a solid matrix is a key step that must embrace some important factors such as easy operation, yield, extraction time, matrix effects etc. Indeed, extraction of these antibiotics has not been without some levels of difficulties as there are different adsorption mechanisms such as complexation, hydrophobic interactions, hydrogen bonding etc with which they are retained on the solid surface. The overall effect of this retention decreases the efficiency of any chosen extraction method (Ezzariai et al., 2018). It therefore begs for a more aggressive and efficient technique such as the pressurized-liquid extraction (PLE) method which demonstrates higher precision and extraction yield, easy operation, reduced extraction time as well as smaller amount of extraction solvent than other known extraction methods (such as the ultrasonic-assisted extraction) for the determination of antibiotics from a solid phase. Although PLE could be associated with huge matrix effects, a clean-up step such as the solid phase extraction (SPE) would compensate for this downside.

2.6 Analysis by liquid chromatography – mass spectrometry (LC-MS)

Current analytical method employed in the separation and detection of antibiotics and many other substances involves coupling gas or liquid chromatography and mass spectrometry. In some cases, the tandem mass spectrometry is also used to increase resolution. However, gas chromatography (GC) coupled to MS can be difficult to employ, since most antibiotics are polar and to a very large extent non-volatile (Peysson & Vulliet, 2013). This leaves LC-MS with a clear advantage for the determination of these substances and serves the major reason why it has been widely used (Ezzariai et al., 2018; Lillenberg et al., 2009; Pamreddy et al., 2013). More recently, the use of ultra-high-performance liquid chromatography (UHPLC) which offers an improved method efficiency, reduced solvent consumption, higher speed of analysis over high-performance liquid chromatography (HPLC) have gained wider application in the analysis of pharmaceuticals. Given LC-MS as a method of choice especially with electrospray ionization (ESI) as the atmospheric pressure ionization source, careful attention must therefore be paid to sample preparation due to ESI-MS susceptibility to matrix effects.

2.7 Aim of the study

Consequent upon the several issues associated with increasing level of antibiotics in the environment, the necessity for a multiclass method for their determination becomes increasingly more evident especially in Estonia where little input has been recorded in this light. A specific pressurized-liquid extraction method has been developed for simultaneous

determination of fluoroquinolones, sulfonamides and tetracyclines but no work (in the UT testing center) has included any amphenicol as well as the fluoroquinolones, marbofloxacin and enrofloxacin in sewage sludge from Estonian wastewater treatment plant. The selection of these antibiotics (analytes) which includes; florfenicol (FF), sulfamethoxazole (SMX), sulfadimethoxine (SDM), ciprofloxacin (CIP), enrofloxacin (ENR), ofloxacin (OFL), marbofloxacin (MAR) and norfloxacin (NOR) have been made with regards to their persistent nature in residues as well their stability. Consequently, this present work is aimed at developing;

- an extraction (PLE) method for fluoroquinolones, sulfonamides and an amphenicol.
- a specific and sensitive LC-MS method for monitoring the concentration of these antibiotics in sewage sludge from Estonian wastewater treatment plants.

3. EXPERIMENTAL

3.1 Chemicals and Materials

All solvents and chemicals were of analytical grade. Eight antibiotic standards from three classes were used- one amphenicol: florfenicol (FF, Sigma-Aldrich); two sulfonamides: sulfamethoxazole (SMX, Sigma-Aldrich) and sulfadimethoxine (SDM, Sigma-Aldrich); five fluoroquinolones: ciprofloxacin (CIP, Sigma-Aldrich), enrofloxacin (ENR, Dr Ehrenstorfer), ofloxacin (OFL, Sigma-Aldrich), norfloxacin (NOR, Sigma-Aldrich) and marbofloxacin (MAR, Honeywell).

Aqueous solutions were made using Milli-Q water purified by Millipore Milli-Q Advantage A10.

LC eluents includes HFIP buffer (Acros Organics), HPLC grade methanol and formic acid (Sigma-Aldrich).

Other chemicals used includes; acetonitrile (CH_3CN) phosphoric acid (H_3PO_4) citric acid monohydrate ($C_6H_80_7 \cdot H_20$), Ammonium acetate (CH_3COONH_4) and Ammonium hydroxide (NH_4OH); all are Sigma-Aldrich products except Ammonium hydroxide (Merck).

All chemical reagents and their important physical properties are presented in Annex1.

3.2 Preparation of Eluent, Solvent and Calibration solution.

Mobile phase (channel D of the instrument) used for the analytical method was prepared by adding 527 μL of HFIP to Milli-Q water up to 1 L volume. The resulting solution was adjusted

to pH 9 with 600 µL of ammonium hydroxide and was thereafter filtered through a 0.45 µm PVDF membrane filter (Durapore).

Formic acid used for the preparation of calibration solutions was made by the dilution of 1 mL of the concentrated form to 1 L of milli-Q water to achieve a 0.1% solution. This was then filtered through a 0.45 µm PVDF membrane filter (Durapore).

Stock solutions were made by carefully weighing 10 mg each of the antibiotic standards on a 5-digits analytical balance to prepare an approximate solution of 1 mg/g using methanol and formic acid.

Calibration solutions between the range of 0.1 – 1000 ng/g were made by first preparing a sub-stock solution (working standard) of 125 µg/g antibiotic mixture from the stock solution. This is then followed by serial dilutions to obtain calibration solutions of different concentrations.

3.3 Sample Collection and Preparation

3.3.1 Sample collection

Anaerobically digested and dewatered sewage sludge samples used for this analysis were obtained from Estonian wastewater treatment plants (WWTPs). Samples were collected in polypropylene containers and stored in the dark at a temperature of 4 °C.

3.3.2 Pressurized Liquid Extraction (PLE)

In order to achieve an exhaustive extraction from the sewage sludge using elevated pressure and temperature, PLE was chosen following (Ezzariai et al., 2018; Lillenberg et al., 2009). The in-house designed system used for this extraction is represented in Fig. 1. Stainless steel and standard HPLC valves were used to construct the system in order to survive high pressures.

10 g (wet weight) of thoroughly mixed sewage sludge was blended with sand (1:1) in order to increase the contact – surface area between the extraction solvent and the sludge particles. The rigorously mixed blend of sand and sludge was packed into a paper and loaded into the extraction cylinder constructed in the oven chamber. The extraction solvent for the PLE process was a mixture (1:1, v/v) of acetonitrile and citric acid monohydrate (which also functions to maintain analyte stability) brought to pH 2.5 using 0.35% phosphoric acid. Extraction was performed in 5 static cycles each using approximately 20 ml of extraction solvent for 10 min. Pressure and temperature were maintained in the range 100-110 bars and 100-110 °C respectively with an initial 20 min heat up time to allow temperature to reach the desired range for extraction. The system was pressurized using argon gas. The first static cycle was

subsequently followed by the other cycles under the same operating conditions and PLE extract collected for each parallel extraction is to a total volume of approximately 100 ml.

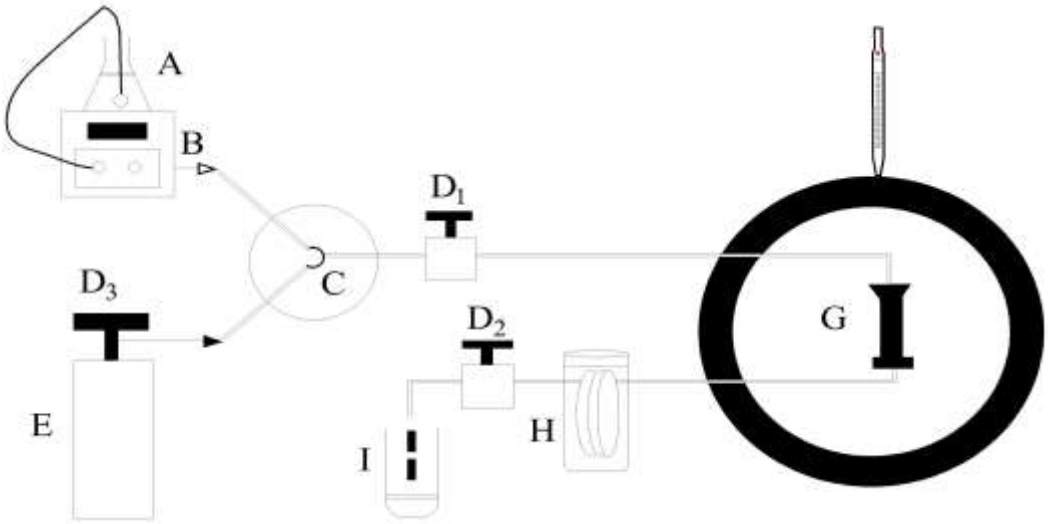


Fig. 1 PLE system: *A, extraction solvent; B, High Performance Liquid Chromatography (HPLC) pump; C, switching valve; D1 and D2, static valves; D3, argon valve; E, argon source; F, extraction chamber (oven); G, extraction cell; H, cooling coil; I, collection vial for PLE extract; J, thermometer. Adapted from (Lillenberg et al., 2009).*

3.3.3 Solid Phase Extraction (SPE)

The clean-up procedure for PLE extract was performed using Oasis HLB extraction cartridges and vacuum manifold (Agilent technologies). The extract obtained was first diluted with milli-Q water until the acetonitrile content is kept at about 10% (100 mL of PLE extract brought to 500 mL). This step was to ensure that antibiotics of interest do not elute from the HLB cartridge when the organic percent in the PLE extract is high. The cartridges were then pre-conditioned with 20ml of methanol and subsequently with 10 mL of milli-Q water. During sample loading and washing procedures, the flow rate was kept at approximately 6 ml per minute and the pressure was maintained in the range 20-30 kPa. After extraction, elution of the analytes from the cartridges was performed with 12 mL of methanol and collected in polypropylene vials. A gentle stream of nitrogen gas was then used to concentrate the SPE extract (samples were not evaporated to complete dryness). The concentrated extract was then reconstituted into a buffer solution (ammonium acetate and 0.1% formic acid regulated to pH 2.8) and methanol (1:1 v/v).

Processed samples are either directly taking for analysis or stored in polypropylene vials at about 6 – 8 °C.

3.4 LC Method Development

Separation of analytes in SPE extract was carried out by LC (Agilent series 1290 MCT) consisting of a flexible pump, an autosampler and a heated column compartment. Autosampler's temperature was kept at 30 °C. Chromatography of antibiotics proceeded with a Waters XBridge reverse phase C18 column (3.0 x 150 mm, 3.5µm) equipped with a guard column. The choice of this stationary phase with a multi-layered organic/inorganic hybrid particle was made due to its high pH tolerance (pH 1-12) as the mobile phase consists of HFIP buffer (solvent D at pH 9) and methanol (solvent B). The use of the regular silica based C18 column at high pH (above pH 8) would leave the silica support dissolved. Also, analysis of analytes with predominant basic properties comes with a careful attention to the pH/pK_a relationship. This particularly applies to this work *(Table 1)* as the basic centers of the listed analytes will be present in their protonated form when the pH is lower than the pK_a. This situation implies a poor retention of the analyte but when the pH is greater than their corresponding pK_a, the analyte's basic center gets deprotonated and gives a better retention, hence, the use of HFIP buffer which provides the basic condition for better separation. The use of HFIP has also been reported to improve ESI signal, peak shape as well as better chromatographic separation of compounds (Kipper et al., 2011).

Prior to making the final choice of eluents as well as optimization of other parameters, acetonitrile (ACN) was first tested with 0.1 % formic acid. However, most of the FQs peaks disappear at high ACN % indicating that these analytes are probably insoluble in the eluent. This was then followed by methanol and 0.1 % formic acid but as a basic pH is desired, HFIP buffer was later chosen instead of formic acid. The first developed gradient for this mixture and the corresponding chromatogram has been shown in table 2.1 and figure 2.1 respectively. In order to decrease run time, improve peak separation and capacitor factor as well as allow for column equilibration the gradient was improved resulting in Figure 2.2. Gradient elution at a flow rate of 0.35 mL/min was performed as presented in table 2.2. Injection volume was 1 µL with a post time of 10 minutes to allow for column equilibration. The final LC method (developed in collaboration with a colleague under the same project but different aims) used for the analysis of eight (8) antibiotics is as shown in Fig.2.2.

Table 2.1 Initial LC gradient for the analysis of 8 antibiotics (B, methanol and D, HFIP buffer)

Time (min)	0	5	15	23	26	28	30	40	42	45
B (%)	6	6	20	20	40	40	100	100	6	6
D (%)	94	94	80	80	60	60	0	0	94	94

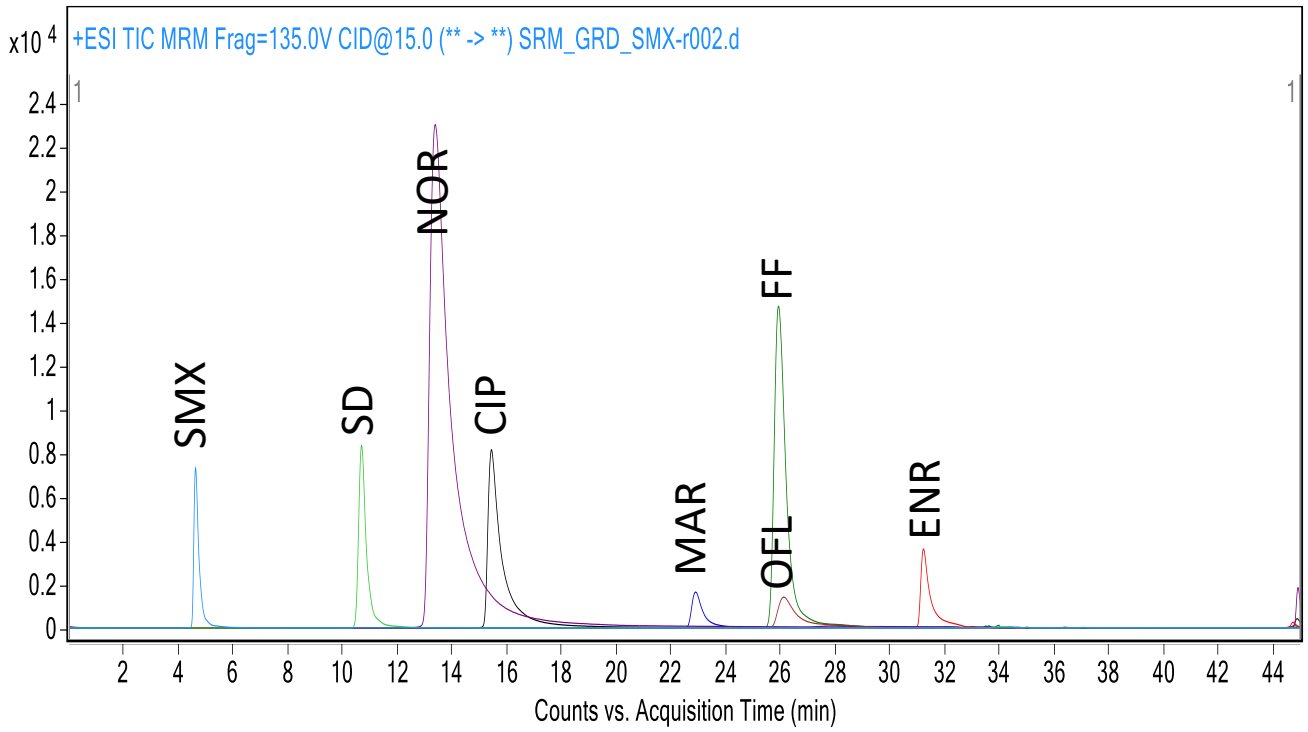


Fig.2.1 Initial LC method showing MRM chromatogram of eight (8) antibiotics standards

Table 2.2 The final LC Gradient for the analysis of 8 antibiotics (B, methanol and D, HFIP buffer)

Time										
(min)	0	5	8	15	18	22	24	30	32	35
B (%)	3	3	20	20	40	40	100	100	3	3
D (%)	97	97	80	80	60	60	0	0	97	97

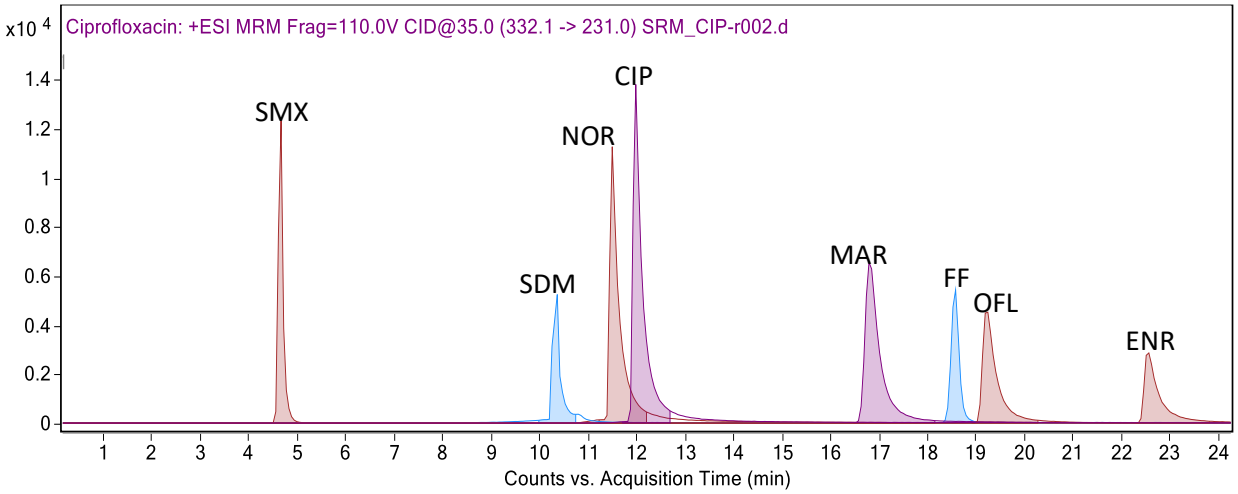


Fig.2.2 The final LC method showing MRM chromatogram of eight (8) antibiotics standards

3.5 ESI-MS/MS

Quantification of analytes was performed using Agilent 6460 Triple Quadrupole mass spectrometer with an electrospray interface (ESI) used in the positive ion mode. Multiple reaction monitoring and the following ionisation source parameters were used: gas temperature; 300 °C, gas flow; L/min, nebulizer gas pressure; 45 psi (310.26 kPa), sheath gas temperature; 350 °C at a flow rate of 11 L/min, capillary voltage was maintained at 3500 V and the nozzle voltage at 500 V. MS/MS scan mode was used to qualify the mass of analytes while the product ion mode served to identify and confirm the analytes. Cell accelerator voltage was 4 V. Protonated, sodium and ammonium adduct were tested. All antibiotics were further analysed in the protonated form except FF which showed a higher intensity in the ammonium adduct form. Optimization of necessary parameters was also compared with the result of Agilent Mass Hunter optimization software to confirm the collision energies as well as the abundances of analytes. Optimised parameters are included in table 3.

Table 3. Precursors, recorded transitions and collision energies of eight (8) antibiotics in order of retentions times (RT).

Compound	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)		Collision Energy (V)	
			Quantifier	Qualifier	Quantifier	Qualifier
SMX	4.82	254	108	156	15	15
SDM	10.36	311	156	108	18	18
NOR	11.49	320	302	282	18	30
CIP	12.20	332	314	231	18	35
MAR	16.79	363	72	345	26	26
FF	18.57	375	340	241	10	22
OFL	19.24	362	318	261	18	18
ENR	22.56	360	316	245	25	25

4. RESULTS AND DISCUSSION

4.1 Linearity, Limit of Detection and Quantification

For linearity and linear range estimation, dilution of the working standard was made in approximate concentration range of 0.1 – 500 ng/g for all antibiotics except SDM with a range of 5.0 – 100.7 ng/g which was not detected at the lower concentration level. The calibration curve showed an excellent linearity in the ranges of concentration studied and the linear regression analysis of representative curves resulted in the intercepts and slopes shown in table 4. Equations (1) and (2) below were used for the determination of LOD and LOQ respectively with the average LOQ of antibiotics found to be 1.2 ng/g except for SDM.

$$\text{LOD} = 3.3 * \frac{S}{b} \qquad (1)$$

$$\text{LOQ} = 10 * \frac{S}{b} \qquad (2)$$

Where *b* is the slope of the calibration graph and *S* is the residual standard deviation of points around the graph.

Table 4. Linearity of external calibration graph, LOD and LOQ

Analyte	Slope	Intercept	Regression Co-efficient	Linear range (ng/g)	LOD (ng/g)	LOQ (ng/g)
SMX	229.58	-86.27	0.9999	0.5 - 189.4	0.3	0.9
SDM	141.34	37.18	0.9996	5.0 - 100.7	3.1	9.3
NOR	652.36	-1919.10	0.9971	0.5 - 214.9	0.2	0.6
CIP	695.95	-2073.50	0.9944	1.0 - 230.1	0.6	1.8
MAR	215.82	-276.45	0.9953	1.0 - 101.8	0.2	0.6
FF	115.01	57.07	1.0000	1.0 - 508.7	0.3	0.9
OFL	348.52	-2275.10	0.9929	1.0 - 208.2	0.4	1.2
ENR	98.42	-229.28	0.9925	1.0 - 100.6	0.8	2.4

4.2 Method Suitability, Matrix Effect, Process efficiency and Recovery

In the present study, the extraction of analytes from the complex solid matrix requires a technique that can yield maximum amount of target analyte without alteration of these compounds in chemical or physical forms. Hence in order to obtain a good ratio between the yield from extraction and analyte preservation, PLE was a method of choice. This was also made considering the physico-chemical properties of target compounds (Table 1) and following the paper, (Lillenberg et al., 2009). The extraction conditions **(3.3.2)** were chosen to enable the breakdown of the strong interaction between sludge organic as well as mineral matter and antibiotics of interest at the same time preserving antibiotics of interest. In order to eliminate possible interferents, PLE was subsequently followed by SPE as a clean-up step.

4.2.1 Recovery

The overall method recoveries were obtained by spiking the PLE extracts using a solution containing approximately 500 ng/g (EU directive; EMEA/CVMP/005) of the eight (8) antibiotics in triplicate (Ezzariai et al., 2018). The recovery results calculated from equation (3) (Taylor, 2005) are in approximate range 80 – 166 % (except SDM) as presented in table 6 and the relative standard deviations (RSDs) were in the range 0.7 – 3.1 % at the same spiked level. The highest recoveries obtained were those of MAR, NOR and CIP (86 – 166 %). Overall, the recoveries obtained is in the required range (80 - 110 %) following AOAC guideline except for SDM and CIP. Similar results were reported for fluoroquinolones using the same extraction solvents (Ezzariai et al., 2018; Lillenberg et al., 2009), however the recovery rates in this work were significantly higher than that of (Lillenberg et al., 2009). Interestingly, the recovery of CIP was seen to be largely higher than 100 % which could be as a result of strong adsorption interaction between the analyte and the sludge organic matter (Ezzariai et al., 2018; Uslu et al., 2008). This is further strengthened by the report of (Polesel et al., 2015) whose findings demonstrated a non-linear relationship of the sorption of CIP onto sludge which implies that there is no absorption/adsorption equilibrium of the analyte on the sludge matrix. This phenomenon would greatly influence the homogeneity of the sample and could therefore lead to an increased recovered CIP amount especially in the presence of strong extracting solvent like acetonitrile. It is also important to mention that high spiking concentrations have been reported (Ezzariai et al., 2018) to greatly influence CIP recovery with lower concentrations having very little or no effect.

$$\text{Recovery} = \frac{Pre\ Extraction\ Addition}{Post\ Extraction\ Addition} * 100 \qquad (3)$$

$$\text{Matrix Effect} = \frac{Post\ \ Extraction\ Addition - Pure\ Solution}{Pure\ Solution} * 100 \quad (4)$$

$$\text{Process Efficiency} = \frac{Pre\ \ Extraction\ Addition}{Pure\ Solution} * 100 \quad (5)$$

Table 6. Overall method recovery, matrix effect and process efficiency for antibiotics spiked at 500 ng/g

Analyte	Recovery (%)	Repeatability (RSD, %)	Matrix Effect (%)	Process Efficiency (%)
SMX	80	1.4	-21	63
SDM	69	1.7	-33	45
NOR	101	0.8	-23	78
CIP	166	1.3	-31	114
MAR	86	1.4	-25	64
FF	81	0.7	-21	64
OFL	79	3.1	-35	51
ENR	82	2.5	-34	54

4.2.2. Matrix Effect (ME) and Process efficiency (PE)

The post extraction addition method (Taylor, 2005) was used for the assessment of matrix effect as shown in equation 4. A calculated value indicating a negative number for matrix effect implies the loss of analytical signal usually due to ionization suppression by co-eluting substances. Therefore, the ideal situation would give a matrix effect value of 0 %. For the range of antibiotics analysed, ME % is negative which means that some signals were supressed in mass spectrometry with the values in the range, 21-35 %. This could mean that there were some co-extracted organic substances during the PLE process with OFL showing the highest effect. Studies (Ezzariai et al., 2018; Pamreddy et al., 2013) have shown that some organics such as humic acids can cause significant suppression of antibiotic signals of up to 50 %. Indeed, the peak area of most analytes especially SDM, OFL and ENR showed significant signal suppression when compared with the pure solution. More so, it has been reported (Ezzariai et al., 2018) that ME % is considered significant when the value is higher than 20 %. Therefore, the standard addition method was further employed to minimize the observed effects. The

external calibration curve prepared in solvent was then compared to the standard addition calibration curve according to equation 6 (Ezzariai et al., 2018; Lu et al., 2019) and the result obtained is shown in table 7. Comparing the post extraction addition technique to the standard addition method, a significant decrease of matrix effect was observed (Fig. 3) thereby bringing the effect to acceptable range except for CIP and NOR. Most probably, the use of internal standard would greatly compensate for the CIP and NOR values. Additionally, whilst considering the possible sources of this matrix effect, the PLE extracts of the last two cycles (3.3.2) were then analysed. Interestingly, there was no analyte detected. This implies that the last two cycles of the extraction process only contributed to matrix content, however, this should be tested and confirmed.

The process efficiency which takes into account both recovery of analytes from the matrix as well as the influence of the matrix on the analyte was calculated using equation 5 (Taylor, 2005). The results obtained for all analytes were in the range 51.8 – 78.1 % except SMD and CIP with PE value of 114 % (Table 6). This high value could be attributed to the over-abundance of co-extracted organic compounds during sample processing; however, this hypothesis must be tested. The low PE values observed for SDM, OFL and ENR could be further explained by their limits of quantification (Table 4) which confirms that low PE values can have a deleterious impact on a method LOQ (Taylor, 2005).

$$\text{ME (\%)} = \left(\left(\frac{Slope\ of\ matched\ calibration\ curve}{Slope\ of\ external\ calibration\ curve}\right) - 1\right) * 100 \qquad (6)$$

Table 7. Calculated ME % from the method of standard addition

	External Calibration	Matched Calibration	
Analyte	**Slope**	**Slope**	**ME (%)**
SMX	229.58	189.97	-17
SDM	141.34	112.69	-20
NOR	652.36	476.76	-26
CIP	695.95	495.01	-28
MAR	215.82	178.24	-17
FF	115.01	97.95	-14
OFL	348.52	293.41	-15
ENR	98.42	82.08	-16

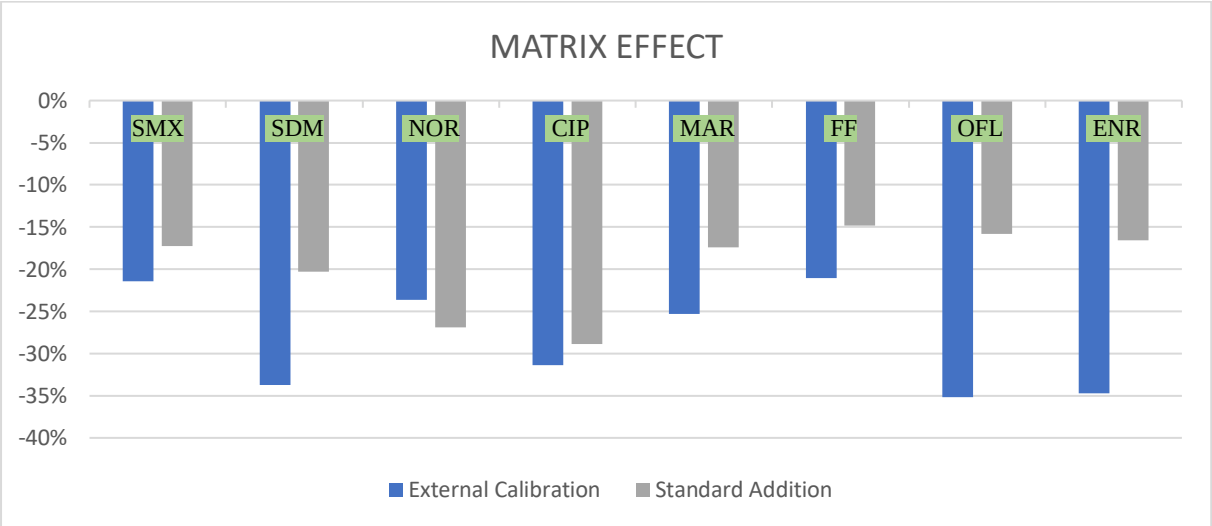


Fig. 3 Percentage of matrix effect obtained from post extraction addition technique and standard addition method.

4.3 Precision and Accuracy

Precision or repeatability shows the closeness of agreement between independent test results under a given condition and it expressed as the relative standard deviation of the test result according to AOAC guideline. The concentrations of analytes expressed as per dry weight (dw) were back calculated considering the dilution factors before converting from wet weight (ww) to dry weight (equation 7). The relative standard deviations (RSDs) of analytes (Table 8.1) falls between 1.7 – 9.6 % (except FF) which is within acceptable range of target RSDs as the AOAC requirement sets a maximum value of 11 %.

$$\text{Concentration}_{\text{dry}} = \frac{Wet\ mass}{Dry\ mass} * \text{Concentration}_{\text{wet}} \quad (7)$$

Also, the calculated RSDs (Table 8.2) of the analytes' retention times (RTs) shows an excellent repeatability as all obtained values; below 1.0 %.

Quantitatively, the accuracy of the method was determined using the Nordtest approach of uncertainty estimation (see equation below). Three replicate samples spiked at the same concentration level were analysed and used as there was no certified reference material (CRM). The bias estimation was then achieved by the recovery obtained. Within laboratory reproducibility of non-spiked samples was taking into account and that of spiked sample was converted to standard uncertainty (u) by assuming the rectangular distribution and the uncertainty result obtained is as shown in table 8.0.

Table 8.0 Accuracy expressed as measurement uncertainty

Analyte	Concentration ± U (ng/g) k = 2
SMX	55 ± 21
SDM	144 ± 53
NOR	264 ± 91
CIP	256 ± 81
FF	33 ± 12
OFL	336 ± 103
ENR	139 ± 50

Nordtest main equation for accuracy quantification

$$U_c = \sqrt{u\,(Rw)^2 + u\,(bias)^2}$$

Within-lab reproducibility

Uncertainty estimate of possible method bias

Table 8.1 Repeatability of test result expressed as relative standard deviation

Analytes	**SMX**	**SDM**	**NOR**	**CIP**	**MAR**	**FF**	**OFL**	**ENR**
Concentration (ng/g)	55.4	144.1	264.5	256.2	71.7	33.6	336.1	139.5
SD	5.3	5.5	5.8	7.2	4.6	4.5	5.7	5.7
RSD, %	9.6	3.8	2.2	2.8	6.4	13.3	1.7	4.1

Table 8.2 Repeatability of retention time of analytes.

Analyte	**SMX**	**SDM**	**NOR**	**CIP**	**MAR**	**FF**	**OFL**	**ENR**
Average RT (mins)	4.80	10.31	11.39	11.88	16.50	18.46	18.99	22.37
SD	0.03	0.04	0.03	0.08	0.13	0.13	0.11	0.07
RSD, %	0.6	0.4	0.3	0.6	0.8	0.7	0.6	0.3

4.4 Selectivity

Selectivity was evaluated by analysing different blank samples for each set of calibration solutions. The objective is to ensure that analytes' peaks are not due to interfering substances. The chromatogram of an extracted representative blank (Fig 4.) indicates the presence of a potential interferent of negligible intensity with a retention time (RT) of 11.4 min. However,

this substance would have zero influence on analytes of interest as none of their RTs overlaps. Also, considering the specific m/z of each analyte with a method in Multiple Reaction Monitoring (MRM) mode, the tendency of maintaining a highly selective method is assured.

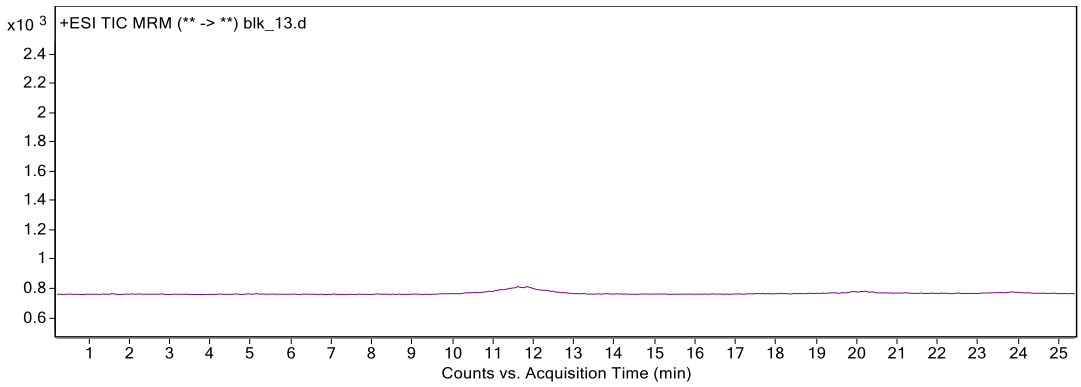


Fig. 4. Representative blank chromatogram.

4.5 Carry over

Carry over was assessed by analysing the concentration of blank samples which were injected after calibration solutions especially those of high concentration. The values of concentrations obtained were significantly above LOQ (Table 9.2) except for SMX, MAR and FF which were not detected. SDM, CIP and NOR were more retained in the column than ENR and OFL.

In order to get rid or at least minimize this effect, the column was first washed thoroughly with 50% HFIP buffer and 50% methanol for 1 hour but there was very little improvement. The isocratic program was then changed to 100 % methanol for 2 hours, but it then appears that only a basic condition might not elute the retained analytes effectively. Therefore, an isocratic elution of methanol and 0.1 % formic acid was employed before it was finally changed to gradient program from ratio 90:10 (0.1 % formic acid and methanol). The program (Table 9.1) was set for 1 hour (sometimes repeated 3 times) by slowly decreasing the water phase to 10:90. An additional step of 50% methanol and 50% HFIP buffer was added to condition the column for subsequent analysis.

Table 9.1. Gradient for washing program.

Time (min)	0	10	20	30	40	45	50	55	60
0.1% F.A	90	80	70	60	50	40	30	20	10
Methanol (%)	10	20	30	40	50	60	70	80	90

Table 9.2 Concentration of antibiotics in blank reagent sample before and after washing.

BEFORE WASHING

Analyte	SMX	SDM	NOR	CIP	MAR	FF	OFL	ENR
Concentration (ng/g)	nd	9.9	11.3	6.9	nd	nd	6.8	3.7

AFTER WASHING

Analyte	SMX	SDM	NOR	CIP	MAR	FF	OFL	ENR
Concentration (ng/g)	nd	nd	nd	nd	nd	nd	Nd	nd

*nd = not detected

4.6 Method Application to Sewage Sludge

Different batches of sewage sludge samples collected from the wastewater treatment plant (WWTP) were analysed. The samples were anaerobically digested and dewatered before being processed. The samples which were processed by PLE and subsequently cleaned up by SPE were then analysed by the developed method (Fig 2.2). Concentrations of detected analytes (Fig 5) found are summarised in Table 10 and their chromatograms are shown in Figure 5. The results are expressed both as wet and dry weight.

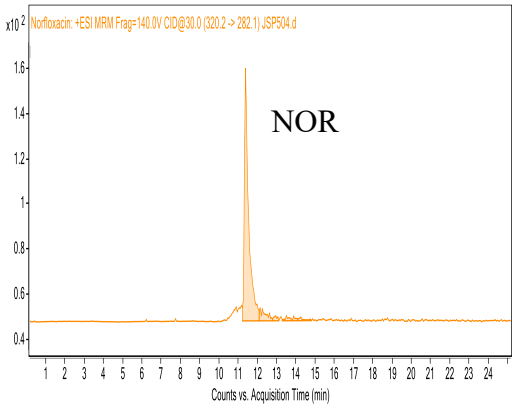
For the dry weight (dw) estimation, a measured amount of the wet sample is oven-dried at 105 °C for 24 hours. The resulting matter is then weighed for the estimation of the dry content of

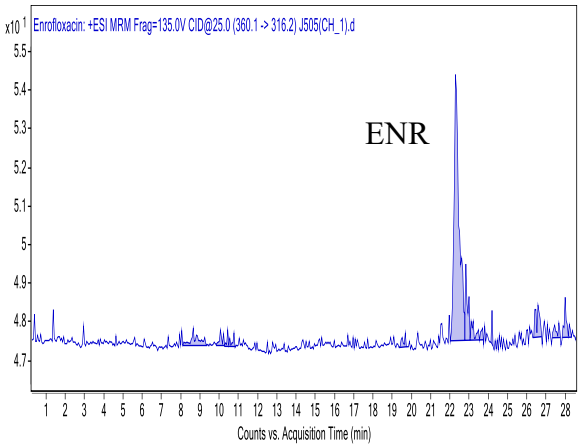
the sludge sample. In order to convert the wet mass to dry mass, equation 7 was used. An extended table for the overall data is given in annex 2.

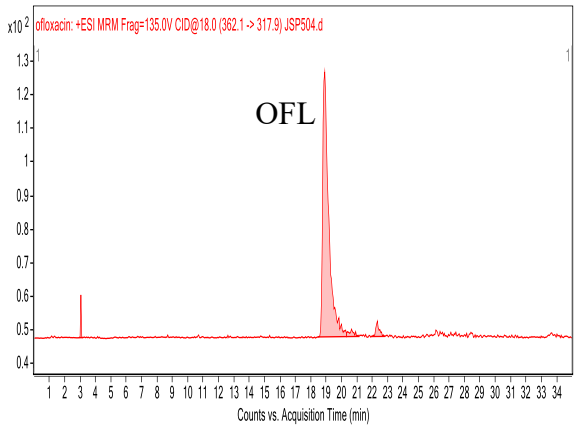
The average concentrations found was in the range 55.4 – 336.1 ng/g (dw) for all analytes with OFL being the highest (Table 10). It should be noted that all analytes were present in every batch of sample except MAR. The concentrations of these analytes (ww) are below the trigger level of drug residues in manure which is 100 μg/kg according to EU directive EMEA/CVMP/055. These results when compared with the average concentration obtained from the studies by (Lillenberg et al., 2009) shows the ww content of NOR and CIP almost two bigger than the present result; NOR,109.8 μg/kg, CIP, 110,8 μg/kg. However, the content of OFL, SDM and SMX were by far lesser than this present result. Also, the contents of all analytes are considerably higher than those obtained from Estonian river water (a research by another colleague under the same project). When compared with the content found in dried sewage sludge from WWTPs in Spain (Pamreddy et al., 2013) the concentration of SMX (84.4 ng/g) is significantly higher than that of this work. Another research conducted in Granada (Dorival-García et al., 2013) for selected antibiotics including fluoroquinolones (FQs) indicates the following values; CIP, 20 – 95 ng/g, NOR, 25 – 115 ng/g, OFL, 15 -129 ng/g all expressed in wet weight. The content of these FQs in the present work falls within the ranges.

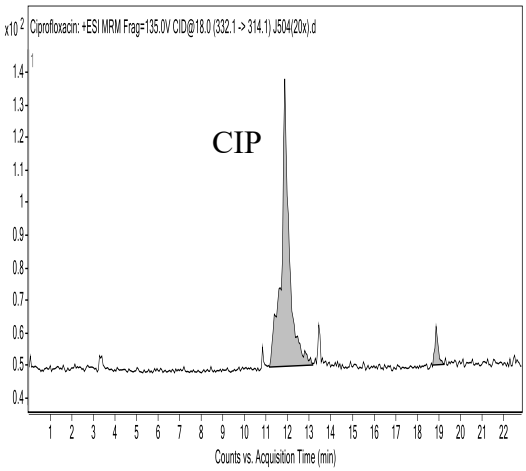
Table 10. Concentration of analytes found in Estonian sewage sludge

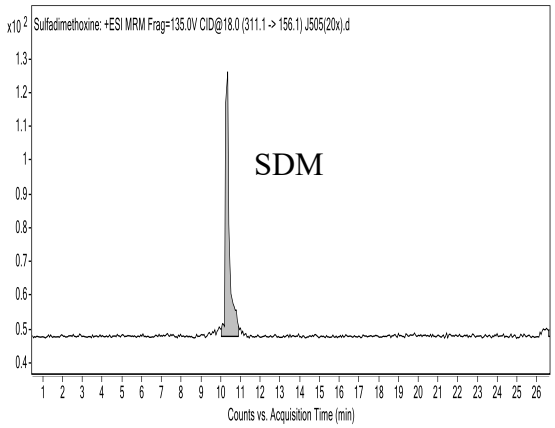
Analytes	**SMX**	**SDM**	**NOR**	**CIP**	**MAR**	**FF**	**OFL**	**ENR**
Wet weight (ng/g)	13.2	34.3	62.9	60.9	nd	8	79.9	33.2
Dry weight (ng/g)	55.4	144.1	264.5	256.2	nd	33.6	336.1	139.5

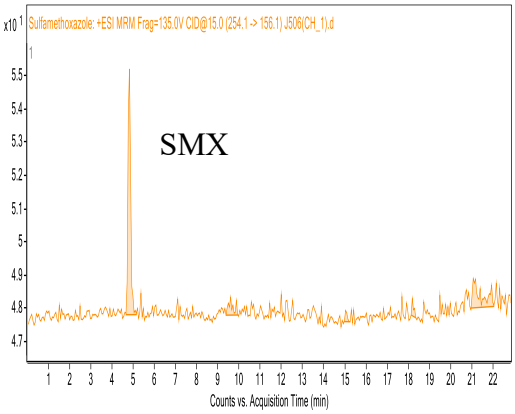

Norfloxacin: +ESI MRM Frag=140.0V CID@30.0 (320.2 -> 282.1) JSP504.d
NOR
Counts vs. Acquisition Time (min)


Enrofloxacin: +ESI MRM Frag=135.0V CID@25.0 (360.1 -> 316.2) J505(CH_1).d
ENR
Counts vs. Acquisition Time (min)


ofloxacin: +ESI MRM Frag=135.0V CID@18.0 (362.1 -> 317.9) JSP504.d
OFL
Counts vs. Acquisition Time (min)


Ciprofloxacin: +ESI MRM Frag=135.0V CID@18.0 (332.1 -> 314.1) J504(20x).d
CIP
Counts vs. Acquisition Time (min)


Sulfadimethoxine: +ESI MRM Frag=135.0V CID@18.0 (311.1 -> 156.1) J505(20x).d
SDM
Counts vs. Acquisition Time (min)


Sulfamethoxazole: +ESI MRM Frag=135.0V CID@15.0 (254.1 -> 156.1) J506(CH_1).d
SMX
Counts vs. Acquisition Time (min)

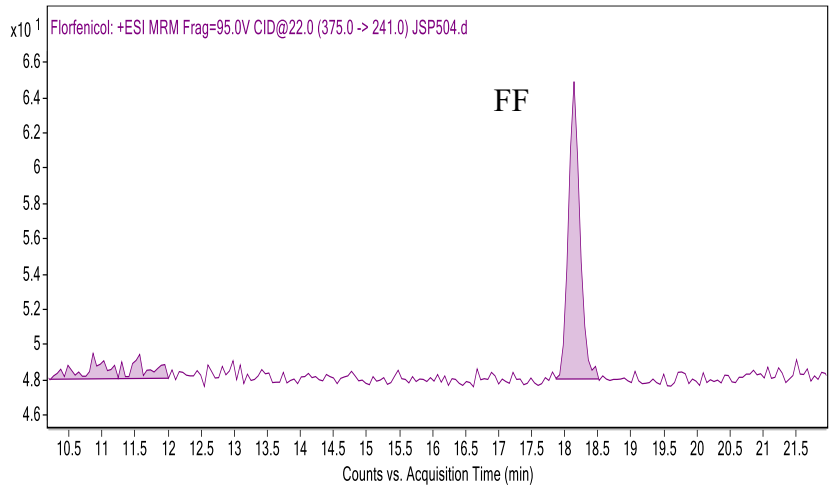


Fig. 5 Chromatograms of detected analytes

5. SUMMARY

In the present study, the aim was to develop firstly; an extraction (PLE) method for eight (8) antibiotics belonging to three different families (fluoroquinolones, sulfonamides and amphenicol) and secondly; a specific and sensitive LC-MS method for monitoring the concentration of these antibiotics in sewage sludge from Estonian wastewater treatment plants. The pressurized liquid extraction method (5 cycles per parallel extraction) used showed a very good analyte recovery and can be said to be very efficient especially for the three antibiotics that haven't been investigated in UT Testing centre laboratory, namely Marbofloxacin (MAR), Enrofloxacin (ENR) and Florfenicol (FF). The choice of solvent used in the extraction is remarkably good as it includes a pharmaceutical excipient (citric acid monohydrate) which confers stability to target analytes under exhaustive extraction conditions (high temperature and pressure). The recovery rates of analyte, though, considered good, also comes with high matrix effect which was greatly minimised by the subsequent clean-up step (SPE). This was further improved by the standard addition method. Since, the aim of any analyst would be to approach the ideal situation of completely (if possible) eliminating matrix effect in this regard, the use of internal standard would help a great deal.

In the same vein, the analytes in the extracts were chromatographically separated using a C18 column and a mobile phase consisting of aqueous buffer with 1,1,1,3,3,3-hexafluoro-2-propanol (pH 9) and methanol. Analysis of samples using a triple quadrupole mass spectrometer in MRM mode showed considerable amounts of antibiotics which indicates that Estonian sewage sludge has some content of antibiotics, although, at a level below the trigger value for manure. It should also be noted that analysis of extract from the last two cycles of the PLE showed zero concentration of target analytes, however, this should be further investigated for confirmation. If confirmed, it will to a large extent, decrease sample processing time. In order to further decrease sample processing time, attempts can also be made to eliminate the clean-up step (SPE) but this must also include some other considerations to eliminate possible interferents. Overall, the developed method showed excellent linearity ($\geq$ 0.998), good selectivity, sensitivity, precision and LOQ. Hence, this method can be applied in monitoring the concentration of similar antibiotics and can also be tested for other antibiotics of different families.

Mõnede antimikroobsete ainete määramine reoveesettemudas survestatud vedelikekstraktsiooni ja LC-MS/MS meetodil

Joshua Onyeka Osagu

Kokkuvõte

Käesoleva töö eesmärgiks oli survestatud vedelikekstraktsioonil (PLE – *pressurized liquid extraction*) ja vedelikkromatograafia-massispektromeetria (LC-MS) meetodil baseeruva selektiivse ja tundliku analüüsimetoodika väljatöötammine kaheksa eri rühma (florokinoloonid, sulfoonamiidid, amfenikool) antibiootikumi määramiseks Eesti reoveepuhastite reoveesettemudast. Kasutatud PLE (5 tsükliga) metoodika saagis oli kõigi analüütide kohta väga hea ja seda võib pidada igati efektiivseks ka nende kolme analüüdi jaoks, mida varem TÜ Katsekojas analüüsitud ei ole: marbofloksatsiin, enrofloksatsiin ja florfenikool. Ekstraheerimise solvendi üheks komponendiks oli sidrunhape, mida kasutatakse ka ravimite lisaainena. Ka sellel võis olla oma roll analüütide stabiilsuse tagamisel, kui ekstrakstsiooni viid läbi kõrgel temperatuuril ja rõhul. Kuigi analüütide saagised olid kõrged, tuli maatriksiefektide vähendamiseks siiski rakendada ekstraktide täiendavat puhastamist tahke faasi ekstraktsiooni (SPE) teel. Olukord paranes veelgi lisamismeetodi kasutamisel. Kui eesmärgiks võtta maatriksiefektide täielik arvesse võtmine, siis tuleks kasutusele võtta isotoopmärgistatud sisestandard.

Ekstraktide kromatograafiliseks lahutamiseks kasutati C18 kolonni ja mobiilfaasi, mis koosnes metanoolist ja 1,1,1,3,3,3-heksafluoro-2-propanooli sisaldavast puhverlahusest (pH 9). Proovide analüüs viidi läbi kolmekordse kvadrupooliga massispektromeetril mitme ülemineku jälgimise (MRM) režiimis. Eesti reoveesettemuda sisaldas mitmeid antibiootikume, kuid alla sõnnikule kehtestatud piirväärtuse.

PLE protsessi uurimiseks analüüsiti eraldi ka kahe viimase ekstraheerimise tsükli ekstrakti. Nendes antibiootikume ei tuvastatud. See tulemus avab võimaluse, kui täiendavad uuringud seda kinnitavad, ektraheerimise etapi oluliseks lühendamiseks. Proovide ettevalmistuse täiendavaks kiirendamiseks võib kaaluda ka SPE etapist loobumist, kuid selle käigus ei tohi unustada segavate komponentide eraldamise vajadust.

Kasutatud metoodikat iseloomustab hea lineaarsus (≥ 0.998), selektiivsus, tundlikkus, täpsus ja määramispiir. Metoodika on sobiv uuritud ainete analüüsiks ja seda võiks laiendada täiendavate analüütide määramiseks.

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Annex 1.

Reagent/Eluent	Producer	Purity, %	Mtw [g/mol]	Density [g/cm3]	Bioling point [°C]	Melting point [°C]	CAS No
Florfenicol	Sigma-Aldrich	99.0	358.2	1.5	618.0	153.0	73231-34-2
Sulfamethoxazole	Sigma-Aldrich	99.0	253.3	1.4	482.0	166-169	732-46-6
Sulfadimethoxine	Sigma-Aldrich	99.0	310.3	1.4	265.5	202-204	122-11-2
Ciprofloxacin	Sigma-Aldrich	99.0	331.3	1.5	581.0	255-257	85721-33-1
Enrofloxacin	Dr Ehrenstorfer	99.8	359.4	1.4	560.5	225.0	93106-60-6
Ofloxacin	Sigma-Aldrich	99.8	361.4	1.3	571.5	270 - 275	82419-36-1
Norfloxacin	Sigma-Aldrich	98.0	319.3	1.3	555.8	220.0	70458-96-7
Marbofloxacin	Honeywell	99.5	362.4	1.6	571.0	268-269	115550-35-1
Methanol	Honeywell	99.9	32.0	0.8	64-65	-98.0	67-56-1
Formic Acid	Sigma-Aldrich	99.9	46.0	1.22	100.8	8.4	
HFIP	Acros Organics	99.5	168.0	1.6	59.0	-4.0	920-66-1
Acetonitrile	Honeywell	99.9	41.1	0.8	81.6	-45.7	75-05-8
Citric acid Monohydrate	Sigma-Aldrich	99.0	210.1	0.8	56.0	-94.0	5949-29-1
Ammonium Acetate	Sigma-Aldrich	100.0	77.1	1.1	138.5	110-112	631-61-8

ANNEX 2

SAMPLE EXTRACTION DATA

Sample	Sand (g)	Sludge (g)	Mixture (g)	g sludge/g mixture	Mass of vial (g)	Mass_buffer (g)	Mass_v_buf_ext (g)	Mass_extract (g)	g DM/kg sludge
1	10.207	10.125	10.140	5.050	5.1043	0.9504	6.4343	0.3796	223.93
2	10.085	10.082	10.014	5.006	5.3724	0.9502	6.6753	0.3527	213.93
3	10.063	10.019	10.162	5.070	5.4740	0.9382	6.8007	0.3885	243.03
4	10.039	10.036	10.116	5.057	5.3641	0.9507	6.7589	0.4441	214.16
5	10.087	10.104	10.161	5.085	5.3012	0.9377	6.6050	0.3661	241.23
6	10.034	10.097	10.091	5.061	5.4010	0.9302	6.8044	0.4732	223.93
7	10.028	10.043	10.012	5.010	5.2910	0.9199	6.7577	0.5468	189.78
8	10.035	10.065	10.099	5.057	5.3621	0.9077	6.6890	0.4192	226.93
9	10.045	10.079	10.050	5.033	5.3380	0.9355	6.5820	0.3085	223.93
10	10.053	10.059	10.085	5.044	5.3650	0.9234	6.6340	0.3456	187.08
11	10.081	10.004	10.068	5.015	5.4566	0.9386	6.8940	0.4988	243.76
12	10.012	10.082	10.052	5.044	5.3245	0.9275	6.6074	0.3554	223.93
13	10.043	10.029	10.072	5.032	5.0012	0.9372	6.4977	0.5593	230.88
14	10.032	10.002	10.111	5.048	5.5002	0.9331	6.7794	0.3461	221.93
15	10.016	10.055	10.098	5.059	5.3455	0.9301	6.7321	0.4565	184.44

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INFORMATION SHEET

Simultaneous determination of selected antimicrobial agents in sewage sludge by pressurized liquid extraction and LC -MS/MS

In the present study, an efficient analytical method for the simultaneous determination of an amphenicol, sulfonamides (SAs) and fluoroquinolones (FQs) in a sewage sludge matrix was developed by high performance liquid chromatography in tandem with mass spectrometry (LC-MS). The selected antibiotics were extracted from the sewage sludge sample by pressurized liquid extraction (PLE) followed by solid phase extraction (SPE) as a clean-up step. Compounds separation was achieved using a mobile phase gradient composition of hexafluoro-isopropanol buffer and methanol. Recoveries of all eight compounds were in the range from 69% to 166%. Limit of quantification ranged from 0.6 ng/g for FQs to 9.3 ng/g for SAs. As most of the compounds showed significant matrix effects, the method was validated using the standard addition method which reduced this effect very significantly.

Key words: antibiotic residue analysis, LC-MS, method development, pressurized liquid extraction

CERCS: P300 analytical chemistry

INFOLEHT

Mõnede antimikroobsete ainete määramine reoveesettemudas survestatud vedelikekstraktsiooni ja LC-MS/MS meetodil

Käesolevas töös arendati kõrgefektiivse vedelikkromatograafia – massispektromeetrial (LC-MS) baseeruv metoodika fluorokinoloonide, sulfoonamiidide ja amfenikoolide rühma kuuluvate antibiootikumide määramiseks reoveesettemudast. Valitud antibiootikumid ekstraheeriti proovi maatriksist kõrgsurve vedelikekstraktsiooni (PLE) teel ja puhastati tahke faasi ekstraktsioonil (SPE). Kromatograafiline lahutus saavutati gradientelueerimisel heksafluoroisopropanooli baasil puhverlahuse ja metanooliga. Kõigi kaheksa analüüsitud aine saagised jäid vahemikku 69%-166%, määramispiirid jäid vahemkku 0,6 kuni 9,3 ng/g. Kuna kõigi analüütide korral täheldati olulisi maatriksiefekte, siis valideeriti metoodika kasutades lisamismeetodit, mis võimaldas maatriksiefekte oluliselt alandada.

Märksõnad: antibiootikumijääkide analüüs, LC-MS, metoodika arendus, survestatud vedelikekstraktsioon

CERCS: P300 analüütiline keemia