Quantitative analysis of selected antibiotic drug residues in honey and manure samples using modern analytical techniques

by

Nakisani Babra Moyo

A dissertation submitted to the Department of Chemistry, University of the Venda in fulfilment of the requirements for the degree of
Master of Science in **Chemistry**

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Declaration

I, **Nakisani Babra Moyo [student number: 19023357]**, declare that the entirety of the work contained therein is my own, original work and that l have not previously submitted it in its entirety or in part for any other qualification. The dissertation is being submitted for the degree of Master of Science in Chemistry at the University of Venda, Thohoyandou, South Africa.

Nakisani Babra Moyo

List of publications

This dissertation is based on the following papers, hereafter referred to by their Roman numerals (I-V):

1. Babra Moyo and Nikita T Tavengwa (2019) **Modern extraction and cleanup methods of veterinary drug residues in food samples of animal origin.** *In: Ince M, Ince OK, editors. Recent advances in Analytical Chemistry. Vol. 1. London, UK: IntechOpen; p. 23-44.*

2. Babra Moyo, Mugera Gitari and Nikita T Tavengwa (2020) **Application of sorptive micro-extraction techniques for the pre-concentration of antibiotic drug residues from food samples.** *Food Contaminants and Additives. 37:1865-1880.*

3. **Recent applications of solid phase extraction in the pre-concentration of antibiotic residues from livestock and poultry manure**
   Babra Moyo and Nikita T Tavengwa (submitted to the Journal of Environmental Science and Health, Part B)

4. **Enrichment of tetracycline residues from honey samples using carrier mediated hollow fibre liquid phase micro-extraction and quantification by the LC-Q-TOF/MS**
   Babra Moyo and Nikita T Tavengwa (submitted to Journal of Food Analytical Methods)

5. **Pre-concentration of sulfonamides from manure samples using molecularly imprinted polymer miniaturized pipette-tip extraction**
   Babra Moyo and Nikita T Tavengwa (to be submitted)

Supplementary paper

6. Babra Moyo, Vhahangwele Matodzi, Malebogo A Legodi, Vusumzi E Pakade, Nikita T Tavengwa (2020) **Heavy metal accumulation in fruits, vegetables and soils in the Thohoyandou town area, South Africa.** *Journal of Water SA. 46:285-290.*

Contribution of the authors

Paper I. Principal author, involved in planning and writing of the article. Co-author revised the draft manuscript and made suggestions for improvement.

Paper II. Principal author, involved in planning and writing of the article. Co-authors revised the draft manuscript and made suggestions for improvement.

Paper III. Principal author, involved in planning and writing of the review manuscript. Co-author revised the draft manuscript and made suggestions for improvement.

Paper IV. Principal author, involved in planning, performed the HFLPME experiments, evaluation of the results and writing of the manuscript. Co-author revised the draft manuscript and made suggestions for improvement.

Paper V. Principal author, involved in planning, performed the m-MIP-PTE experiments, evaluation of the results and writing of the manuscript. Co-authors revised the draft manuscript and made suggestions for improvement.

Paper VI. Principal author, involved in planning, performed the heavy metal analysis experiments, evaluation of the results and writing of the article. Co-authors revised the draft manuscript and made suggestions for improvement.

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Abstract

In this study, modern clean-up and pre-concentration techniques, namely hollow fibre liquid phase micro-extraction (HFLPME) and miniaturized pipette-tip extraction based on molecularly imprinted polymers (m-MIP-PTE), were used for the extraction of antibiotic residues from honey and manure samples, respectively. The work is divided into five papers.

In **paper I**, modern extraction and clean-up methods of antibiotic residues from various food samples were reviewed. One of the challenges in veterinary drug residue analysis is their occurrence in trace amounts that are normally below limits of detection of most analytical instruments. Consequently, various efficient, economical, miniaturized and environmentally friendly extraction methods have been developed in recent years to pre-concentrate these drugs in an attempt to overcome the limitations of conventional extraction methods such as liquid-liquid extraction and solid phase extraction. In this work, the application of techniques including quick, easy, cheap, effective, rugged and safe (QuEChERS), molecularly imprinted polymers, dispersive liquid-liquid microextraction and hollow fiber liquid-phase micro-extraction in the pre-concentration of various antibiotic residues in animal derived food samples was discussed.

The recent applications of sorptive micro-extraction techniques in the pre-concentration of antibiotic residues from food samples were reviewed in **Paper II**. The current trends of analytical sample preparation methods are favouring extraction and pre-concentration techniques that are compliant to the principles of green analytical chemistry. In this paper, the origin and principles of solvent-free sample preparation techniques, known as green techniques, including solid phase micro-extraction, stir bar sorptive extraction, stir cake sorptive extraction and fabric phase sorptive extraction were briefly discussed. Their application in the extraction of antibiotic residues from food samples in the years 2015 to 2020 was critically reviewed. Furthermore, possible sorption mechanisms facilitating the adsorption of these drugs on sorbents were dicussed.

In **paper III,** recent applications of solid phase extraction in the pre-concentration of antibiotic residues from livestock and poultry manure were collated and critically discussed. Application of livestock manure contaminated with antibiotics and their metabolites to farming land may pose a threat to agro-ecosystems, plant growth, aquatic life and soil life, with antibiotic resistance increasingly becoming ubiquitous. Therefore, monitoring of manure for antibiotic residues is of vital importance in order to assess the risks of environmental pollution by these drugs. Several sample pre-treatment techniques have been developed for the extraction of antibiotic residues from complex matrices including manure over the years. However, SPE is the still the commonly used clean-up

technique for this matrix. Therefore, a critical overview of studies that have been conducted in the past 6 years on the extraction of antibiotic residues from manure employing SPE were given in this review.

The presence of tetracycline residues in honey samples was investigated in **paper IV.** Carrier mediated HFLPME was employed for the extraction and pre-concentration of these drugs prior to analysis on the LC-Q-TOF/MS. An acceptor phase solution employed in this study was 0.1 M H_3PO_4 containing 1.0 M NaCl (pH = 1.0), whereas 0.05 M Na_2HPO_4 (pH = 9.5) was used as the donor phase. Parameters that could affect the extraction efficiency of this method, such as the pH of the acceptor and donor solution, salt addition, agitation speed and extraction time were optimized before the application of the developed method to honey samples. Calibration curves showed good linearity, in the range of 20-500 µg L^{-1} with correlation coefficients ranging between 0.9918 and 0.9998, under the optimized conditions. The recoveries of blank honey samples at three spiking levels (75, 200 and 300 µg L^{-1}) ranged from 56.1% to 120.8% with RSDs between 0.37% and 11.21%. LODs and LOQs were in the ranges of 0.042 to 0.661 µg kg^{-1} and 0.127 to 2.002 µg kg^{-1}, respectively. Doxycycline residues were detected in a commercial honey sample at a concentration of 0.20 µg kg^{-1}.

The pre-concentration of sulfonamides from manure samples using miniaturized pipette-tip extraction based on molecularly imprinted polymers (MIPs) was studied in **paper V**. MIPs were synthesized by precipitation polymerization using sulfamethoxazole as a template. Characterization of the MIPs was then done using FTIR. Parameters that could influence the extraction efficiency of this technique including loading cycles, desorption cycles, type of elution solvent, sample pH, salt addition and sorbent amount were optimized. The optimized method was applied to manure samples prior to analysis on the LC-Q-TOF/MS where sulfachlorpyridazine, sulfadiazine, sulfamethoxypyridazine, sulfamethoxazole and sulfaquionoxaline were investigated. The developed method was validated and linearity in the range of 20-400 µg L^{-1} was obtained with regression coefficients ranging between 0.9881 and 0.9990, under the optimized conditions. The recoveries of blank manure samples at three spiking levels (100, 200 and 300 µg L^{-1}) ranged from 67.73% to 120.48% with RSDs between 0.08% and 15.3%. LODs and LOQs were in the ranges of 0.111 to 0.319 µg kg^{-1} and 0.336 to 0.966 µg kg^{-1}, respectively. Finally, the proposed method was successfully applied for the extraction of the five sulfonamides from cattle manure samples. Sulfamethoxazole and sulfamethoxypyridazine were detected at concentration levels of 0.1083 and 0.065 µg kg^{-1}, respectively.

Dedications

This dissertation is dedicated:

- To my loving husband, Richard, for the patience, unlimited support and encouragement. I am truly thankful for having him in my life, especially for being there for me throughout this research.
- To my son, Olwethu, for the motivation to work harder.
- To my parents, for their continuous and unconditional support, and for teaching and encouraging me to work hard for the things I aspire to achieve.
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- The University of Venda for its financial support.

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List of abbreviations

ACN	Acetonitrile
AIBN	Azobisisobutyronitrile
3-APTMS	3-aminopropyl trimethoxysilane
DVB	Divinylbenzene
EDMA	Ethylene dimethacrylate
EGDMA	Ethyleneglycol dimethacrylate
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FQ	Fluoroquinolones
HLB	Hydrophilic-Lipophilic Balance
HPLC	High-Performance Liquid Chromatography
HPLC-DAD	High-Performance Liquid Chromatography Diode Array Detector
HPLC-FLD	High-Performance Liquid Chromatography-Fluorescence Detection
HPLC-UV	High-Performance Liquid Chromatography-Ultraviolet Detection
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MAA	Methacrylic acid
MAAM	Methacrylamide
MAE	Microwave-assisted extraction
MIP	Molecularly imprinted polymer
m-PTE	Miniaturized pipette tip extraction
MNC	Minocycline
MRLs	Maximum residue levels
OTC	Oxytetracycline
PTES	Propyl triethoxysilane
PVP	Polyvinylpyrrolidone
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RAFT	Reversible addition fragmentation chain transfer
SAs	Sulfonamides

SDZ	Sulfadiazine
SLE	Solid liquid extraction
SMT	Sulfamethazine
SMX	Sulfamethoxazole
SN	Sulfanilamide
SPE	Solid phase extraction
SPME	Solid phase micro-extraction
SQX	Sulfaquinoxaline
TC	Tetracycline
TEOS	Tetraethylorthosilica
TCA	Trichloroacetic acid
WHO	World Health Organization

Chapter 1

Introduction and background

The introduction and background of this study as well as the problem statement and objectives are given in this chapter. The chapter concludes by giving an outline on how the work is presented in this dissertation.

1. Introduction

1.1. Background of study

Antibiotics are used worldwide in veterinary medicine to treat and prevent bacterial infections as well as improve feed efficiency, and hence promote animal growth (Moyo and Tavengwa, 2019; Zhang et al., 2020). The use of antibiotics in food-producing animals may result in trace quantities of these compounds or their metabolites being present as residues in food samples, and this might be due to the inappropriate or excessive use of these drugs (Beyene, 2016). Various antibiotics have been reported to be retained in meat and milk of food producing animals (Gómez-Ramírez et al., 2018; Mookantsa et al., 2016; Gao et al., 2017) and this might pose health risks to humans who consume these food products. Moreover, these drugs are excreted by animals and end up in manure if not fully metabolized (Jiang and Wang, 2017). Residues of antibiotics excreted in animal manure may enter the environment either by spreading livestock waste in agricultural fields as fertilizer or in the form of sludge after manure collection and storage (Jansen et al., 2019). There are few reports, if any, of monitoring antibiotic residues in animal waste or in the environment at the moment (Patyra et al., 2020; Yévenes et al., 2019; Rasschaert et al., 2020).

The widespread use of antibiotics in livestock farming have spread into developing countries resulting in adverse effects on human health and food contamination (Zhang et al., 2020). The impact may vary somewhat among countries and regions, based on the interaction between human populations, land use, contaminated water sources, animal demography, national policies (production, trade, food security and animal health), and national or international trade (WHO, 2012). Many micro-organisms have become resistant to antibiotic drugs over the years and the possibility of these drug-resistant bacteria passing from animals to humans through food or being discharged into the environment is a concern globally (Blanco et al., 2017a; Blanco et al., 2017b; Pitarch et al., 2017).

Antibiotics in the form of premixes used in livestock production in South Africa have been estimated to be 29% by Eagar (2008), and this constitutes the largest proportion to the percentage of all the registered antimicrobials. Picard and Sinthumule (2002) and Eagar (2008) reported that the commonly used antibiotics, as indicated by sales, were those for treating and preventing diseases in poultry and pigs, as well as growth promotants. Tylosin, a growth

promotant was found to be the mostly used and sold antibiotic in South Africa followed by tetracyclines, sulfonamides and penicillins, respectively, in a survey conducted by Henton et al. (2011).

It was found that South Africa does not have a regulated process of antibiotic residue testing in food in a study carried out by McDermid (2012). Furthermore, currently there is limited information on the magnitude of antibiotic residues in food worldwide (Beyene, 2016) and that does not leave South Africa out. Information on the amount of antibiotics used in livestock production is scarce in South Africa. Henton et al. (2011) reported that there is a gap on the patterns of antibiotic consumption in food animals. Insufficient information on the total amount of antibiotics produced indicates that there is inadequate data on the quantities used for specific purposes in agriculture and human medicine. Therefore, there is a need to monitor antibiotics in food products in order to ensure food safety, and hence more studies should be done on this subject. In this context, this study will contribute towards building a database on the prevalence of antibiotic residues in food samples from food producing animals since currently there is limited research that has been done in South Africa.

Widely used sample treatment techniques for the extraction of some antibiotic drug residues in food products include liquid-liquid extraction (LLE) (Hernández-Mesa et al. 2017; Moreno-González et al. 2017) and solid-phase extraction (SPE) (Lan et al. 2019; Kechagia et al. 2018). Despite the good analytical performance of LLE, toxic organic solvents and relatively high amounts of the sample are needed for this technique. Moreover, LLE is an expensive technique in terms of costs and time. SPE uses lower solvent volumes compared to LLE, but it is also a time-consuming and expensive technique (Rodríguez-Gómez et al. 2018).

Antibiotic drug residues occur at trace levels as low nanogram per gram (Gómez-Ramírez et al., 2018; Tajabadi et al., 2016) hence the need to pre-concentrate them. Promising extraction and pre-concentration techniques for antibiotic drug residues that have been explored recently by many reseachers include dispersive liquid-liquid micro-extraction (DLLME) (Mookantsa et al., 2016; Gao et al., 2017; Karami-Osboo et al., 2014; Arroyo-Manzanares et al., 2014), hollow fibre based liquid phase micro-extraction (HFLPME) (Tajabadi et al., 2016; Shariati et al., 2009; Sehati et al., 2014; Xu et al., 2017) and Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) (Gómez-Ramírez et al., 2018; da Costa et al., 2015; Wang et al., 2015; Li et al., 2016), solid phase micro-extraction (Liu et al. 2017; Lirio et al. 2016; Lu et al. 2020) and miniaturized pipette-tip extraction (Yan et al., 2014) where the general trend is compliance

with green analytical chemistry principles. A general overview of these pre-concentration techniques is given in Chapter 2. Furthermore, in **Papers I** and **II**, recent applications of some of these techniques for the clean-up of various antibiotic drugs in different food samples was reviewed in detail. In **paper IV**, HFLPME was employed for the pre-concentration of tetracycline from honey samples, whereas m-MIP-PTE was used for the selective extraction of sulfonamides from manure samples in **paper V**. Despite new developments in recent years in separation science where the common trend is miniaturization and green approaches, SPE is still the most widely used technique in the extraction of antibiotics from various matrices including manure (**Paper III**).

1.2. Problem statement

Antibiotic residues in food samples might have very low concentration levels because low doses of antimicrobials are administered by livestock traders in order to maximise the number of doses available (Hoelzer et al., 2017). Hence there might be a problem of sensitivity during analysis. Mookantsa et al. (2016) detected concentrations of chlortetracycline and oxytetracycline in bovine muscle samples between 38.4 and 82.3 μg kg^{-1}. In a study done by Karami-Osboo et. (2016), florfenicol was detected in one of the milk samples at a concentration of 62.4 μg kg^{-1}. The analysis of tetracycline residues by Feng et al. (2016) showed that one milk sample contained tetracycline residues (52 ng mL^{-1}) and another milk sample contained oxytetracycline residue (87 ng mL^{-1}). Tajabadi et al. (2016) detected danofloxacin at a concentration of 24.8 ng g^{-1} in chicken liver and tetracycline at a concentration of 37.5 ng g^{-1} in lamb liver. Consequently, there is a need to do pre-concentration using extraction methods such as HFLPME (**Paper IV**) and m-MIP-PTE (**Paper V**) prior to analysis on a chromatographic instrument. Moreover, analysis of antibiotic residues by liquid chromatography followed by fluorescence or ultra-violet detection systems can give low detection limits (Khaled et al., 2019; Rodriguez-Romez et al., 2018).

Multi-residual extraction might be difficult due to the varying chemistry of antibiotic drugs from different classes (Moyo and Tavengwa, 2019). Moreover, the main difficulty in the extraction of animal samples is the co-extraction of lipidic and proteinic material which can be incompatible with the instrument detector, so the laborious aspect in the pre-treatment procedure is the pre-concentration of the analytes and clean-up of the extract (Song et al., 2016; Huang et al., 2006). Sorbents that are highly selective such as MIPs can be used to overcome challenges of multi-residual analysis and co-extraction (**Paper V**).

There is insufficient information about the occurrence, fate and risks associated with antibiotics entering the environment after being used for human and veterinary purposes worldwide (Jansen et al., 2019) and there are no much studies that have been done in Thohoyandou, Limpopo, South Africa, if any at the moment. Therefore, more research should be done in this area (**Paper IV** and **Paper V**).

1.3. Broad and specific objectives

1.3.1. Broad objectives

- Enrichment of tetracycline residues from honey samples using carrier mediated hollow fibre liquid phase micro-extraction and quantification on the LC-Q-TOF/MS (**paper IV**)
- Pre-concentration of sulfonamides in manure samples using molecularly imprinted polymer miniaturized pipette-tip extraction (**paper V**)

1.3.2. Specific objectives

Paper IV

- ❖ Optimization of parameters that may affect the extraction efficiency of carrier mediated HFLPME
    - pH of the donor solution
    - pH of the acceptor solution
    - Salt addition
    - Agitation speed
    - Extraction time
- ❖ Method validation and application to honey samples

Paper V

- ❖ Synthesis of the MIP and NIP
- ❖ Characterization of the MIP and NIP using FTIR
- ❖ Optimization of parameters that may affect the extraction efficiency of the m-MIP-PTE technique

- Number of aspirating cycles
- Number of desorption cycles
- Sample pH
- Sorbent mass
- Salt addition
- Type of elution solvent

❖ Method validation and application to cattle manure samples

1.4. Outline of the dissertation

The outline of the dissertation (comprising of five chapters) is presented as follows:

Chapter 1: A general introduction to food contamination and environmental pollution by antibiotic drug residues is briefly discussed. Furthermore, in this chapter the problem statement and objectives are given and hence, setting up the context, relevance and aim of this research.

Chapter 2A: A comprehensive overview of antibiotic drugs as food contaminants and environmental pollutants is given.

Chapter 2B: A critical overview of the presence of antibiotic drugs in various food samples and manure is given in this chapter (**papers I - III**).

Chapter 3: In this chapter the materials and methods are set out.

Chapter 4: Manuscripts (**papers IV** and **V**) prepared for the MSc examination are listed in this section. Each paper presents the work executed, results and discussion.

Chapter 5: General conclusions and future research based on experimental findings are discussed in this chapter.

References: References cited in the introduction and literature review (Chapter 1 and 2A) are listed at the end of the dissertation.

Appendix: This section is composed of supplementary information of the experiments performed and a supplementary paper.

Chapter 2A

Literature review

This chapter gives the literature survey of antibiotic drugs as food contaminants as well as environmental pollutants. The application of modern extraction and pre-concentration techniques for the clean-up of these drugs was particularly highlighted.

2.A. Antibiotics

Antibiotics, also called antibacterials, are a type of antimicrobial drugs used to treat as well as prevent bacterial infections in humans, commercially reared animals, aquaculture and apiculture. An antimicrobial is any substance of natural, semi-synthetic or synthetic origin that kills or inhibits the growth of micro-organisms but causes little or no damage to the host. They are either used as feed additives or administered therapeutically by injection in animals (Mondal et al., 2019; Mo et al., 2015; Khoobi et al., 2019). Growth promotion is another common use of antibiotics in food-producing animals and they are essential for an economically sustainable animal industry (Zhang et al., 2020; Lu et al., 2020).

2.A.1. Classification of antibiotic drugs

The main antibiotics used in both human and veterinary medicine are classified according to their chemical structure or mechanisms of action. Classes of antibiotics are defined and described below. Physico-chemical properties and structures of selected antibiotic drugs are shown on Table 1.

2.A.1.1. Sulfonamides

Sulfonamides (SAs) have a functional group, $-S(=O)_2-NH$, a sulfonyl group connected to an amine group. SAs, as one of the important and oldest classes of antibiotics, have been widely used as bacteriostatic agents due to their function in preventing the growth of bacteria, treating the microbial infections and promoting rapid growth for fish cultures, poultry and other animals (Shuo et al., 2018; Georgiadis et al., 2019; Khaled et al., 2019). These drugs show impartially low sorption capacity to solids compared to other veterinary drugs (Moyo and Tavengwa, 2019). Examples of sulfonamides include sulfadiazine, sulfamethazine, sulfamethoxazole and sulfaquinoxaline.

2.A.1.2. Tetracyclines

Tetracyclines (TCs), including tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC), are broad-spectrum antibiotics in wide use in modern animal husbandry (Lu et al., 2020). Tetracycline antibiotics have a linearly arranged naphthacene ring structure, with hydroxyl, carbonyl, amine and amide functional groups. They are amphoteric compounds soluble in polar and moderately polar solvents (Antep et al., 2017). Among the different

antibiotics, TCs are most commonly used in livestock farming and often prescribed for human therapy, owing to their lower cost and higher antimicrobial activity (Du et al., 2019; Lu et al., 2020)

2.A.1.3. Quinolones

Quinolones are synthetic antibiotics with broad-spectrum antibacterial effects (Mei and Huang, 2016; Tang et al., 2017). This antibiotic class consists of plain quinolones, such as oxolinic acid and nalidixic acid, and fluorinated quinolones, known as fluoroquinolones, such as ciprofloxacin, flumequine and sarafloxacin. Quinolones have a dual heterocyclic aromatic ring structure with the first ring having a nitrogen atom at position 1, a carboxyl group at position 3 and a carbonyl group at position 4. Fluoroquinolones result from the addition of a fluorine atom at position 6 of the second ring. Substitution at position 1 and 7 results in new enhanced fluoroquinolones.

2.A.1.4. Amphenicols

Amphenicols are a broad-spectrum antibiotic group that include chloramphenicol and its metabolites, thiamphenicol and florfenicol. Florfenicol has its own metabolite, florfenicol amine. Chloramphenicol is the most common member of this antibiotic class. It is effective against many bacterial strains, but its toxicity and unwanted effects have restricted its use over the years (Manivasagan et al., 2013). Chloramphenicol, florfenicol and thiamphenicol have strong UV absorption and they can be determined directly by HPLC (Samanidou et al., 2015).

2.A.1.5. Macrolides

Macrolides are a class of semi-synthetic medium-spectrum with a macrolyclic lactone ring with isolated or conjugated double bonds attached with amino sugars. The most commonly used macrolides have 12-16 membered structures. In this class, the most common antibiotic is tylosin (da Costa et al., 2015). The antibacterial activity of macrolides is due to their binding to the subunit 50S in the bacterial ribosome; as a result, it prevents the bacterial protein synthesis (Dinos, 2017). Examples include erythromycin, tylosin, spiramycin, tilmicosin and tulathromycin.

2.A.1.6. Beta lactams

Beta lactam antibiotics consist of several groups of compounds, cephalosporins and penicillins, are among the most important. Both classes contain bulky side chains attached to the 7-aminocephalosporanic acid or 6-aminopenicillanic acid nuclei, respectively. Penicillins are commonly used for their microbial activity against both gram-positive and gram-negative organisms (Lirio et al., 2016). Beta lactams show high complexity of the sample matrix, very low wavelength of the UV absorption maximums, particularly penicillins, hence the main clean-up is pre- and postcolumn derivatisation and detection methods are and LC-MS and LC-UV (Samanidou et al., 2016; Khoobi et al., 2019). Examples of penicillins are amoxicillin, ampicillin, oxacillin and cloxacillin. Examples of cephalosporins are cephapyin, ceftiofur and cefadroxil.

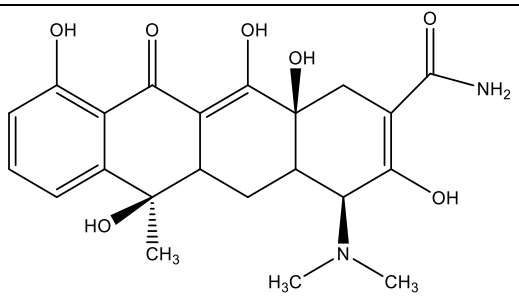
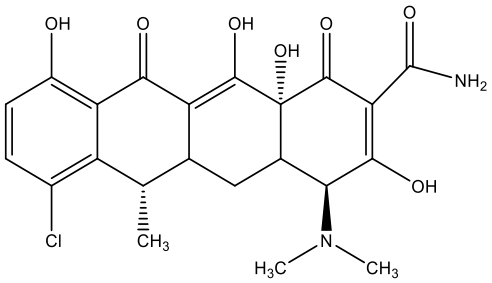
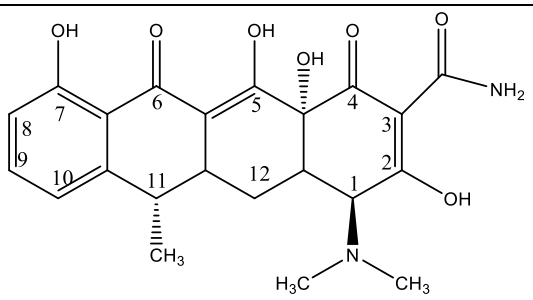
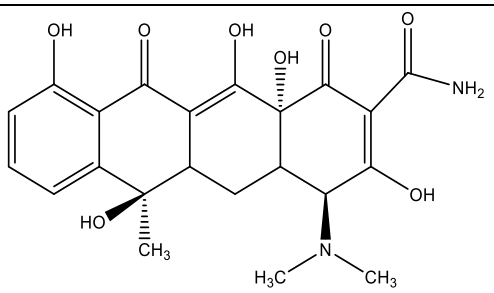
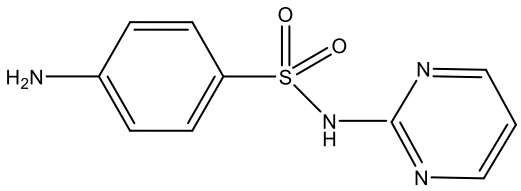
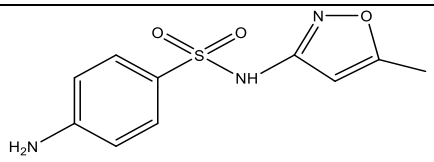
2.A.1.6. Aminoglycosides

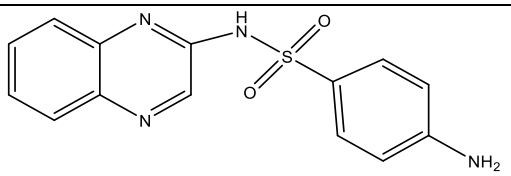
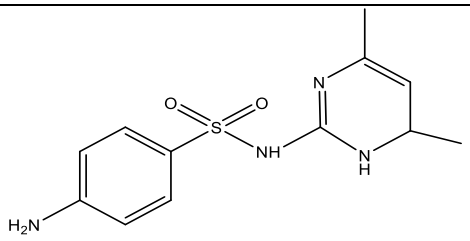
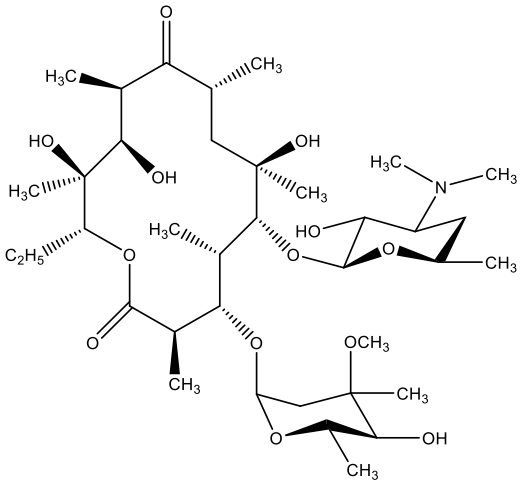
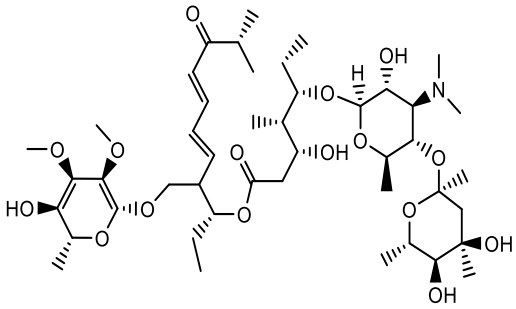
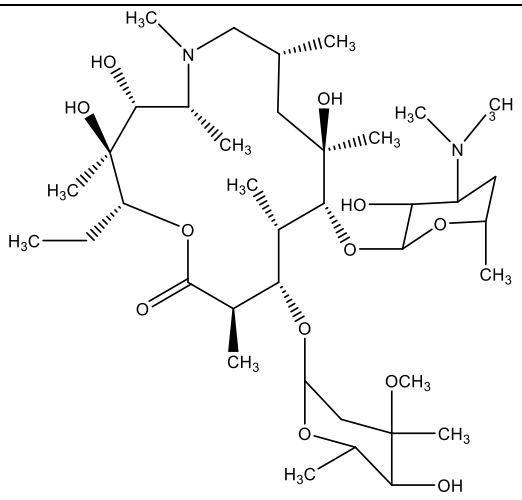
Aminoglycosides are broad spectrum with antibacterial and antifungal activities produced by *Streptomyces* and *Micromospora*. They consist of an aminocylitol ring (2-deoxystreptamine) connected to two or more amino sugars in aglycosidic linkage. Aminoglycoside use has been clinically limited to severe infections because of its toxicity (Avent et al., 2011). The more toxic antibiotics in this class have been restricted to topicalor oral use for the treatment of infections caused by *Enterobacteriaceae* (Dowling, 2013). The less toxic aminoglycosides are used for parenteral treatment of severe sepsis caused by Gram negative aerobes (Harris and Ferguson, 2012). Streptomycin, kanamycin, tobramycin and gentamicin are examples of aminoglycosides

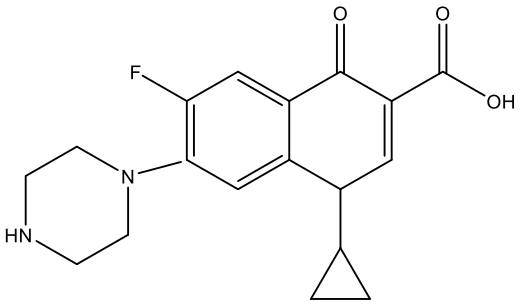
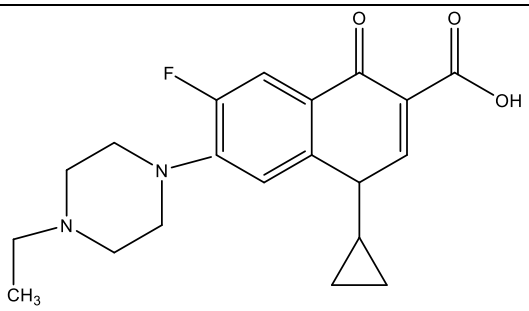
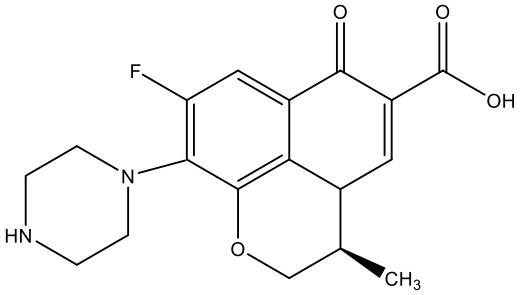
2.A.1.8. Nitrofurans

Nitrofurans are synthetic chemotherapeutic agents used in the prevention and treatment of gastrointestinal infections caused by *Escherichia Coli* and *Salmonella* (Torre et al., 2015). They are widely used in cattle, cows, pigs and poultry, and are administered orally and usually as feed additives. However, the use of nitrofurans has been banned in veterinary medicine within the European Union because of their toxic influence on the health of consumers of animal origin food (Sniegocki et al., 2018). The defining structural component is a furan ring with a nitro group. Examples of nitrofurans include: furazolidine, furaltadone, nitrofurantoin and nitrofurazone.

Table 1 Physico-chemical properties of selected antibiotic drugs from different classes

Class of antibiotic drugs	Name of antibiotic	Structure of antibiotic	Solubility in water (mg L^{-1})
Tetracyclines	Tetracycline		231
	Chlortetracycline		8600
	Doxycycline		50 000
	Oxytetracycline		313
Sulfonamides	Sulfadiazine		77
	Sulfamethoxazole		500

	Sulfaquinoxaline		45.1
	Sulfamethazine		1500
Macrolides	Erythromycin		2 000
	Tylosin		5000
	Azithromycin		<1000

Fluoroquinolones	Ciprofloxacin		300
	Enrofloxacin		146
	Ofloxacin		230

2.A.2. Food contamination and environmental pollution by antibiotic drugs

If antibiotic drugs are used accordingly, they should not result in residues at slaughter (Okocha et al., 2018). Possible reasons for the presence of residues in food products, include, not adhering to recommended label directions or dosage (extra-label usage), not observing the recommended withdrawal times, administering a large volume of the antibiotic at a single injection site, use of drug-contaminated equipment, not cleaning equipment used to mix or administer drugs, exposure of animals to spilled chemicals or medicated feeds and the animal age (Beyene, 2016; Beyene and Tesega, 2014; Du et al., 2019).

Antibiotic drugs normally accumulate in the liver or kidney rather than in other tissues. However, it has been observed that different residue levels can be found in different tissue positions such as site and route of administration (Mookantsa et al., 2016; Tajabadi et al., 2016; Karami-Osboo et al., 2014; Zhang et al., 2020). Previous studies suggest that antimicrobial use in food animals at low dosages for prolonged periods is a significant risk factor for the

development of antimicrobial resistance amongst bacterial pathogens (Hoelzer et al., 2017; Varga et al., 2009).

After administration of a drug to an animal under normal physiological conditions, most drugs are metabolized in order to facilitate elimination. Most of the parent product and its metabolites are excreted through urine and to a less extent as faecal discharge (Jiang and Wang, 2017; Okocha et al., 2018; Hou et al., 2015). Although most compounds are excreted mainly by the kidneys, some drugs are partially or completely excreted through the bile (Beyene, 2016).

Antibiotics used for veterinary purposes or as growth promoters are excreted by the animals and end up in manure (Quaik et al., 2019; Awad et al., 2014). Spreading livestock waste containing residues of antibiotics in agricultural fields as fertilizer may result in these drugs seeping through the soil and entering ground water and posing health risks to aquatic organisms (Zhou et al., 2020; Sun et al., 2017). Antibiotic residues can also be absorbed by plants (Ben et al., 2019; Gros et al., 2019), thereby introducing potential risks of effects on growth, reproduction or survival of plants as well as terrestrial fauna dependent on these plants (Hanna et al., 2018). Moreover, if these antibiotic residues in the environment are not completely degraded, they may lead to the development of antibiotic resistant microbial populations (Tao et al., 2014; Beyene, 2016).

2.A.3. Health effects of antibiotic drug residues

The threat of food contamination by antibiotic residues is one of the main challenges worldwide to public health (Moyo and Tavengwa, 2019; Du et al., 2019). Food contamination by low levels of antibiotic residues generally may not generate a negative impact on public health (Beyene et al., 2016). However, the substantial use of drugs may increase the risk of adverse effects of residues on humans including hypersensitivity, carcinogenicity, mutagenicity, teratogenicity, disruption of intestinal normal flora, allergic reactions, liver damage and yellowing of teeth (Yao et al. 2015; Georgiadis et al. 2019; Rodríguez-Gómez et al. 2018; Du et al., 2019). Continuous ingestion of antibiotics can promote the development of resistance to antimicrobials in an individual, resulting in resistance to treatment with the same antibiotics when the need arises (Duan et al., 2020; Zhang et al., 2020). To ensure food safety, and hence prevent consumers from potential health problems, regulatory bodies such as the European Union (EU, 2010) and FAO/WHO (FAO/WHO, 1992) have set maximum residue limits (MRLs) of antibiotic drug residues in food samples. Table 2 shows MRLs of different food products stipulated by the EU (2010).

Table 2 Maximum residue limits of antibiotic drug residues in food samples

Class of antibiotic drugs	Target antibiotic drug	Matrix	MRL ($\mu g\ kg^{-1}$)
Sulfonamides	Sulfonamides	Milk, fish and other seafood	100
	Sulfonamides	Eggs	Not allowed
Quinolones	Danofloxacin, enrofloxacin-ciprofloxacin and oxolinic acid	Muscle	100
	Enrofloxacin and ciprofloxacin	Eggs	Not allowed
	Enrofloxacin and ciprofloxacin	Liver	200
	Enrofloxacin and ciprofloxacin	Kidney	300
Macrolides	Macrolides	Muscle, liver and kidneys	200
	Macrolides	Milk	40
	Macrolides	Eggs	150
Tetracyclines	Tetracycline, oxytetracycline, chlortetracycline and doxycycline	Muscle, milk	100
	Tetracycline, oxytetracycline, chlortetracycline and doxycycline	Eggs	200

2.A.4. Extraction and pre-concentration techniques of antibiotics from food samples

There is no doubt that reliability, precision and accuracy of the results of any analytical procedure are strongly dependent on sample preparation methods, particularly analysing trace and ultratrace levels of the analytes in complex matrices (Sharifi et al., 2016). The presence of antibiotic drug residues in food of animal origin is of great concern currently globally due to the health risk that they may pose to consumers. Therefore, there is a need for reliable, rapid, environmentally friendly and selective techniques that can be used for the extraction of these residues in order to ensure food safety (Lu et al., 2015; Liu et al., 2017; Chi et al., 2019). Common techniques that are used in the extraction of antibiotic drug residues from food samples include LLE (Hernandez-Mesa et al., 2017; Moreno-Gonzalez et al., 2017; Jank et al., 2015) and SPE (Sichilongo et al., 2015; Lan et al., 2019; Kechagia et al., 2018). However, these methods suffer a number of drawbacks, even though they perform their tasks adequately. Both LLE and SPE are environmentally unfriendly due to the large amounts of organic solvents they use. Moreover, these methods are time consuming and labour intensive. Another disadvantage of SPE is that cartridges are costly. Consequently, extraction techniques that address shortcomings of these conventional methods such as QuEChERS (Gómez-Ramírez et al., 2018; da Costa et al., 2015; Wang et al., 2015; Li et al., 2016), DLLME (Mookantsa et al., 2016; Gao et al., 2017; Karami-Osboo et al., 2014; Arroyo-Manzanares et al., 2014), HFLPME (Tajabadi et al., 2016; Sehati et al., 2014; Xu et al., 2017, SPME (Lirio et al., 2016; Mondal et al., 2019; Tang et al., 2017; Lu et al., 2020) and stir bar sorptive extraction (SBSE) (Khaled et al., 2019; Khoobi et al., 2019; Rodriguez-Gomez et al., 2018) have emerged in recent years. Principles and application of these methods will be discussed below.

2.A.4.1. QuEChERS

The Quick Easy Cheap Effective Rugged Safe (QuEChERS) method is an extraction technique that employs an organic solvent and phase separation using high salt content, in some cases followed by dispersive solid phase extraction (d-SPE) for sample clean-up (Khaled et al., 2019; Zhang et al., 2019). The QuEChERS method, which was originally developed for pesticide analysis in fruits and vegetables (Anastassiades, 2003; Berendsen et al., 2013), has recently been proposed for the analysis of antibiotic drugs in different food matrices (Gomez-Ramírez et al., 2018; da Costa et al., 2015; Li et al., 2016; Machado et al., 2013). The advantages of this technique include easy operation, environmental friendliness, high reproducibility and stability, the applicability of this method in multi-residual extraction and it is also fast (Zhang

et al., 2019). As a result, the QuEChERS method is now employed in routine analysis by testing laboratories in the food industry (Khaled et al., 2019). Table 3 summarises recent studies that have been done where this method was used for the extraction of antibiotic residues from food samples. Generally acidified acetonitrile is used for fat and protein precipitation to prevent column damage. Furthermore, further clean-up steps were necessary to remove possible matrix interferences in order to achieve better chromatographic peak shapes and recoveries (Schwaiger et al., 2018; Jin et al., 2017; Rocha et al., 2017). The common sorbents used in the d-SPE method are primary and secondary amine exchange material (PSA) for removing sugars and fatty acids and C_{18} sorbent (for elimination of nonpolar interferences) (Li et al. 2016; Wang et al., 2017). The common salting-out agents include magnesium sulfate (Konak et al., 2017; Grabsk et al., 2019), sodium chloride (da Costa et al., 2015) and sodium sulfate (Jin et al., 2017; Rocha et al., 2017).

Table 3 Application of the QuEChERS method in the extraction and pre-concentration of antibiotic drug residues from food samples

Target antibiotic	Food matrix	Extraction procedure	Method of detection	Concentration of antibiotic detected	LOD	LOQ	Recovery (%)	Reference
3 Sulfonamides	Chicken breast	QuEChERS based on ACN/H_2O with 1% CH_3CO_2H followed by a cleanup using d-SPE Oasis HLB as a sorbent.	HPLC-DAD	-	10 and 13 µg kg^{-1}	25 - 30 µg kg^{-1}	75.4 - 98.7	Machado et al. (2013)
6 multiresidues	Bovine milk	QuEChERS based on acetonitrile followed by a cleanup with d-SPE based C_{18}, PSA and sodium acetate	LC-MS/MS	-	-	-	84.18-15.99	Wang et al. (2015)
7 macrolides	Milk	QuEChERS based on acetonitrile extraction + a mixture of salts (sodium sulphate, sodium chloride and potassium carbonate)	LC-MS/MS	-	0.84 µg kg^{-1}	2.79 µg kg^{-1}	89 - 97	Da Costa et al. (2015)
Sixteen multi-residues	Preserved eggs	QuEChERS based on water, acetonitrile with 1% acetic acid followed by a cleanup using d-SPE with C_{18} and PSA as sorbents	UHPLC-MS/MS	-	0.1- 0.9 µg kg^{-1}	0.3-3.0 µg kg^{-1}	73.8 - 127.4	Li et al. (2016)
47 multi-residues	Honey and royal jelly	QuEChERS based on acetonitrile containing 1% acetic acid, anhydrous Na_2SO_4 and NaCl followed by a clean-up using d-SPE with C_{18} and PSA as sorbents	LC-MS/MS	-	0.14 - 2.91 ng g^{-1}	0.50-9.70 ng g^{-1}	80.4-118.4	Jin et al. (2017)
25 multi-residues	Porcine and poultry kidneys	ACN acidified with 5% v/v of glacial acetic acid, sodium sulfate, sodium acetate was used as the extraction phase followed	LC-MS/MS	-	-	-	82.7 to 115.5%	Rocha et al. (2017)

		by d-SPE using with C_{18} and PSA as sorbents						
30 multi-residues	Dairy products	The extraction phase was Na_2EDTA-McIlvaine buffer solution followed by d-SPE based on C_{18}	UHPLC-MS/MS	-	-	-	-	Schwaiger et al. (2018)
12 Sulfonamides	Baby food	The extraction phase was 1% aceticacid in acetonitrile, sodium acetate and anhydrous magnesium sulfate followed by d-SPE based on C_{18} and PSA	UHPLC-Orbitrap-MS	-	-	0.10-0.55 µg kg^{-1}	60.9-85.9	Konak et al. (2017)
Ceftiofur, cloxacillin and enrofloxacin	Milk	The extraction phase ACN with 1% acetic acid (v/v), anhydrous $MgSO_4$ and anhydrous NaAc followed by d-SPE based on C_{18}, PSA and sodium acetate	UHPLC-MS/MS	-	1.4-6.8 µg L^{-1}	1.5-8.7 µg L^{-1}	95-99	Grabsk et al. (2019)

- Data not available

2.A.4.2. Liquid phase micro-extraction

Liquid phase micro-extraction (LPME) is a solvent-minimized sample pre-treatment procedure of LLE, in which only several microlitres of solvent are required to concentrate analytes from various samples rather than hundreds of millilitres needed in traditional LLE (Sharifi et al., 2016; Moyo and Tavengwa, 2019; Bahrami et al., 2017). The advantages of LPME are simplicity of operation, rapidity, low cost, high recovery and high enrichment factors (Gao et al., 2017; Mookantsa et al., 2016; Tajabadi et al., 2016).

LPME can be divided into four main modes which include solidified floating organic drop micro-extraction, single-drop micro-extraction, hollow-fiber liquid phase micro-extraction (HFLPME) and dispersive liquid-liquid micro-extraction (DLLME). Among these modes of LPME, HFLPME and DLLME have received much attention and they will be discussed below. The application of these two LPME techniques in the extraction of antibiotic residues from food samples is summarised in Table 4. Problems of the drop dislodgement and instability limits the applicability of single drop micro-extraction (Sharifi et al., 2016).

2.A.4.2.1. Dispersive liquid-liquid micro-extraction

DLLME was introduced by Rezaee et al. (2006) as a new LLE technique for determination of polyaromatic hydrocarbons and pesticides. The application of DLLME in the extraction of veterinary drugs in literature has increased over the years (Karami-Osboo et al., 2014; Arroyo-Manzanares et al., 2014; Gao et al., 2017; Mookantsa et al., 2016). This technique is based on a ternary component solvent system including an extraction solvent, disperser solvent and an aqueous sample, and is known as traditional DLLME. The advantages of traditional DLLME are the microliter level volumes required for extraction and dispersive solvents and short extraction times. However, the disadvantage of traditional DLLME is the use of organic solvents such as the extraction and dispersive solvents. As a result, modified modes of DLLME have been invented recently and they include, low-density solvent based DLLME (LDS-DLLME) (Seebunrueng et al., 2014; Chaiyamate et al., 2018), solidified floating organic drop DLLME (SFO-DLLME) (Thongsaw et al., 2017; Jalbani and Soylak, 2015), effervescence assisted DLLME (Wu et al., 2017; Sorouraddin et al., 2019), air assisted dispersive liquid-liquid micro-extraction (AA-DLLME) (Ferrone et al., 2018), surfactant assisted DLLME (SA-DLLME) (Sadeghi et al., 2018; Bişgin, 2019) and ionic liquid DLLME (IL-DLLME) (Gao et al., 2017; Unsal et al., 2019; Lawal et al., 2018) to address the disadvantages associated with

traditional DLLME. Despite these disadvantages, DLLME is more advantageous in terms of short total time, low cost and feasibility compared with other liquid-phase micro-extraction techniques (Al-Saidi and Emara, 2013). Moreover, the application of these new modes of DLLME in the extraction of antibiotic drug residues from food samples is still limited.

2.A.4.2.2. Hollow fibre liquid phase micro-extraction

HFLPME is a mode of LPME that uses a porous polypropylene hollow fiber for the immobilization of the organic solvent in its pores. Figure 1 shows the set up of this method. The main components of this technique are the donor phase, porous polypropylene hollow fiber, and the acceptor phase (Bahrami et al., 2017; Gjelstad et al., 2015). The different modes of HFLPME are static, dynamic, two and three phase (Sharifi et al., 2016; Khan et al., 2019). The advantages of HFLPME are high enrichment of analytes, high degree of sample clean-up and low solvent consumption. The disadvantage of HFLPME procedure is that it is slow with extraction times ranging from 15 to 45 min and target analytes may partly be trapped in the supporting liquid membrane (SLM) (Shariati et al., 2009; Ncube et al., 2016). Another disadvantage is that there is no commercially available equipment for this method although hollow fibres are commercially available (Gjelstad and Pedersen, 2013). There are a few studies that have been done recently on the application of HFLPME on antibiotic drugs in food samples (Shahi et al., 2020; Sehati et al., 2014).

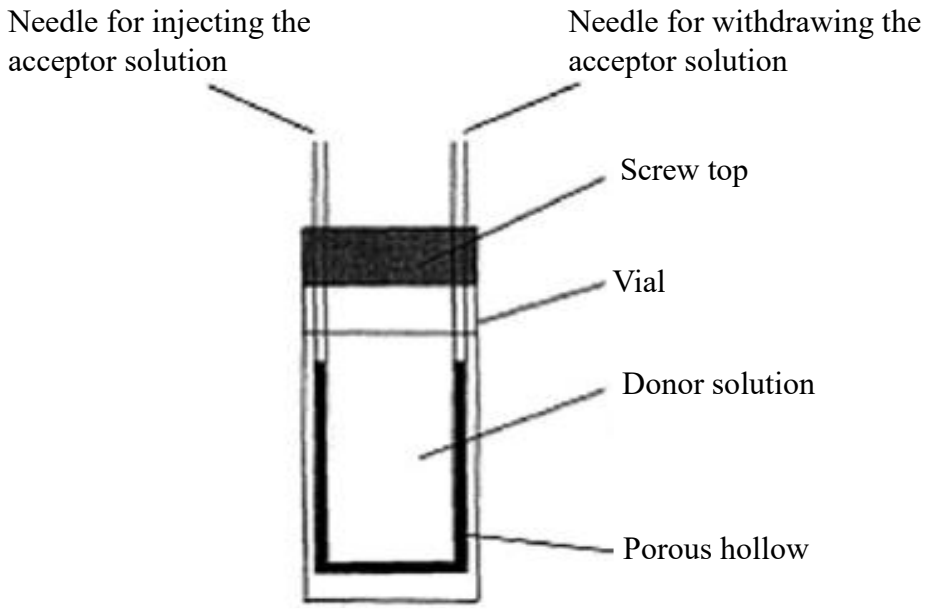


Figure 1 Set up of HFLPME

Table 4 Application of LPME for the extraction of antibiotic residues from food samples

Target antibiotic	Food matrix	LPME technique	Extraction procedure	Method of detection	Concentration detected	LOD	LOQ	Recoveries (%)	Reference
Oxytetracycline, doxycycline, penicillin G and chloramphenicol	Milk	DLLME	ACN (extraction solvent) and deep eutectic solvent (disperser)	HPLC-DAD	-	2.0-2.8 µg L^{-1}	6.5-9.3 µg L^{-1}	65 - 81	Mohebi et al. (2020)
7 Tetracyclics	Beef	DLLME	MeOH (disperser) and CH_2Cl_2 (extracting solvent)	LC-MS/MS	38.4 and 82.3 µg kg^{-1}	2.2-3.6 µg kg^{-1}	7.4-11.5 µg kg^{-1}	80 - 105	Mookantsa et al. (2016)
4 Tetracyclines	Egg supplements	US-DLLME	EtOH (disperser) and $CHCI_3$ as extraction solvent	Flow injection analysis	-	6.4 - 11.1 µg L^{-1}	21.3 - 37.0 µg L^{-1}	85.7 - 96.4	Rodríguez et al. (2016)
Sulfonamides	Milk	DLLME	Extraction solvent ($CHCI_3$) and dispersive solvent (ACN)	HPLC-FD	-	0.73 - 1.21 µg L^{-1}	-	92.9 - 104.7	Arroyo-Manzanares et al. (2014)
6 FQs	Milk	DLLME	-	HPLC-UV	-	-	2.5 - 15 µg kg^{-1}	74.1 - 101.4	Karami-Osboo et al. (2014)
4 Tetracyclines	Milk and eggs	IL-DLLME	$[C_6MIM][PF_6]$ as an extraction solvent, FIL-NOSM a disperser solvent)	HPLC-UV	-	0.08 - 1.12 μg kg^{-1}	-	94.1 - 102.1	Gao et al. (2017)
Florfenicol and Chloramphenicol	Pasteurized Milk	DLLME	$CHCl_3$ as an extracting solvent and water as a dispenser solvent	HPLC-UV	62.4 µg kg^{-1}	12.2 and 12.5 µg kg^{-1}	36.6 and 37.5 µg kg^{-1}	-	Karami-Osboo et al. (2016)

5 Quinolones and 4 tetracyclines	Milk, honey, fish, liver and lamb muscle	HFLPME	1.0 mol L^{-1} NaOH and and NaCl was the AP, 10% w/v Aliquat-336 in 1-octanol was the SLM	HPLC-DAD	24. 8 ng g^{-1} TC	0.5 - 20 ng g^{-1}	1.25 - 40 ng g^{-1}	-	Tajabadi et al. (2016)
4 Tetracyclines	Milk	HF-DLLME	$CHCI_3$ as an extracting solvent and water as a dispenser solvent	HPLC-UV	-	0.95 - 3.6 µg L^{-1}	5 - 15 µg L^{-1}	92.38 - 107.3	Xu et al. (2017)

-Data not available

2.A.4.3. Sorbent-based sorptive micro-extraction techniques

Solvent-free sorbent-based sorptive micro-extraction techniques such as solid phase micro-extraction (SPME) and its different modifications, stir bar sorptive extraction (SBSE), stir cake sorptive and fabric phase sorptive extraction (FPSE) have emerged in recent years in an attempt to address limitations of conventional extraction methods such as SPE. These techniques utilize a solid/semi-solid organic polymer as the extractive phase immobilized on a substrate such as fused silica fibre (Chen and Huang, 2016; Lirio et al., 2016), stainless steel wire (Lu et al., 2020; Tang et al., 2017; Khaled et al., 2019), glass-coated bar magnet (Yao et al., 2015; Rodríguez-Gómez et al., 2018) and cellulose/polyester (Samanidou et al., 2017; Samanidou et al., 2015). The applications of SPME and SBSE in the extraction of antibiotic drug residues from food samples is summarised on Table 5.

2.A.4.3.1. Solid phase micro-extraction

Solid phase micro-extraction, which was developed by Arthur and Pawliszyn (1990), has gained great attention due to its simplicity, reduced analysis time and it is solvent free. SPME has been widely accepted for its usefulness in the analysis of food samples containing antibiotic residues (Liu et al., 2017; Lirio et al., 2016; Lu et al., 2020). The coating of SPME fiber plays a key role in effectively extracting trace levels of target analytes from complex matrices (Liu et al., 2017). Common commercial coatings for SPME fibres are PDMS, polyacrylate, carboxen/PDMS and PDMS/divinylbenzene. Although these coatings are effective in sample pre-treatment, they lack selectivity towards target analytes, which results in competitive adsorption of other organic pollutants, particularly in animal food samples (Lin et al., 2015; Canellas et al., 2016). In an attempt to address this disadvantage, sorbents that are highly selective such as molecularly imprinted polymers have been developed in recent years and applied as coating materials in sorptive micro-extraction methods (Lu et al., 2020; Yang et al., 2017).

2.A.4.3.2. Stir bar sorptive extraction

Stir bar sorptive extraction (SBSE) is a rapid, green and low-cost method suitable for volatile and semi-volatile compounds that was developed by Baltussen et al. (1999), and it is based on the same principles as SPME. However, SBSE has a higher extraction capacity than SPME due to the larger volume and surface area of the sorptive phase. Consequently, SBSE gives higher recoveries compared to SPME (Cárdenas and Lucena, 2017; Telgheder et al., 2018). Moreover, SBSE allows the extraction of numerous samples simultaneously, with a low solvent consumption and less sample manipulation (Rodríguez-Gómez et al., 2018). However, the thickness of the coating on the stir bar affects the extraction kinetics since diffusion of the analytes in the polymeric coating can be hindered (Wells, 2003).

Polydimethylsiloxane (PDMS) is a common stir bar coating material for SBSE and it is commercially available in the market. The main disadvantage of this material is that it is not efficient for polar compounds such as antibiotic drugs. However, other stir bar coatings such as PEG (Rodríguez-Gómez et al., 2018), graphene oxide (Fan et al., 2015), zeolitic imidazolate frameworks (Khoobi et al., 2019) and molecularly imprinted polymers (MIPs) (Tang et al., 2018; Yang et al., 2017) have extended the range of applicability of this technique. The application of SBSE employing these coatings for the extraction of various antibiotics from food samples is summarised on Table 5.

Table 5 Application of sorptive micro-extraction techniques for the pre-concentration of antibiotic drugs in food samples

Target analyte	Matrix	Sorptive micro-extraction technique	Sorbent	Instrument of analysis	LOD	Recovery (%)	Reference
6 Penicillins	Milk	SPME	Al-MOF	UPLC-UV	0.06-0.26 µg L^{-1}	90.5-95.7	Lirio et al. (2016)
2 Quinolones, 2 sulfonamides, 1 macrolide and 1 pyrimethamine	Fish	SPME	MIL-101(Cr)NH_2-polyacrylonitrile coated on a quartz fiber	LC/MS-MS	0.2 - 4.6 ng L^{-1}	-	Mondal et al. (2019)
5 Fluoroquinolones	Fish	SPME	C_{18} particles coated on stainless steel wires	LC-MS/MS	0.3-1.5 ng g^{-1}	-	Tang et al. (2017)
3 Chloramphenicols	Milk	SPME	AuNPs@Au wire-ITO fiber	HPLC-DAD	0.262-0.293 ng mL^{-1}	75.7-94.5	Liu et al. (2017)
7 Flouroqinolones	Milk and honey	SPME	Poly(apronal-co-divinylbenzene/ethylenedi methacrylate based monolithic fiber	HPLC/MS-MS	0.0019-0.018 µg kg^{-1} and 0.0010-0.0028 µg kg^{-1}	74.5-116 and 80.2-113	Chen and Huang (2016)
2 Tetracyclines	Milk, chicken and fish	SPME	MIPs coated on a stainless steel wire	HPLC-DAD	0.38-0.72 µg kg^{-1}	77.3-104.4	Lu et al. (2020)
Sulfamethoxazole and trimethoprim	Milk and eggs	PT-SPME	Phenyl-boronic acid polymer monolith	UHPLC/MS-MS	1.5 and 0.3 ng mL^{-1}	92.4-100.5 and 92.7-102.6	Chi et al. (2019)
8 Sulfonamides	Fish and chicken	IT-SPME	NH_2-MIL-53(Al) MOF in a capillary column	UHPLC/MS-MS	1.3-4.7 ng L^{-1}	85.7-113	Zhang et al. (2020)
Several antibiotics	Chicken and beef	Direct immersion SPME	Polyacrylonitrile/ hydrophilic-lipophilic balance coated on stainless steel rods	LC-MS/MS	-	70-120	Khaled et al. (2019)
17 Quinolones	Cow's milk	SBSE	PEG coated a polar stir bar	UHPLC-MS/MS	0.1-1.0 µg kg^{-1}	88-114	Rodríguez-Gómez et al. (2018)
11 Quinolones	Bovine milk	Immunoaffinity-SBSE	Monoclonal antibodies coated on a glass bar	HPLC-FLD	0.05-0.1 ng g^{-1}	11.8-40.0	Yao et al. (2015)
5 Fluoroquinolones	Chicken muscle and liver	SBSE	Graphene oxide /PEG	HPLC-FLD	0.0045-0.0079 µg kg^{-1}	-	Fan et al. (2015)
9 Fluoroquinolones, 1 tetracycline, 1	Chicken, pork and fish	SBSE	MIPs coated on magnetic stir bar	HPLC-DAD	0.1-0.3 ng g^{-1}	67.4-99.0	Yang et al. (2017)

penicillin and 1 nitrofuran							
Benzylpenicillin	Milk	SBSE	Zn-Al-LDH/ZIF-8 nanostructures coated on a magnetic aluminium oxide stir bar	HPLC-UV	0.05 μg L^{-1}	96.0-110.0	Khoobi et al. (2019)

-Data not available

2.A.4.4. Molecular imprinting technology

Sorbents play a critical role in any SPE based procedure. The selectivity of a sorbent has a significant influence on the accuracy of results during the pre-concentration step (Li et al., 2017). The efficiency of SPE-based clean-up and pre-concentration techniques is dependent on the affinity that sorbents have for target analytes (Chisvert et al., 2019). Traditional sorbents such as octadecylsilane (C_{18}) and Oasis HLB are the commonly used commercially available materials for SPE based techniques. However, these sorbents are susceptible to interferences by impurities and suffer competitive adsorption in multi-residual extraction, and they cannot be reused. Various sorbents that overcome these drawbacks have been developed in SPE and SPE based techniques for the extraction of antibiotic residues in recent years. These include carbon nanotubes (de Faria et al., 2017; Nasir et al., 2019), metal-organic frameworks (Bagheri and Ghaedi, 2020; Du et al., 2019), graphene/graphene oxide (He et al., 2017; Wei et al., 2017) and molecularly imprinted polymers (MIPs) (Ji et al., 2018; Kechagia et al., 2018; Moreno-Gonzalez et al., 2017). Out of these techniques, MIPs are briefly discussed below and their application in the extraction of antibiotic residues from food samples is summarised on Table 6.

Molecular imprinting is a technique where binding cavities are introduced by co-polymerising functional monomers such as methacrylic acid and cross-linkers in the presence of a template molecule (Figure 2). Examples of monomers include acrylamide, methacrylic acid (MAA) and poly(vinyl pyrrolidone) (PVP). Ethylene glycol dimethacrylate and divinylbenzene (DVB) are some common cross-linkers. Polymerization is usually facilitated by thermal or UV activation of the initiator (Azizi and Bottaro, 2020). Specific recognition sites remain in the polymeric matrix complementary in size, shape and position of the template functional groups to the imprinted molecule after template removal (Zhao et al., 2018; Mohsenzadeh et al., 2018).

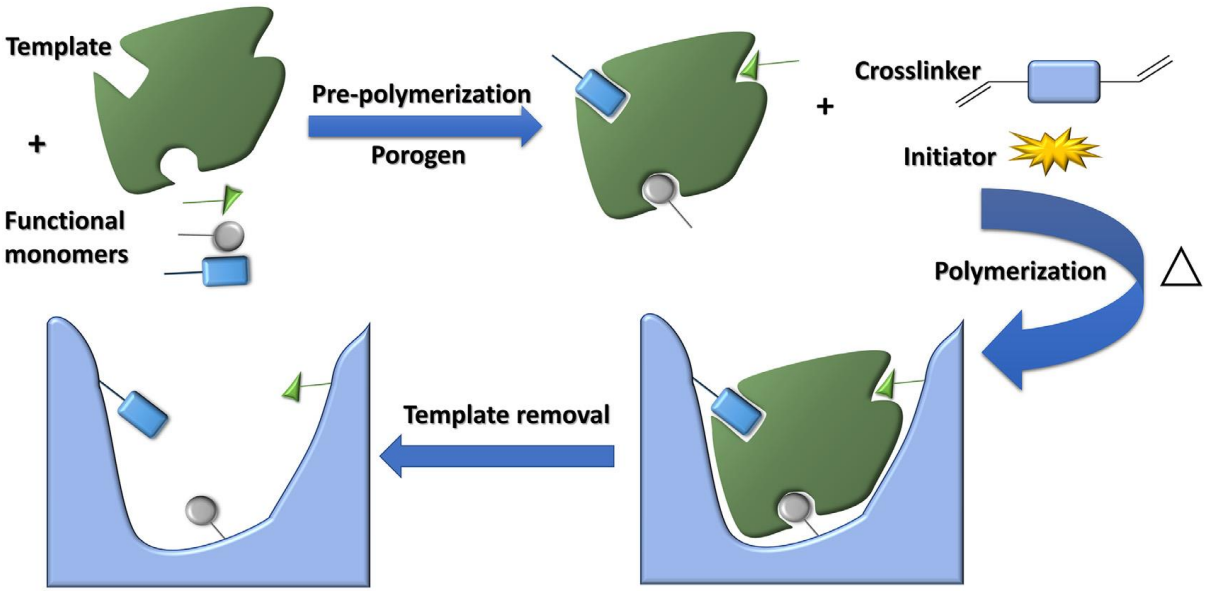


Figure 2 General preparation procedure of MIPs

2.A.4.4.1 Synthesis strategies of MIPs

Three general approaches, namely, covalent, non-covalent, and semi-covalent approaches, have been described for the synthesis of MIPs (Gama and Bottoli, 2017; Ansari and Karimi, 2017; Speltini et al., 2017). The covalent approach involves the formation of reversible covalent bonds between the template molecule and monomers before polymerization. Subsequently, the template removal from the polymer is done by the cleavage of the corresponding covalent bonds, which reform upon rebinding of the target compound (Turiel and Esteban, 2020). Due to the high stability of template-monomer interactions, this approach results in a polymer with a homogenous population of binding sites and the existence of nonspecific sites is minimized (Chen et al., 2016). However, the challenge associated with this approach is the difficulty in designing an appropriate template-monomer complex where covalent bond formation and cleavage can be readily reversible under mild conditions (Bitas and Samanidou, 2018). In this case, the semi-covalent strategy can offer an alternative intermediate approach, where the template rebinding is based only on non-covalent interactions, although, the template is also covalently bound to a functional monomer (Speltini et al., 2017; Tartaglia et al., 2019). Finally, the non-covalent approach is based on the formation of relatively weak non-covalent interactions such as hydrogen bonds and ionic interactions between the template molecule and selected monomers before polymerization. Owing to its simplicity and the availability of a wide variety of monomers, this approach is by far the most common for the preparation of MIPs (Chen et al., 2011; Aggarwal et al., 2016; He et al., 2016).

2.A.4.4.2 Polymerization techniques in MIP synthesis

The most popular techniques used in the synthesis of MIPs include free radical polymerization and controlled radical polymerization (Refaat et al., 2019; Azizi and Bottaro, 2020).

2.A.4.4.2.1 Free radical polymerization

There are four main methods that proceed via the free radical polymerization mechanisms, namely, bulk, suspension, emulsion and precipitation polymerization (Figure 3). Bulk polymerization is a fast and simple MIP synthesis method that does not require special instrumentation. Therefore, it is the most widely used free radical polymerization method among the other three approaches. Nevertheless, this method requires increased template amount and the synthesized MIP bulk should be ground, sieved and sedimented (Bitas and Samanidou, 2018). This is time consuming and results in particles with irregular shape and size, decreased number of imprinted sites and, binding capacity, while template bleeding can be observed (Boulanouar et al., 2018; Ashley et al., 2017). Suspension polymerization is also a simple method which involves a single-step reaction where a polymerization mixture is suspended in a continuous aqueous, mineral oil or perfluorocarbon liquid phase and results in spherical porous MIP particles. However, the sythesized MIP particles size may have decreased recognition capability and are unsuitable for SPE applications (Speltini et al., 2017; Chen et al., 2016). Like suspension polymerization, the precipitation method is also a single-step polymerization method that provides high quality, spherical MIP particles with homogenous size in high yields. However, this method requires an increased amount of organic solvents and meticulous monitoring of the polymerization conditions such as solvent polarity, temperature and stirring speed that may affect the MIP particle size (Gama and Bottoli, 2017). Lastly, emulsion polymerization takes place in an oil/water diphasic system with the addition of surfactants that prevents diffusion and favors the formation of small, homogeneous emulsion droplets. This method can provide mono-dispersed MIP particles, in high yield, however surfactant residues can interfere with the imprinted sites, thus reduce binding capacity of the MIPs (Ashley et al., 2017).

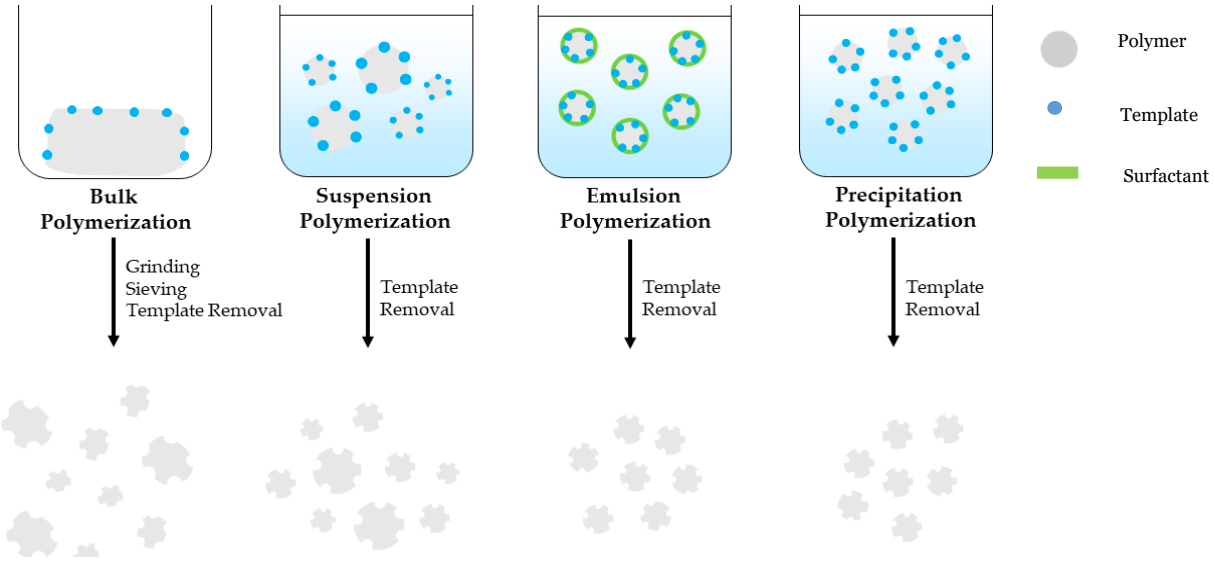


Figure 3 Free radical polymerization techniques in MIP synthesis (Bitas and Samanidou, 2018).

2.A.4.4.2.2 Controlled radical polymerization

The most common controlled radical polymerization approaches are atom transfer radical polymerization (ATRP), nitroxide-mediated polymerization (NMP) and reversible addition-fragmentation chain transfer (RAFT) polymerization (Bitas and Samanidou, 2018). In ATRP, a reversible redox reaction is catalyzed by a metal-ligand complex and the resulting MIP particles have functional groups that allow surface modification. However, MIP synthesis using this technique is limited because the functional groups of the template molecule and the recognition unit of the functional monomer can inhibit the metal-ligand complex (Haupt et al., 2011). Meanwhile, NMP involves a thermally reversible termination reaction that produces a nitroxyl radical that allows control over the polymerization reaction, RAFT polymerization involves reversible addition-fragmentation reactions initiated by an initiator and chain propagation is facilitated by a chain transfer agent. This technique can be used with a variety of functional groups and is suitable for non-covalent imprinting purposes (Ye, 2015).

2.A.4.4.3 Application of MIPs in the extraction of antibiotics from food samples

MIPs are ideal for the selective extraction of compounds at trace levels, particularly when the sample is complex due to the strong interaction between MIPs and target molecules. This is a major advantage that MIPs have over conventional sorbents, hence they are suitable for multi-residue analysis of antibiotics. Moreover, the high selectivity of MIPs can increase the sensitivity and repeatability of antibiotic analysis by reducing co-extraction of lipidic and proteinic material in food products. As a result, overlapping of chromatographic peaks and matrix effects in mass spectrometry may be reduced (Grimmett and Munch, 2013). The use of MIPs as SPE sorbents (MISPE) for the selective extraction of antibiotics from food samples has grown significantly in the past few years (Moreno-Gonzalez et al., 2017; Kechagia., 2018; Zhao et al., 2018). The MISPE method exhibits excellent enrichment factors (He at al., 2016).

Table 6 Application of MIPs as sorbents in the extraction of antibiotic residues from food samples

Target antibiotic	Food matrix	Extraction method	Type of polymerization	Pre-polymerization reagants	Instrument of analysis	Concentration of antibiotics detected	LOD	Recovery (%)	Reference
Lincomycin	Pasteurized milk	SPE	Core-shell	MAA (monomer), EGDMA (cross-linker) and lincomycin (template)	HPLC-UV	-	0.02 and 0.08 µg mL^{-1}	80-89	Negarian et al. (2019)
Ampicillin	Eggs	SPE	Surface molecular imprinting	Acrylamide (monomer), EGDMA (cross-linker) and ampicillin (template)	HPLC-UV	-	-	91.5-94.9	Tian et al. (2018)
Aminoglycosides	Honey	SPE	-	Commercial MIPs	CE-MS/MS	-	0.4-28.5 µ kg^{-1}	88.2-99.8	Moreno-Gonzalez et al. (2015)
11 Aminoglycosides	Milk and milk products	SPE	-	Commercial MIPs	UHPLC-MS/MS	-	1.3-14.7 µg kg^{-1}	70.0-106.0	Moreno-Gonzalez et al. (2017)
Sulphadiazine	Eggs	SPE	Suspension	MAA (monomer), EGDMA (cross-linker) and sulfadiazine (template)	HPLC-DAD	-	0.05-0.06 µg L^{-1}	78.22-86.10	He et al. (2016)
6 Sulphonamides	Milk	SPE	Sol-gel	3-APTMS and PTES (monomers), TEOS (cross-linker) and all SAs (template)	HPLC-DAD	-	1.9-13.3 µg kg^{-1}	85.8-115.7	Kechagia et al. (2018)
6 Sulphonamides	Milk, eggs and different types of meat	SPE	RAFT	MAA (monomer), DVB (cross-linker) and sulfadiazine (template)	HPLC-MS/MS	3.57 and 3.79 g µkg^{-1}	0.02 -0.1 µg L^{-1}	63.49-115.72	Zhao et al. (2018)
Cefquinome	Milk, honey and eggs	SPE	Magnetic imprinting	PVP (monomer), EGDMA (cross-linker) and cefquinome (template)	-	-	-	74-93.7	Aggarwal et al. (2016)

Note: –Data not given

2.A.4.5. Miniaturised pipette tip extraction

One of the well-known sample preparation techniques is miniaturised pipette-tip extraction (m-PTE), which is an extraction technique based on SPE (Tavengwa et al., 2016; Yan et al., 2014). This method overcomes limitations of the conventional SPE cartridges while retaining its advantages (Seidi et al., 2019). m-PTE consists of a solid phase extraction cartridge in which the sorbent is dispersed in a pipette tip, which allows a quick and dynamic contact between the aspirated analyte from the sample and the solid phase (Bordin et al., 2016). The standard procedure of this technique consists of four steps normally done in SPE, which include, conditioning of the sorbent to activate adsorption sites followed by sample aspiration which is facilitated by air aspiration to allow the dynamic mixing between the sorbent and the sample. Thereafter, a cleaning step is done using a suitable solvent in order to remove matrix interferences and finally the analytes of interest are desorbed from the sorbent (Bordin et al., 2016; Brigante et al., 2017). The dynamic mixing of the solid sorbent with the sample using air aspiration provides fast equilibrium between the analyte and sorbent, thus the extraction time is reduced. Another advantage is the lower consumption of solvent and sorbent phase compared to SPE (Cozarra et al., 2017). Moreover, on-site sampling can be possible with the aid of these devices and generally the sample pre-treatment time is noticeably reduced (Seidi et al., 2019).

Like any other extraction method, this technique suffers some drawbacks and these include, clogging of the pipette-tips and the low amounts of sample throughputs in every single extraction due to the small quantities of the sorbents that can be packed (Seidi et al., 2019). As a result, this method cannot be efficient enough for large quantities of samples. Although, C_{18} is the commonly used sorbent in m-PTE, in recent years other extractive phases including MIPs (Yang et al., 2018; Du et al., 2014), graphene oxide (Yan et al., 2014; Wang et al., 2014), polyacrylonitrile nanofibres (Tavengwa et al., 2016) and carbon nanotubes (Wang et al., 2017; Kahkha et al., 2016; Fresco-Cala et al., 2018) were used. The application of this method for the extraction of antibiotics from food and manure samples is still limited.

Chapter 2B

Critical overview of literature

This chapter gives a critical overview of literature on the presence of antibiotic drugs in various food samples and manure. Recent applications of modern extraction and pre-concentration techniques for the clean-up of these drugs were discussed. This section consists of a book chapter and two review papers.

2.B.1. Paper I

This book chapter “Modern extraction and clean-up methods of veterinary drug residues in food samples of animal origin” was published on IntechOpen in the book *Recent advances in Analytical Chemistry*. It briefly discussed the general principles of modern extraction techniques including dispersive liquid-liquid micro-extraction, hollow fibre liquid phase micro-extraction, QuEChERS and molecularly imprinted polymers. The extensive application of these techniques in the extraction and pre-concentration of antibiotic drugs from food samples was also outlined.

Modern Extraction and Cleanup Methods of Veterinary Drug Residues in Food Samples of Animal Origin

Babra Moyo and Nikita Tawanda Tavengwa

Abstract

Extensive research on the presence of veterinary drug residues in food samples has been conducted and is still underway. The inappropriate or excessive use of veterinary drugs in food producing animals may result in trace quantities of these drugs or their metabolites in food samples. Food contamination by veterinary drug residues is one of the main challenges worldwide to public health with drug resistance being the biggest threat. One of the challenges in veterinary drug residue analysis is their occurrence in trace amounts that are normally below limits of detection of most analytical instruments. Various efficient, economical, miniaturized and environmentally friendly extraction methods have been developed in recent years to pre-concentrate these analytes before instrumental analysis to enhance their detection and also to overcome the limitations of traditional extraction methods such as liquid-liquid extraction and solid phase extraction. These methods include quick, easy, cheap, effective, rugged and safe (QuEChERS), molecularly imprinted polymers, dispersive liquid-liquid microextraction and hollow fiber liquid-phase microextraction, and they will be discussed in this chapter.

Keywords: veterinary drug residues, food samples, modern extraction methods, pre-concentration, miniaturization

1. Introduction

Food is an indispensable part of human life and supplies the energy and nutrients needed for the development and growth of the neonate [1]. However, food safety is an important issue regarding residues of veterinary drugs in foods from food producing animals. Veterinary drugs are used to prevent and treat bacterial infections as well as improve feed efficiency and to promote animal growth worldwide [2]. The use of veterinary drugs in food producing animals may result in residues of the drugs or their metabolites being present in food samples, and this might be due to the inappropriate or excessive use of these drugs [3]. Various veterinary drugs have been reported to be retained in meat and milk of food producing animals [4–6] and this might be a health problem to humans who consume these food products.

Various pre-treatment methods have been described for the extraction of veterinary drug residues in food samples, such as liquid-liquid extraction (LLE) [7–9], solid-phase extraction (SPE) [10], solid-phase micro extraction [11–14]. Pre-concentration is necessary because veterinary drug residues often occur in trace amounts. However, some of these methods are laborious and time consuming, like LLE and SPE. It is very important to develop simple, rapid and efficient methods for the determination of veterinary drug residues in foods samples. In recent years, extraction and pre-concentration techniques that are compliant to the green chemistry methods have been developed and they will be discussed in Section 6. Moreover, several countries and different international organizations such as the World Health Organization (WHO), the Food Agriculture Organization (FAO) and the European Union (EU) have set maximum residue limits (MRLs) of veterinary drug residues in food to ensure food safety.

2. Physicochemical properties and uses of veterinary drugs

Physicochemical properties and uses of different veterinary drug classes are described below. A few examples of the physicochemical properties of selected veterinary drugs are shown on **Table 1**. Sulfonamides (SAs) show impartially low sorption capacity to solids compared to other veterinary drugs. These are used for the treatment of bacterial infections in animal husbandry and also act as growth promotants. Sulphonamides are also used in farm animal feeds and fish cultures [15]. Examples include sulfadiazine, sulfamethazine, sulfamethoxazole and sulfaquinoxaline.

Tetracyclines (TCs), including tetracycline, oxytetracycline, chlortetracycline and doxycycline are broad-spectrum veterinary drugs with broad use in animal husbandry. They are amphoteric compounds. Generally, TCs are more stable in acidic conditions.

Quinolones (QNs) are synthetic veterinary drugs with broad-spectrum antibacterial effects. This veterinary drug class consists of plain quinolones, such as oxolinic acid and nalidixic acid and fluorinated quinolones, known as fluoroquinolones (FQs), such as ciprofloxacin, flumequine and sarafloxacin.

Amphenicols are a broad-spectrum veterinary drug group that include chloramphenicol and its metabolites, thiamphenicol and florfenicol. Florfenicol has its own metabolite, florfenicol amine. The most common member of this veterinary drug class is chloramphenicol which is effective against many bacterial strains. Its toxicity and unwanted effects have restricted its use over the past years [16, 17].

Macrolides are a class of semi-synthetic medium-spectrum veterinary drugs. The most commonly used macrolides have 12–16 membered structures. Erythromycin is the most common veterinary drug in this class. Generally, macrolides have weak characteristics and thus are unstable under acidic conditions. Examples include erythromycin, tylosin, spiramycin, tilmicosin and tulathromycin.

Beta lactam veterinary drugs consist of several groups of compounds with cephalosporins and penicillins among the most important. Penicillins are commonly used for their microbial activity against both gram-positive and gram-negative organisms. The main clean-up method of penicillins for their analysis is pre- and post-column derivatization and the commonly used detection methods are LC-MS and LC-UV. Examples of penicillins are amoxicillin, ampicillin, oxacillin and cloxacillin, and examples of cephalosporins are cephapyin, ceftiofur and cefadroxil.

Aminoglycosides are broad spectrum veterinary drugs with antibacterial and antifungal activities produced by *Streptomyces* and *Micromospora*. The use of aminoglycosides has been clinically limited to severe infections because of its toxicity. More toxic veterinary drugs in this class have been restricted to topical or oral use for the treatment of infections caused by *Enterobacteriaceae*. Less toxic aminoglycosides are

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Class of veterinary antibiotic drugs	Name of antibiotic	Solubility in water (mg L^{-1})	pK_a
Tetracyclines	Tetracycline	231	3.3
	Chlortetracycline	~8600	3.3
	Doxycycline	50,000	—
	Oxytetracycline	1×10^6	3.27
Sulphonamides	Sulfadiazine	77	6.5
	Sulfamethoxazole	500	8.8
	Sulfaquinoxaline	7.5	—
	Sulfamethazine	1500	8.43
Macrolides	Erythromycin	2000	8.8
	Tylosin	5000	—
	Azithromycin	<1000	—
Quinolones	Ciprofloxacin	Insoluble	—
	Enrofloxacin	146	—
	Oxolinic acid	Insoluble	6.87

–, Not available.

Table 1.
Physicochemical properties of selected veterinary drugs from different classes.

used for treatment of severe sepsis caused by gram-negative aerobes. Examples of aminoglycosides are streptomycin, kanamycin, tobramycin and gentamicin.

Nitrofurans are synthetic chemotherapeutic agents used in the treatment and prevention of gastrointestinal infections caused by *Escherichia coli* and *Salmonella*. They are broadly used in cattle, cows, pigs and poultry and are administered orally or as feed additives. Examples of nitrofurans include are furazolidine, furaltadone, nitrofurantoin and nitrofurazone.

In summary of this section, generally, veterinary drugs are compounds characterized by a complex chemical structure that have very variable water solubilities, low volatilization potential, several ionizable functional groups (amphoteric molecules) and different pKa values hence they have a low bioaccumulation potential [18]. Veterinary drugs may have different functionalities within the same molecule, making them either neutral, cationic, anionic, or zwitterionic under different pH conditions. Different functionalities within a single molecule may result in its physicochemical and biological characteristics such as, sorption behavior, photo reactivity and toxicity changing with pH. Solubility and hydrophobicity are also are pH dependent. The pH dependency of antibiotic solubility can affect the extraction and quantification by analytical techniques [19].

3. Contamination of food by veterinary drugs

The use of veterinary drugs in food producing animals can result in the presence of residues in animal derived products such meat, milk, eggs and honey. This poses a health hazard to the consumers [3]. Veterinary drugs such as macrolides, tetracyclines, sulfonamides and penicillins are also used as antibiotics in humans [20, 21]. Physicochemical properties of drugs, pharmacokinetic characteristics or biological processes of animals are factors that affect the presence of drug residues in food of animal origin. Improper drug usage and failure to observe withdrawal periods may be a reason for the occurrence of veterinary drug residues in foods derived from animals.

4. Health effects

The threat of food contamination by veterinary drug residues is one of the major challenges to public health worldwide [3]. The presence of low levels of veterinary drug residues may not have a negative impact on public health. However, the substantial use of drugs may increase the risk of adverse effects of these residues to humans [3, 22, 23]. Continuous ingestion of veterinary drug residues can promote the development of drug resistance bacterial strains in an individual, resulting in resistance to treatment with the same antibiotics when need arises [24–26]. Veterinary drug traces also have harmful effects on humans, such as allergic reactions, liver damage, yellowing of teeth and gastrointestinal disturbance [27]. Sulphonamides can cause drug intoxication and hypersensitivity. Signs of hypersensitivity and intoxication are fever and anemia respectively.

Manuring, treatment of animals and disposal of carcasses, offals, urine, feces and unused products can contaminate the environment with veterinary drugs [28]. An excessive use of antibiotics in commercially reared animals does not only affect humans, it can also affect the food chain leading to ecological imbalances. For example, a deficient management of the livestock carcasses can lead to antibiotic resistance in the scavengers that ingest them, like vultures [24–26]. The disposal of medicated animals should be regulated to minimize the risk in scavenger birds.

5. Maximum residual limits

The MRL values for food products result from calculations based upon the acceptable daily intake. MRL values depend on chronic toxicity of the antibiotic in question. More toxic drugs have lower MRL values compared to moderately toxic drugs. Prohibited substances are pharmacologically active substances for which an MRL cannot be established because of their toxicity and these include substances such as chloramphenicol, nitrofurans and nitroimidazoles. The kidney is the most important organ of drug excretion and that might be the reason why for most drugs it is allocated a higher MRL. For example, in the European Union (EU), countries have established a MRL of 200, 100, and 300 μg kg^{-1} for liver, muscle and kidney tissues, respectively for enrofloxacin and ciprofloxacin. The MRL set by the EU Committee for veterinary medicinal products is 200 μg kg^{-1} in muscles, liver and kidneys of animal origin, 40 μg kg^{-1} in milk, and 150 μg kg^{-1} in eggs for the macrolide drugs. **Table 2** shows some MRL values for different foods of animal origin.

Class of veterinary drugs	Target veterinary drug	Matrix	MRL (μg kg^{-1})
Sulphonamides	Sulphonamides	Milk, fish and other seafood	100
	Sulphonamides	Eggs	Not allowed
Quinolones	Danofloxacin, enrofloxacin-ciprofloxacin and oxolinic acid	Muscle	100
	Enrofloxacin and ciprofloxacin	Eggs	Not allowed
	Enrofloxacin and ciprofloxacin	Liver	200
	Enrofloxacin and ciprofloxacin	Kidney	300
Macrolides	Macrolides	Muscle, liver and kidneys	200

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Class of veterinary drugs	Target veterinary drug	Matrix	MRL ($\mu g\ kg^{-1}$)
	Macrolides	Milk	40
	Macrolides	Eggs	150
Tetracyclines (single/total)	Tetracycline, oxytetracycline, chlortetracycline and doxycycline	Muscle, milk	100
Tetracyclines (single/total)	Tetracycline, oxytetracycline, chlortetracycline and doxycycline	Eggs	200

Table 2.
Maximum residue limit for veterinary drug residues in food samples according to European Community, Commission Regulation (EU) No. 37/2010.

6. Pre-concentration techniques

Veterinary drug residues in food of animal origin are of great concern to regulatory agencies and consumers, hence reliable extraction methods for rapid, selective and sensitive detection of these residues are necessary to ensure food safety [29]. There are various extraction methods that have been used in veterinary drug residues analysis in food samples, such as liquid-liquid extraction (LLE) [30–32] and solid phase extraction (SPE) [33, 34]. These methods suffer a number of drawbacks even though they perform their tasks adequately. Both LLE and SPE are environmentally unfriendly due to the large amounts of organic solvents they use, they are time consuming and labor intensive. Another disadvantage of SPE is that cartridges are costly.

Promising extraction and pre-concentration techniques for veterinary drug residues that have been explored recently by many researchers include dispersive liquid-liquid microextraction (DLLME) [5, 6, 35, 36], hollow fiber based liquid-phase microextraction (HFLPME) [37–40] and quick, easy, cheap, effective, rugged and safe (QuEChERS) [4, 41–43] where the general trend is compliance with green chemistry principles. Veterinary drug residues occur at trace levels as low nanogram per gram [4, 37] hence the need to pre-concentrate. The application of QuEChERS, DLLME, HFLPME and molecularly imprinted polymers (MIPs) for the extraction and pre-concentration of veterinary drug residues in food samples will be discussed below and summarized in **Table 3**.

The food industry also needs the development of new methods that are fast, easy and cheap for routine analysis of residues in food samples. The latest trend in drug residue analysis is the development of generic methods that are capable of monitoring a wide variety of compounds, belonging to different veterinary drug classes. This has proven to be challenge due to the varying chemistries and physicochemical properties of veterinary drugs from different classes, as a result, multi-class methods for veterinary drugs are still not so widespread although they are strongly required.

6.1 QuEChERS

The quick easy cheap effective rugged safe (QuEChERS) method is an extraction technique that employs an organic solvent and phase separation using high salt content, in some cases followed by dispersive solid phase extraction (d-SPE) for sample clean up. The QuEChERS method, which was originally developed for pesticide analysis in fruits and vegetables [44, 45], has recently been proposed for the analysis of veterinary drugs in different food matrices [4, 41, 43, 46]. Recent applications of this method are discussed below.

Target antibiotic	Food matrix	Analytical technique	Extraction technique	Concentration of antibiotic detected	LOD	LOQ	Recovery (%)	References
Seven macrolides	Milk	LC-MS/MS	QuEChERS based on acetonitrile extraction + a mixture of salts (sodium sulfate, sodium chloride and potassium carbonate)	—	0.84 μg kg^{-1}	2.79 μg kg^{-1}	89–97	[41]
Six multi-residues	Bovine milk	LC-MS/MS	QuEChERS based on acetonitrile followed by a cleanup with d-SPE based C_{18}, PSA and sodium acetate	—	—	—	84.18–115.99	[42]
Sixteen multi-residues	Preserved eggs	UHPLC-MS/MS	QuEChERS based on water, acetonitrile with 1% acetic acid followed by a cleanup using d-SPE with C_{18} and PSA as sorbents	—	0.1–0.9 μg kg^{-1}	0.3–3.0 μg kg^{-1}	73.8–127.4	[43]
Three SAs	Chicken breast	HPLC-DAD	QuEChERS based on acetonitrile and water with 1% CH_3CO_2H followed by a cleanup using d-SPE Oasis HLB as a sorbent.	—	10 and 13 μg kg^{-1}	25–30 μg kg^{-1}	75.4–98.7	[46]
Seven TCs	Beef	LC-MS/MS	DLLME, methanol was a disperser solvent and dichloromethane was an extracting solvent	38.4 and 82.3 μg kg^{-1}	2.2–3.6 μg kg^{-1}	7.4–11.5 μg kg^{-1}	80–105	[5]
Several SAs	Milk	HPLC-FD	Traditional DLLME (extraction solvent (1 mL chloroform) and dispersive solvent (1.9 mL acetonitrile)	—	0.73–1.21 μg L^{-1}	—	92.9–104.7	[36]
Six FQs	Milk	HPLC-UV	DLLME was coupled to QuEChERS	—	—	2.5–15 μg kg^{-1}	74.1–101.4	[35]
Four TCs	Milk and eggs	HPLC-UV	IL-DLLME ($[C_6MIM][PF_6]$ as an extraction solvent, FIL-NOSM a disperser solvent)	—	0.08–1.12 μg kg^{-1}	—	94.1–102.1	[6]

Target antibiotic	Food matrix	Analytical technique	Extraction technique	Concentration of antibiotic detected	LOD	LOQ	Recovery (%)	References
Florfenicol and Chloramphenicol	Pasteurized Milk	HPLC-UV	Traditional DLLME (chloroform as an extracting solvent and water as a dispenser solvent)	62.4 μg kg^{-1} florfenicol	12.2 and 12.5 μg kg^{-1}	36.6 and 37.5 μg kg^{-1}	—	[53]
Five QNs and four TCs	Milk, honey, fish, liver and muscles of lamb	HPLC-DAD	HFLPME (0.1 mol L^{-1} nitric acid and sodium chloride was the acceptor phase, 10% w/v Aliquat-336 in 1-octanol	24. 8 ng g^{-1} danofloxacin 37.5 ng g^{-1} tetracycline	0.5–20 ng g^{-1}	1.25–40 ng g^{-1}	—	[37]
Four TCs	Milk	HPLC-UV	HF-DLLME (chloroform as an extracting solvent and water as a dispenser solvent)	—	0.95–3.6 μg L^{-1}	5–15 μg L^{-1}	92.38–107.3	[38]
Three TCs	Bovine milk	HPLC-UV	Carrier mediated three phase HFLPME (0.1 M phosphoric acid, 1.0 M sodium chloride with pH = 1.6 as an acceptor phase, 0.05 M disodium hydrogen phosphate (pH between 9.1 and 9.5) as donor phase and 10% (w/v) of Aliquat-336 in octanol as an SLM.	6.0–27.4 μg L^{-1}	0.5–1.0 μg L^{-1}	0.5–1.0 μg L^{-1}	-	[39]
Tylosin	Milk	UV/Vis	HFLPME-TiO_2 (TiO_2 was dispersed in 1-octanol)	—	0.21 μg L^{-1}	—	89–99	[40]
Eight FQs, eight SAs and four TCs	Pork	UPLC-PDA	Mixed template MIP-MSPD (0.15 g MMIP, methanol/water (2:8, v/v) as a washing solvent, methanol/acetic acid (9:1, v/v) as an eluting solvent)	—	0.5–3.0 ng g^{-1}	1.5–6.0 ng g^{-1}	92–99	[57]

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Target antibiotic	Food matrix	Analytical technique	Extraction technique	Concentration of antibiotic detected	LOD	LOQ	Recovery (%)	References
Four TCs	Pork, milk and eggs	HPLC-PDA	MIP-SPE (30 mg MIP particles, 0.01 mol L^{-1} trifluoroacetic acid, pH 3.0 as the loading solvent, methanol/acetic acid (9:1, v/v) as the elution solvent)	52 ng mL^{-1}: TC 87 ng mL^{-1}: oxytetracycline in milk only	20–40 ng mL^{-1}	50–80 ng mL^{-1}	74–93	[58]
Ten macrolides	Swine, cattle and chicken muscles	LC-MS/MS	MIP-SPE (20 mg MIP particles, 10% methanol in water as the washing solvent, 5% ammonia in methanol as the elution solvent)	—	0.1–0.4 μg kg^{-1}	0.3–1.0 μg kg^{-1}	60.7–100.3	[56]
Ten FQs	Fish	HPLC-FLD	DMIP-MSPD (50 mg MIP particles, 20% methanol in water as the washing solvent, 1% trifluoroacetic acid in acetonitrile as the elution solvent)	—	0.06–0.22 ng g^{-1}	—	64.4–102.7	[59]
Three FQs	Milk	HPLC-UV	Mini-MISPE (40 mg MIP particles, water as the washing solvent, methanol-acetic acid (19:1, v/v) as the elution solvent)	Ciprofloxacin: 0.21 and 0.25 g mL^{-1}	1.5–2.3 ng mL^{-1}	5.0–7.5 ng mL^{-1}	87.2–106.1	[60]
TCs	Milk	Fluorescent sensing	CDs@MIPs (40 mg MIP particles, 1% (v/v) trichloroacetic acid solution was a solvent)	ND	5.48 nM	—	97.3–105.3	[61]

–, Not mentioned; ND, not detected.

Table 3.
Modern analytical techniques in the analysis of antibiotic residues in food samples.

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da Costa et al. [41] developed a modified QuEChERS extraction technique using acetonitrile, followed by the addition of a mixture of salts (sodium sulfate, sodium chloride and potassium carbonate) for the extraction of seven macrolide drugs in milk followed by analysis on liquid chromatography and tandem mass spectrometry (LC-MS/MS). Sodium sulfate and sodium chloride removed water from samples promoting the salting out effect while acetonitrile was used for deproteination. Potassium carbonate salt was included to elevate the extraction pH to around 9.5 promoting an increase in the recovery, since macrolides have a pKa between 6.6 and 9.2. The limit of detection (LOD) and limit of quantification (LOQ) were 0.84 and 2.79 μg kg^{-1} respectively and recoveries were ranging between 89 and 97%. No further clean-up step such as an additional d-SPE step was required, hence reducing time, cost and labor.

In another study by Wang et al. [42], a modified QuEChERS extraction technique based on octadecylsilane (C_{18}), primary secondary amine (PSA) and sodium acetate for six multi-residue veterinary drugs in bovine milk followed by analysis using LC-MS/MS. The QuEChERS method was optimized for use in the determination of multi-class veterinary drug residues in fatty foods (milk) using response surface methodology. The amounts of C_{18}, PSA, and sodium acetate used in this study were determined by the response surface methodology variables. PSA, C_{18} and sodium acetate have a dissolving effect on milk-fat globules and hence, resulting in higher recoveries (84.18–115.99%) compared to da Costa et al. [41]. Organic solvents, such as acetonitrile, methanol and ethanol, are commonly employed in the precipitation of proteins in biological matrices. For all residues, the LOQs were low enough to quantify the analytes below their MRLs.

Li et al. [43] employed the QuEChERS method followed by d-SPE coupled to ultrahigh-performance liquid chromatography tandem mass spectrometry for the multi-residue analysis of 16 veterinary drugs belonging to three classes (macrolides, quinolones, and sulfonamides) in preserved eggs. Graphitized carbon black was used as a comparative d-SPE sorbent. The recoveries of all veterinary drugs decreased with the addition of graphitized carbon black, while purification with a conjugation of PSA and C_{18} in the presence of magnesium sulfate resulted in better results. The results demonstrated good linearity, accuracy, precision, LOD (0.1–0.9 μg kg^{-1}) and LOQ (0.3–3.0 μg kg^{-1}) which indicated that the proposed method was highly sensitive and could efficiently determine trace amounts.

Machado et al. [46] developed a QuEChERS method followed by analysis on the high performance liquid chromatography (HPLC) with a diode array detector (DAD) for the simultaneous determination of sulfadiazine, sulfamethoxazole and sulfamethoxypyridazine in chicken breast samples. The LODs ranged between 10 and 13 μg kg^{-1} and the LOQs ranged between 25 and 30 μg kg^{-1} while recoveries ranged between 75.4 and 98.7%. SPE was done for comparison and recoveries lower than 70% were obtained. However, SPE, proved to reduce the matrix effect compared to the QuEChERS method.

6.2 Liquid phase microextraction

Traditional sample preparation techniques such as liquid-liquid extraction (LLE) have drawbacks in spite of the substantial use of this method over the years. The LLE method is tedious, time consuming and uses large amounts of toxic organic solvents which are non-compliant to the green analytical chemistry (GAC) principles. In order to overcome these drawbacks, new extraction techniques that are simple, rapid and inexpensive, miniaturized and have the ability of automation have been developed in recent years [47]. The efforts of various researchers in this area have resulted in the development of a new extraction technique known as

liquid-phase microextraction (LPME). LPME offers an alternative to SPME [48]. LPME can be divided into three main modes which are single-drop microextraction (SD-LPME), hollow fiber liquid phase microextraction (HFLPME) and dispersive liquid-liquid microextraction (DLLME). Among these modes of LPME, HFLPME and DLLME have been the most used because of the advantages that they offer [47]. SD-LPME is the least used mode because excessive stirring tends to break up the droplet, extraction is time consuming and reaching equilibrium can be a challenge. This disadvantage overrides the advantage that this method has, which is the enormous reduction of volumes of organic solvent it uses [49].

These methods are cheap and do not have sample carryover problems that are associated with SPME [48]. LPME offers advantages such as high recovery and high enrichment factors, simplicity of operation, rapidity and they are also environment friendly [50]. Below is a summary of some studies that have used DLLME and HFLPME for the extraction of veterinary drug residues from food samples.

6.2.1 Dispersive liquid-liquid microextraction

Rezaee and co-workers [51] introduced DLLME as a new LLE technique for the determination of polyaromatic hydrocarbons and pesticides. The application of DLLME in the extraction of veterinary drugs in literature has increased over the years [5, 6, 35, 36]. This technique is based on a ternary component solvent system including an extraction solvent, disperser solvent and an aqueous sample and is known as traditional DLLME. The advantages of traditional DLLME are the microliter-level volumes required for extraction and dispersive solvents and short extraction times. However, the disadvantage of traditional DLLME is the use of organic solvents as the extraction and dispersive solvents.

Modified modes of DLLME have been invented recently and they include, low-density solvent based DLLME (LDS-DLLME), solidified floating organic drop DLLME (SFO-DLLME), effervescence assisted DLLME, air assisted dispersive liquid-liquid microextraction (AA-DLLME), surfactant assisted DLLME (SA-DLLME) and cloud point DLLME (CP-DLLME), ionic liquid DLLME (IL-DLLME) [6] to address the disadvantages associated with traditional DLLME. Despite these disadvantages, DLLME is more advantageous in terms of short total time, low cost and feasibility compared with other liquid-phase microextraction techniques [52]. Below is research that has been done recently on veterinary drugs in food samples using DLLME.

Mookantsa et al. [5] employed traditional DLLME for the extraction of seven tetracyclines from beef where methanol was a disperser solvent and dichloromethane was an extracting solvent followed by LC-MS/MS. Recoveries of spiked blank muscle samples at three levels (50, 100 and 150 μg kg^{-1}) ranged from 80–105%. LODs and LOQs ranged from 2.2 to 3.6 μg kg^{-1} and from 7.4 to 11.5 μg kg^{-1} respectively. Concentrations of chlortetracycline and oxytetracycline were detected in bovine muscle samples to be between 38.4 and 82.3 μg kg^{-1} which is lower than the stipulated European Union MRL of 100 μg kg^{-1}. DLLME was compared to a South African National Accreditation System accredited d-SPE method and the t-test showed that the results obtained by the methods had no significant difference. However, DLLME was simple, fast, inexpensive and uses very low volumes of organic solvents hence more greener compared to d-SPE.

In a study done by Karami-Osboo et al. [35], DLLME was coupled to QuEChERS for the determination of six fluoroquinolones using HPLC with ultra-violet (UV) detection. The dried supernatant from the QuEChERS method was resuspended in 1.0 mL of a 10% acetic acid-acetonitrile mixture, combined with 200 μL of chloroform and rapidly injected into 4 mL of deionized water. The cloudy solution

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was centrifuged for 5 minutes at 4500 rpm. By coupling QuEChERS to DLLME, the authors removed matrix interference, which is a common problem with the detection of fluoroquinolones. The method showed good recoveries (74.1–101.4% for all analytes) and low LOQs (below 2.5 μg kg^{-1} for danofloxacin and below 15 μg kg^{-1} for all other FQs).

Arroyo-Manzanares et al. [36] used traditional DLLME for the determination of several sulfonamides in milk. The analytes were detected by HPLC with fluorescence detection. The authors also compared their optimized DLLME procedure to QuEChERS. Proteins were precipitated using trichloroacetic acid and then filtered. The DLLME extraction procedure was optimized using a central composite design. The optimum volumes for chloroform as an extraction solvent and acetonitrile as a dispersive solvent were 1 and 1.9 mL, respectively. DLLME resulted in lower LODs (0.73–1.21 μg L^{-1}) than QuEChERS (1.15–2.73 μg L^{-1}) and higher recoveries (92.9–104.7% compared to 83.6–97.1%, when samples were spiked with sulfonamides at 150 μg L^{-1}. However, QuEChERS proved to be more reproducible than DLLME with lower relative standard deviation values of 2.9–7.1 and 3.0–9.7%, respectively.

In another study by Karami-Osboo et al. [53], traditional DLLME coupled to HPLC- UV was used for the determination of chloramphenicol and florfenicol residues in milk samples where chloroform was used as extraction solvent and the deproteinized milk as a disperser solvent. The blank milk samples were spiked at three levels, 150, 300 and 600 μg of each chloramphenicol and florfenicol per kg of milk and recoveries were between 69.1 and 79.4%. The LODs for chloramphenicol and florfenicol were 12.5 and 12.2 μg kg^{-1} respectively whereas the LOQs were 37.5 and 36.6 μg kg^{-1} respectively. Despite the use of florfenicol not being permitted for milk producing animals from which milk is produced for human consumption, it was detected in one of the samples at a concentration of 62.4 μg kg^{-1}.

Ionic liquids (ILs), consisting of organic cations and inorganic or organic anions with melting points at or below 100°C, have been widely applied as green solvents to improve extraction and enrichment performance as compared to the traditional use of organic solvents. A significant advantage of this method is that the metathesis reaction and extraction are accomplished in one step making it rapid and suitable for high-throughput analysis. Gao et al. [6] used functionalized ionic liquid-based non-organic solvent microextraction (FIL-NOSM) based on 1-butyl-3-methylimidazolium naphthoic acid salt ($[C_4MIM][NPA]$) with strong acidity for the determination of TCs in milk and eggs. The use of $[C_4MIM][NPA]$ in the FIL-NOSM method eliminated the pH adjustment step because of its strong acidity which saves as a pH regulator. This proposed method provided high extraction efficiency, less pretreatment time and requires non-organic solvents for determination of trace TC concentrations in complex animal-based food matrices. Moreover, no organic solvent was utilized in this IL-based DLLME procedure making this method more environmentally friendly. The LODs were between 0.08 and 1.12 μg kg^{-1} in milk and egg samples. The recoveries ranged from 94.1 to 102.1%.

6.2.2 Hollow fiber liquid phase microextraction

Hollow fiber liquid phase microextraction is a mode of LPME that uses a porous polypropylene hollow fiber for immobilization of organic solvent in its pores. The development of HFLPME provides a way to stabilize the extraction droplet in SD-LPME by placing it in a hollow fiber [54]. The main consumable material is the hollow fiber membrane, which is lower than other methods in cost and sample consumption [38]. The different modes of HFLPME are static, dynamic, two and three phase. The advantages of HFLPME are high enrichment, high degree of sample clean-up and low solvent consumption. The disadvantage

of HFLPME procedure is that it is slow with extraction times ranging from 15 to 45 minutes and target analytes may partly be trapped in the supporting liquid membrane (SLM) [39]. Another disadvantage is that there is no complete setup commercially available for this method although hollow fibers are commercially available [55]. Below are some recent studies on veterinary drug residues that have been carried out using HFLPME.

Tajabadi et al. [37] used a carrier mediated three phase HLFPME prior to analysis on the HPLC-DAD for the simultaneous determination of the veterinary drug residues of four TCs and five QNs in a wide range of animal source food samples such as fish, milk and honey as well as the liver and muscles of lamb and chicken. Multivariate curve resolution-alternative least squares was used for resolving some overlapped peaks in multivariate data of HPLC-DAD and made possible the simultaneous analysis of nine TCs and QNs in minimum time. LODs and LOQs for the different veterinary drugs ranged between 0.5–20 and 1.25–40 ng g^{-1}. Danofloxacin was detected at a concentration of 24.8 ng g^{-1} in chicken liver, tetracycline was detected at 37.5 ng g^{-1} in lamb liver which are less than the stipulated MRLs according to EU 37/2010 and the rest of the veterinary drugs were not detected.

Xu et al. [38] employed a carrier mediated three phase hollow fiber membrane based dynamic liquid-liquid microextraction coupled with HPLC-UV detection for the residue analysis of TCs in milk samples without deproteinization and defatting, but the milk samples were diluted five folds. A peristaltic pump was used to promote mass transfer between the carrier and the operated solution. The standard addition method was used to eliminate the matrix effect. Octanol containing 20% (w/w) Aliquat-336 was used as a SLM, 0.05 M disodium hydrogen phosphate, pH 9.0 containing the sample was a donor phase and solutions of 1.0 mol L^{-1} sodium chloride and phosphoric acid (pH = 1.0) were used as the acceptor solvent. The LOD and LOQ were in the range of 0.95–3.6 and 5–15 μg L^{-1} respectively. The recoveries in spiked samples ranged from 92.38 to 107.3%.

A similar study was carried out by Shariati et al. [39] where tetracycline, oxytetracycline and doxycycline were extracted from bovine milk, human plasma and water samples using a carrier mediated three phase HFLPME prior analysis on the HPLC-UV. The acceptor solvent was 0.1 M phosphoric acid, 1.0 M sodium chloride with pH = 1.6, 0.05 M disodium hydrogen phosphate (pH between 9.1 and 9.5) containing the sample as the donor phase and 10% (w/v) of Aliquat-336 in octanol as a SLM. The LOD and LOQ were 0.5–1.0 and 0.5–1.0 μg L^{-1} respectively which are lower compared to the ones obtained by Xu et al. [38] proving that fiber membrane-based dynamic liquid-liquid microextraction is a more efficient extraction method. All the milk samples contained TCs in the range of 6.0–27.4 μg L^{-1} that was below the MRL as set by the EU.

From the two studies that are above it can be concluded that passive transport of TCs in the absence of the carrier is difficult because of existence of TCs as zwitterionic forms (at the studied conditions) in solution and hence they have a very small tendency to pass through the impregnated organic solvent. A unique advantage of the carrier mediator Aliquat-336 is that it stays in a cationic form in all pH ranges.

Sehati et al. [40] coupled HFLPME to nanomaterials, where TiO_2 nanomaterials were dispersed in 1-octanol and used it to fill the lumen of a HF. Then, they sealed the two ends of the HF with orthodontic stainless steel wires. The LPME took place by putting the HF into the milk samples for the extraction of tylosin. This method allowed obtaining recovery percentages in the range 89–99% and despite using an ultraviolet- visible spectrophotometer for the determination of tylosin, an LOD of 0.21 mg L^{-1} was achieved which proves the efficiency of the extraction method that was used.

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6.3 Molecularly imprinted polymers

Molecularly imprinted polymers (MIPs) are synthesized using a template, functional monomer, cross-linker and an initiator. MIPs are selective towards the target molecules, allowing them to be eluted from the SPE cartridge almost free of co-extracted compounds compared to classical sorbents used for clean-up procedures [56]. SPE sorbents such as C_{18}, hydrophilic lipophilic balanced (HLB) material, diatomite, N-propylethylenediamine, alumina and Florisil are susceptible to interferences by impurities in biological samples and the cartridges can only be used once [57]. Therefore, it is important to develop simple, rapid and environmentally friendly methods. MIPs overcome the above-mentioned drawbacks of traditional SPE sorbents. MIPs are stable under different harsh conditions (extreme pH, high pressures and high temperatures) and can be reused several times [58]. Below are a few studies where MIPs were applied in the solid phase extraction of veterinary drug residues in food samples.

In a study conducted by Song et al. [56], a MIP-SPE method combining LC-MS/MS was developed to determine the residues of macrolide drugs in animal derived foods. Tylosin was used as a virtual template and the synthesized MIPs were used as the selective sorbent for packing SPE cartridge. A system of sodium borate buffer solution (pH = 10.0) and ethyl acetate was selected for the extraction of the residues of macrolides from muscle samples. Mean recoveries of 10 target analytes were in the range of 60.7–100.3%. Compared with the conventional SPE cartridges (approximately 60–90%), the MISPE cartridge was highly selective and obtained higher recoveries for the 10 macrolides drugs. The LOD and LOQ values ranged between 0.1–0.4 and 0.3–1.0 μg kg^{-1} respectively. The results indicated that the sensitivity of the proposed method for the determination of 10 macrolide drugs residues in animal muscle samples was acceptable.

Wang et al. [57] used a mixed-template molecularly imprinted polymer (MMIP) coupled with matrix solid phase dispersion (MSPD) to recognize eight FQs, eight SAs and four TCs from pork samples following analysis with ultraperformance liquid chromatography with a photo diode array detector. The LOD and LOQ were 0.5–3.0 and 1.5–6.0 ng g^{-1} respectively. The recoveries ranged between 92 and 99%. MMIPs were compared to C_{18} and diatomaceous earth dispersing sorbents. The obtained chromatograms showed that the two sorbents were able to achieve the satisfactory purification effects, but the recoveries of the 20 drugs from the two sorbents (70–95%) were lower than that from MMIP.

In another study by Feng et al. [58], a MIP-SPE method combining HPLC was developed to determine the residues of TC drugs in animal derived foods. A template for MIP synthesis was selected among doxycycline, oxytetracycline and chlortetracycline for enhanced enrichment factors. Results showed that one milk sample contained TC residue (52 ng mL^{-1}) and another milk sample contained oxytetracycline residue (87 ng mL^{-1}), but the residue levels were lower than their MRLs in milk (100 ng mL^{-1}). Results of other samples were negative. In order to compare the purification effect of MIP-SPE with conventional SPE, the extracts of TCs fortified blank milk (100 ng mL^{-1}) were purified with three commercial SPE cartridges containing different sorbents (strong cation exchange phase, HLB and C_{18}) and there were different interfering peaks around TCs peaks in the chromatograms, revealing inferior purification performances of these sorbents. MIP-SPE proved to be specific, sensitive and accurate for the extraction of TCs residues.

Dummy molecularly imprinted polymers (DMIPs) based on the matrix solid phase dispersion method for the extraction of FQs from fish prior to analysis on the HPLC with fluorescence detection were used by Sun et al. [59]. The use of

DMIPs was to prevent any possible template leakage which could still happen even after thorough washing steps. Template leakage could have a serious impact on the accuracy of the analytical method or made it not suitable for simultaneous analysis of the whole class of FQs. This problem has become one of the major area of concern in sample pre-treatment methods of MIPs. Good recoveries, low LODs and excellent accuracy demonstrated the suitability of the DMIP sorbent for pre-treatment of FQs in fish samples. The use of DMIP resulted in less matrix interferences compared to directly extracted samples and no co-eluted peaks were observed in the chromatogram. The LOD was 0.06–0.22 ng g^{-1} and recoveries ranged between 64.4 and 102.7%.

Wang et al. [60] used an inorganic-organic co-functional monomer, methacrylic acid-vinyltriethoxysilane (MAA-VTES) for the synthesis of molecularly imprinted microspheres (MIMs). The obtained MAA-VTES based MIMs exhibited good recognition and selectivity to FQs and were successfully applied as selective sorbents of a miniaturized home-made solid phase extraction device for the determination of ofloxacin, lomefloxacin and ciprofloxacin in milk samples. The LODs and the LOQs of FQs were 1.5–2.3 and 5.0–7.5 ng mL^{-1}, respectively. The average recoveries for the analyte were in the range of 87.2–106.1%. Ciprofloxacin was detected in two samples as 0.21 and 0.25 ng mL^{-1} which were below the MRL established by EU (100 g kg^{-1}). Due to the efficiency of the developed co-functional monomer based mini-MISPE-HPLC method, it was possible to analyze the target compounds in milk samples at ng mL^{-1} level.

A selective and eco-friendly sensor for the detection of tetracycline by grafting imprinted polymers onto the surface of carbon quantum dots was used by Hou et al. [61]. A simple microwave-assisted approach was utilized to fabricate the fluorescent imprinted composites rapidly for the first time, which could shorten the polymerization time which normally takes 8–24 hours and simplify the experimental procedure. In this study polymerization took about 1 hour. The development of fluorescent molecularly imprinted composites might be a promising method for rapid analysis in complex samples in future. TCs were not detected in milk samples. Recoveries ranged from 97.3 to 105.3%.

7. Challenges and future trends

In high-fat foods like milk and meat, veterinary drug residues may bind to lipoproteins and extraction solvents forming emulsions and foam, especially polar veterinary drugs which may decrease recoveries and hence, affecting separation and analysis [56, 62]. Extracting analytes from biological samples using modern extraction techniques like DLLME has some challenges. In traditional DLLME, prior to a DLLME procedure on a complex matrix such as milk, lipids and proteins must be eliminated since they can act like surfactants and disrupt the interfacial tension at the droplet surface, constraining phase separation [63]. During the sample pre-treatment step, salts are added for analyte partitioning, phase separation, buffering and for reducing the amounts of co-extracted matrix that could hinder the transfer of analytes from the aqueous phase to the organic phase [5].

TCs are challenging drugs for analytical analysis because they are hydrophilic compounds with high solubility in aqueous media. They have both acidic and basic functionalities, and therefore exist in various forms at different pH conditions [39]. Moreover, they can form complexes with divalent metal ions and silanolic groups on the HPLC column which may result in severe peak tailing [64]. Reverse phase-HPLC with mobile phases containing acids such as phosphoric, acetic and tartaric acids can be used to reduce peak tailing or an RP-amide

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column can be used. The ability of the RP-amide column to separate TCs might be explained by the hydrogen bonding between the amide functionality of the column and the hydroxyl functionality of TCs. Another challenge is that TCs are prone to photo-degradation.

Overlapping peaks during multi-residual analysis when using HPLC-DAD is a challenge. Multivariate curve-resolution coupled to alternating least squares to calculate the exact peak area of overlapping compounds was used by Tajabadi et al. [37], hence more sensitive analytical instruments such as the LC-MS/MS are required for multi-residual analysis. Moreover, the solubilization procedure of veterinary drug residues is a rate-limiting step in multi-residual analysis.

The matrix effect still remains an issue when extracting veterinary drug residues using the QuEChERS method from complex samples such as meat, and hence reducing the sensitivity of chromatographic instruments [46].

8. Future trends

The world is moving towards the use of greener solvents and hence promoting the principles of GAC, therefore, it can be envisioned that most extraction methods still making use of organic solvents may be completely eliminated in future. Currently greener solvents such ionic liquids are widely used in microextraction procedures as dispersive or extraction solvents according to their different solubilities in DLLME.

Electrochemical sensors and their relative detection strategies, with the advantages of high sensitivity, simplicity and rapid response, have attracted considerable attention in recent years. Among them, aptasensors are considered as one of the most promising research directions owing to the employment of an aptamer. Aptamers, with the advantage of high affinity and specificity to targets, low price and easy to be synthetic in vitro, have provided a broad prospect for developing electrochemical sensing system.

9. Conclusion

Expanding agriculture, aquaculture and apiculture practices have resulted in increased levels of infections among species. Various classes of veterinary drugs including QNs, TCs, β-lactams, SAs and others exhibit activity against both gram-positive and gram-negative bacteria, hence they are widely used to treat or prevent diseases. However, extended use of these veterinary drugs has led to food safety issues worldwide and hence a need for developing sensitive methods for their determination. The focus of this chapter has been to present the trends in modern extraction and clean-up techniques of veterinary drug residues from food samples of animal origin, with milk being the most studied matrix because of its importance on the diet of humans and one of the most consumed foods in the world. Even though some of these veterinary drugs such as chloramphenicols have been banned in some countries due to their dangerous side effects on humans they are still detected in food samples because farmers are not adhering to EU regulations. Generally, in most studies these veterinary drug residues are below stipulated MRLs. Although most extraction methods that are emerging are promising, multi-residual analysis is still a challenge.

Author details

Babra Moyo and Nikita Tawanda Tavengwa*
Department of Chemistry, School of Mathematical and Natural Sciences,
University of Venda, Thohoyandou, South Africa

*Address all correspondence to: nikita.tavengwa@univen.ac.za

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2.B.2. Paper II

This paper “Application of sorptive micro-extraction techniques for the pre-concentration of antibiotic drug residues from food samples” was published in the *Journal of Food Additives and Contaminants A.* The basic principles and recent applications of sorptive micro-extraction techniques including SPME, SBSE, SCSE and FPSE in the clean-up and pre-concentration of antibiotic drug residues from various food samples were extensively discussed.

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Application of sorptive micro-extraction techniques for the pre-concentration of antibiotic drug residues from food samples – a review

Babra Moyo[a], Mugera Gitari[b], and Nikita T. Tavengwa[a]

[a]Department of Chemistry, School of Mathematical and Natural Sciences, University of Venda, Thohoyandou, South Africa; [b]Department of Ecology and Resource Management, School of Environmental Sciences, University of Venda, Thohoyandou, South Africa

ABSTRACT

Antibiotic residues have become a major concern worldwide as food contaminants due to the risk that they may pose to human health. The presence of these residues in food is due to improper veterinary practices. Consequently, rapid and cost-effective clean-up methods prior to analysis for these residues in food matrices are increasingly becoming necessary in order to ensure food safety. Miniaturised extraction and pre-concentration techniques have been developed as alternatives to conventional extraction procedures in recent years. Furthermore, the current trends in analytical sample preparation favour extraction techniques that comply with the principles of green analytical chemistry. Solid phase micro-extraction, stir bar sorptive extraction, stir cake sorptive extraction and fabric phase sorptive extraction methods are very promising sorbent-based sorptive micro-extraction techniques, and they are compliant to the principles of green chemistry. This review critically discusses the application of these techniques in the extraction and pre-concentration of antibiotic residues from food samples in the years 2015 to 2020.

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Sorptive micro-extraction; antibiotic drugs; food contamination; pre-concentration; sample clean-up

Introduction

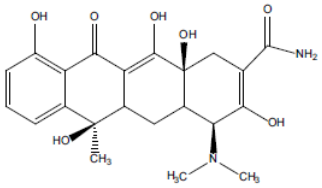
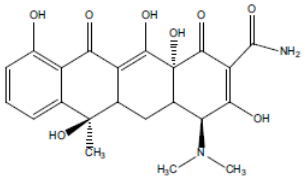
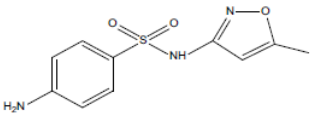
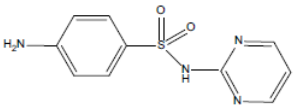
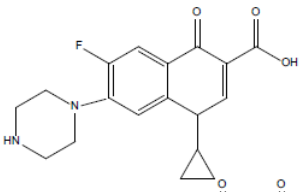
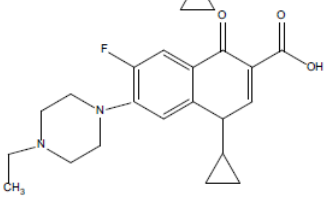
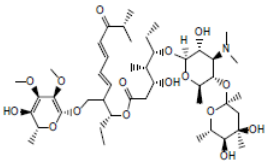
Antibiotics have been detected in food samples such as milk (Chi et al. 2019; Lu et al. 2020) and meat (Khaled et al. 2019; Zhang et al. 2020) as a result of the improper use of these drugs in veterinary medicine. Bacterial resistance, liver damage, allergic reactions, hypersensitivity and gastrointestinal problems are some of the health effects caused by the presence of antibiotic residues above maximum allowable limits (MRLs) (Yao et al. 2015; Rodríguez-Gómez et al. 2018; Georgiadis et al. 2019).

These antibiotics normally occur in the presence of other analytes that are closely or remotely related to them. In recent years, extraction and pre-concentration techniques that are cheap, fast, reliable and compliant to the principles of green chemistry such as fabric phase sorptive extraction (FPSE) and stir bar sorptive extraction (SBSE) have emerged and are used increasingly. These green extraction techniques have overcome the drawbacks of traditional methods that consume large volumes of organic solvents. According to the trends of analytical chemistry on miniaturisation, various approaches of sorptive micro-extraction techniques such as solid-phase micro-extraction (SPME) (Mondal et al. 2019; Lu et al. 2020; Zhang et al. 2020), SBSE (Rodríguez-Gómez et al. 2018; Yang et al. 2017; Khoobi et al. 2019), stir cake sorptive extraction (SCSE) (Mei and Huang 2016; Du et al. 2019) and FPSE (Karageorgou et al. 2016; Samanidou et al. 2017) have been developed for the extraction of antibiotic drug residues from animal-derived food samples. The origins and the principles of these sorptive extraction techniques are discussed briefly below.

A need for sensitive analytical methods and green chemistry compliant clean-up and pre-concentration techniques for antibiotic drug residues cannot be emphasised enough due to the toxicity and occurrence of these analytes at trace levels. Various review articles focusing on the application of sorptive micro-extraction techniques in the clean-up of antibiotics from different biological samples have been published over the years. Kazantzi and Anthemidis (2017) reviewed the application of FPSE in the extraction of antibiotics from milk while Demirhan et al. (2017) reviewed the application of SPME and

CONTACT Nikita T. Tavengwa nikita.tavengwa@univen.ac.za Department of Chemistry, School of Mathematical and Natural Sciences, University of Venda, Thohoyandou 0950, South Africa

Table 1. Chemical structures and physico-chemical properties of selected commonly used antibiotics in food-producing animals.

Class of the antibiotic drugs	Antibiotic name	Antibiotic structure	pK_a	log P	Water solubility (mg L^{-1})
Tetracyclines	Tetracycline		3.32 7.78 9.58	−0.3	231
	Oxytetracycline		3.22 7.46 8.94	−1.3	313
Sulphonamides	Sulfamethoxazole		8.8	0.89	610
	Sulfadiazine		6.5	−0.09	77
Fluoroquinolones	Ciprofloxacin		6.09 8.25	0.28	30,000
	Enrofloxacin		5.70 7.72	−0.39	3400
Macrolides	Tylosin		7.74	–	5000

–, not available.

SBSE in the extraction of various contaminants including antibiotics from several food matrices. Meanwhile, Kabir et al. (2017) focused on the application of SPME, SBSE and FPSE for the extraction of

Table 2. Application of sorptive micro-extraction techniques for the clean-up and pre-concentration of antibiotic residues from food samples.

Target analyte	Matrix	Sample pre-treatment procedure	Sorptive micro-extraction technique	Sorbent	Instrument of analysis	LOD	Recovery (%)	Reference
3 Sulphonamides	Milk	No protein precipitation	FPSE	Cellulose substrate coated with sol-gel PEG	HPLC-UV	–	22.98–49.5	Karageorgou et al. (2016)
4 Penicillins	Milk	No protein precipitation	FPSE	Cellulose substrate coated with sol-gel PEG	HPLC-DAD	3.0–9.0 μg kg^{-1}	20.0–57.0	Samanidou et al. (2017)
3 Amphenicols	Milk	No protein precipitation	FPSE	Cellulose substrate coated with sol-gel PEG	HPLC-DAD	–	44–81.4	Samanidou et al. (2015)
3 Sulphonamides	Milk	Deproteinisation by adding acetonitrile followed by vortexing and centrifugation	CPME	Cotton crochet thread coated with sol-gel PEG	HPLC-DAD	16.7 μg kg^{-1}	12.1–18.1	Georgiadis et al. (2019)
6 Penicillins	Milk	Vortex mixing the sample containing acetonitrile (deproteinisation)	SPME	Al-MOF	UPLC-UV	0.06–0.26 μg L^{-1}	90.5–95.7	Lirio et al. (2016)
4 Tetracyclines	Milk, eggs, chicken muscle and kidney	HCl and MeCN were added to milk and egg samples and then centrifuged. Citrate buffer (pH 5.0) and $CH_3CO_2C_2H_5$ were added to kidney and muscle samples before homogenisation and then centrifugation was done	SCSE	Fe_3O_4@HKUST-1-polyHIPE monolithic cake	HPLC-FLD	1.9–4.6 and 5.5–13.9 ng mL^{-1}	86.6–110.7	Du et al. (2019)
2 Quinolones, 2 sulphonamides, 1 macrolide and 1 pyrimethamine	Fish	In vivo	SPME	MIL-101(Cr)NH_2-polyacrylonitrile coated on a quartz fibre	LC/MS-MS	0.2–4.6 ng L^{-1}	–	Mondal et al. (2019)
5 Fluoroquinolones	Fish	In vivo	SPME	C_{18} particles coated on stainless steel wires	LC-MS/MS	0.3–1.5 ng g^{-1}	–	Tang et al. (2017)
3 Chloramphenicols	Milk	Fat removal by centrifugation followed by deproteinisation using MeCN	SPME	AuNPs@Au wire-ITO fibre	HPLC-DAD	0.262–0.293 ng mL^{-1}	75.7–94.5	Liu et al. (2017)
7 Flouroqinolones	Milk and honey	Spiked honey samples were diluted with water and milk samples were defatted and deproteinised using TFA	SPME	Poly(apronal-co-divinylbenzene /ethylenedimethacrylate based monolithic fibre	HPLC/MS-MS	0.0019–0.018 μg kg^{-1} and 0.0010–0.0028 μg kg^{-1}	74.5–116 and 80.2–113	Chen and Huang (2016)
2 Tetracyclines	Milk, chicken and fish	Na_2EDTA-McIlvaine buffer was added to homogenised samples followed by sonication, centrifugation and filtration	SPME	MIPs coated on a stainlesssteel wire	HPLC-DAD	0.38–0.72 μg kg^{-1}	77.3–104.4	Lu et al. (2020)
Sulfamethoxazole and trimethoprim	Milk and eggs	Extraction and deproteinisation done in MeOH followed by centrifugation	PT-SPME	Phenyl-boronic acid polymer monolith	UHPLC/MS-MS	1.5 and 0.3 ng mL^{-1}	92.4–100.5 and 92.7–102.6	Chi et al. (2019)
7 Fluoroquinolones	Milk	TFA was added and samples were centrifuged	SCSE	Boron-rich monolithic cake	HPLC-DAD	0.11–0.22 μg L^{-1}	68.8–120	Mei and Huang (2016)
8 Sulphonamides	Fish and chicken	Samples were sonicated in MeCN containing Na_2SO_4 followed by deproteinisation of the supernatant using 10.3% potassium ferrocyanide (w/v) and 21.9% zinc acetate.	IT-SPME	NH_2-MIL-53(Al) MOF in a capillary column	UHPLC/MS-MS	1.3–4.7 ng L^{-1}	85.7–113	Zhang et al. (2020)
10 Cephalosporins, 4 tetracyclines, 8 fluoroquinolones, 9 macrolides, 2 phenicols and 13 sulphonamides	Chicken and beef	Water was added to spiked samples and shaken on a vortex mixer followed by dilution	Direct immersion SPME	Polyacrylonitrile/hydrophilic-lipophilic balance coated on stainless steel rods	LC-MS/MS	–	70–120	Khaled et al. (2019)

(*Continued*)

Table 2. (Continued).

Target analyte	Matrix	Sample pre-treatment procedure	Sorptive micro-extraction technique	Sorbent	Instrument of analysis	LOD	Recovery (%)	Reference
17 Quinolones	Cow's milk	Protein/fat precipitation was done in a solution containing MeCN, zinc acetate polyhydrated phosphotungstic acid and CH_3CO_2H	SBSE	PEG coated a polar stir bar	UHPLC-MS /MS	0.1–1.0 µg kg^{-1}	88–114	Rodríguez-Gómez et al. (2018)
11 Quinolones	Bovine milk	Fat removal was done by centrifuging	Immunoaffinity-SBSE	Monoclonal antibodies coated on a glass bar	HPLC-FLD	0.05–0.1 ng g^{-1}	11.8–40.0	Yao et al. (2015)
5 Fluoroquinolones	Chicken muscle and liver	Water was added to the samples followed by sonication and centrifugation	SBSE	Graphene oxide/PEG	HPLC-FLD	0.0045–0.0079 µg kg^{-1}	–	Fan et al. (2015)
9 Fluoroquinolones, 1 tetracycline, 1 penicillin and 1 nitrofuran	Chicken, pork and fish	MeCN/trichloroacetic acid (7:3, v/v) was added to the samples and centrifugation was done	SBSE	MIPs coated on magnetic stir bar	HPLC-DAD	0.1–0.3 ng g^{-1}	67.4–99.0	Yang et al. (2017)
Benzylpenicillin	Milk	Centrifugation was done after the addition of MeCN and TFA for defatting and deproteinisation, and filtration was done thereafter.	SBSE	Zn-Al-LDH/ZIF-8 nanostructures coated on a magnetic aluminium oxide stir bar	HPLC-UV	0.05 µg L^{-1}	96.0–110.0	Khoobi et al. (2019)

–, data not available/provided.

various analytes including antibiotics from different complex matrices, Kechagia and Samanidou (2017) reviewed the application of SBSE and FPSE in the extraction of sulphonamides from milk samples. In this review, the recent application of the sorptive micro-extraction techniques, SPME, SBSE, SCSE and FPSE in the clean-up and pre-concentration of antibiotic drug residues from animal-derived food samples is critically discussed. Table 2 is a summary of the studies reviewed in this paper. Furthermore, the possible sorption mechanisms that facilitate the extraction of these analytes are discussed. To the best of our knowledge, this is the first time that the application of these four techniques for the extraction of antibiotic drug residues from various food matrices is reviewed.

Antibiotics

Antibiotics are widely used in veterinary medicine to treat and prevent bacterial infections (Moyo and Tavengwa 2019; Zhang et al. 2020). The main antibiotics used in both human and veterinary medicine are classified according to their chemical structure or mechanism of action. The main classes include β-lactams, tetracyclines, macrolides, aminoglycosides, amphenicols, quinolones/fluoroquinolones, sulphonamides, lincosamides, glycopeptides and polyether ionophores (Kummerer 2009). Antibiotics from different classes have distinct physico-chemical properties (Table 1) due to the different functionalities that they have within the same molecule; hence, multi-residual analysis might be challenging. Improper use of these drugs, particularly not observing withdrawal times, can result in unacceptable residues of these antibiotics in food products such as milk and meat (Karageorgou et al. 2016). Bacterial resistance that may occur as a result of continuous ingestion of unsafe levels of these drugs in food is currently a major concern worldwide (Duan et al. 2020; Zhang et al. 2020). Consequently, monitoring such residues in food products is vital for the consumer safety (Du et al. 2019). Several national and international regulatory bodies have set MRLs of antibiotics in animal-derived food products in order to protect the consumers' health. For instance, according to the regulation 37/2010/EC of the European Union, MRLs for enrofloxacin and ciprofloxacin in milk are 100 μg kg^{-1} and 75 μg kg^{-1}, respectively (EU 2010), while FAO/WHO has set 100 μg kg^{-1} as the MRL for sulphonamides in poultry tissues and eggs (JECFA 1992).

Sample pre-treatment

A common challenge in the extraction of antibiotic residues from food samples is the complexity of these matrices. These matrices contain biological constituents including proteins, lipids and carbohydrates (Samanidou et al. 2015), hence, several sample pre-treatment steps for the elimination of interferences are required prior to analysis (Rodríguez-Gómez et al. 2018; Georgiadis et al. 2019). Efficient removal of matrix interferences is necessary in order to prevent damage to chromatographic columns as this may affect the performance of analytical instruments (Khaled et al. 2019; Duan et al. 2019). Extraction of target analytes into a liquid phase with a suitable solvent is necessary for solid food matrices prior to a clean-up step using any sorptive technique. Furthermore, pre-treatment procedures may be necessary for liquid food matrices to avoid sorbent clogging and extend its reusability (Pereira et al. 2019). Sample pre-treatment procedures that are commonly done to remove matrix interferents prior clean-up using sorptive techniques include defatting, deproteinisation, sample dilution in order to reduce the viscosity, centrifugation and filtration (Chen and Huang 2016; Du et al. 2019; Lu et al. 2020). Acetonitrile is a common deproteinisation solvent which also serves as an extraction solvent (Lirio et al. 2016; Georgiadis et al. 2019; Du et al. 2019). Other precipitation reagents include trifluoroacetic acid (Chen and Huang 2016; Mei and Huang 2016), zinc acetate (Rodríguez-Gómez et al. 2018; Zhang et al. 2020) and formic acid (Karageorgou et al. 2016).

Sorptive extraction techniques

Widely used sample treatment techniques for the extraction of some antibiotic drug residues in food products include liquid-liquid extraction (LLE) (Hernández-Mesa et al. 2017; Moreno-González et al. 2017) and solid-phase extraction (SPE) (Kechagia et al. 2018; Lan et al. 2019). Despite the good analytical performance of LLE, toxic organic

solvents and relatively high amounts of the sample are needed for this technique. Moreover, LLE is an expensive and time-consuming technique. SPE uses lower solvent volumes compared to LLE; however, it is also expensive and time-consuming (Rodríguez-Gómez et al. 2018). The trend of analytical methods currently favours the use of miniaturised and solvent-minimised techniques for sample preparation. In recent years, solvent-free sample preparation techniques, known as green techniques, have emerged and these include SPME, SBSE, SCSE and FPSE. Their use in the extraction and pre-concentration of veterinary antibiotic drug residues has increased in recent years.

Solid phase micro-extraction

Solid-phase micro-extraction (SPME), which was developed by Arthur and Pawliszyn (1990), has gained great attention due to its simplicity, reduced analysis time, can be automated, compatibility with various analytical instruments and it is solvent free (Kabir et al. 2017; Herrington et al. 2020). Notwithstanding that automation offers advantages such as higher throughput, increased precision and accuracy compared to manual techniques (Zhang et al. 2020), carry-over effects are a major drawback in on-line configurations of any sorbent-based extraction techniques, and hence it is necessary to efficiently clean the extraction system after each analysis. SPME has been widely accepted for its usefulness in the analysis of food samples containing antibiotic residues (Lirio et al. 2016; Liu et al. 2017; Lu et al. 2020). The coating of SPME fibre plays a key role in effectively extracting trace levels of target analytes from complex matrices (Liu et al. 2017). Common commercial coatings for SPME fibres are PDMS, polyacrylate, carboxen/PDMS and PDMS/divinylbenzene. Although SPME fibre coatings containing materials such as carboxen and divinylbenzene are effective in sample pre-treatment, they lack selectivity towards target analytes, which results in competitive adsorption of other analytes, particularly in animal food samples (Lin et al. 2015; Canellas et al. 2016). SPME is a well-established micro-extraction technique, but, due to the small sorbent mass, sample loading capacity and poor precision, it may not provide the desired sensitivity in some applications (Canellas et al. 2016; Duan et al. 2020; Zhang et al. 2020). Consequently, modifications to this method have been done in recent years to address these shortcomings resulting in new versions of SPME such as in-tube SPME (Shuo et al. 2018; Zhang et al. 2020), pipette tip SPME (Chi et al. 2019), thin-film SPME (Grandy et al. 2018; De la Calle et al. 2019) and arrow SPME (Yuan et al. 2019). However, studies on the extraction of antibiotic drug residues from animal-derived food samples employing these modified versions of SPME are still limited.

Stir bar sorptive extraction

Stir bar sorptive extraction (SBSE) is a rapid, green and low-cost method suitable for volatile and semi-volatile compounds that was developed by Baltussen et al. (1999), and it is based on the same principles as SPME. However, SBSE has a higher extraction capacity than SPME due to the larger volume and surface area of the sorptive phase. Consequently, SBSE gives higher recoveries compared to SPME (Cárdenas and Lucena 2017; Telgheder et al. 2018). However, the thickness of the coating on the stir bar affects the extraction kinetics since diffusion of the analytes in the polymeric coating is hindered (Wells 2003).

Polydimethylsiloxane (PDMS) is a common stir bar coating material for SBSE and it is commercially available in the market. The main disadvantage of this material is that it is not efficient for polar compounds such as antibiotic drugs. However, other stir bar coatings such as PEG (Rodríguez-Gómez et al. 2018), graphene oxide (Fan et al. 2015), zeolitic imidazolate frameworks (Khoobi et al. 2019) and molecularly imprinted polymers (MIPs) (Tang et al. 2017; Yang et al. 2017) have extended the range of applicability of this technique. SBSE allows the extraction of numerous analytes simultaneously, with a low

solvent consumption and less sample manipulation (Rodríguez-Gómez et al. 2018).

Stir cake sorptive extraction

Another sorptive extraction method that has been explored in the pre-concentration of antibiotic drug residues in recent years is stir cake sorptive extraction (SCSE) although only few studies have been done so far (Mei and Huang 2016; Du et al. 2019). This technique is an improved version of SBSE, first introduced by Huang et al. (2011), and it combines extraction, enrichment and clean-up in one step (Mei and Huang 2016). The SCSE set-up consists of a monolith placed in a cake-shaped plastic holder that is pierced with a glass protected iron wire on one end to allow magnetic stirring of the device (Kissoudi and Samanidou 2018) as shown on Figure 1. Unlike SBSE, this technique eliminates the direct contact of the sorptive phase with the sample vessel wall during stirring (Chen and Huang 2016), hence extending the life span of the monolith. Moreover, extraction can be done under high stirring rates and monolithic cakes can be reused many times compared to stir bars in SBSE (Cárdenas and Lucena 2017). Monolithic cakes that have been used for the extraction of antibiotic drug residues include boron-rich monoliths (Mei and Huang 2016) and metal-organic frameworks (MOFs) embedded on polymerised high internal phase emulsions (polyHIPEs) (Du et al. 2019).

Fabric phase sorptive extraction

Fabric phase sorptive extraction (FPSE) is a green sample preparation technique that was introduced by Kabir and Furton (2014). The design of this method integrates SPME and SPE into a single technological platform (Samanidou and Kabir 2015). This technique makes use of fabric substrate surface coated with different polymers using the sol-gel technology, which helps forming a large surface area for efficient extraction (Kabir and Furton 2014). The commonly used fabric substrates are cotton cellulose (Karageorgou et al. 2016; Samanidou et al. 2017) and polyester (Montesdeoca-Esponda et al. 2015; Anthemidis et al. 2016). Other substrates that have been used include fibre glass (Alcudia-Leon et al. 2017) and nonwoven propylene (Yang et al. 2018). The selectivity and the polarity of the FPSE media are determined by the nature of the fabric substrates used (Zilfidou et al. 2018). Carbowax 20 M, sol-gel poly(tetrahydrofuran) (PTHF) and sol-gel poly-(dimethyldiphenylsiloxane) are among the sorbents that have been developed for a wide range of analyte polarities. Sol-gel poly(ethyleneglycol) (PEG) is suitable for highly polar analytes including antibiotics (Karageorgou et al. 2016).

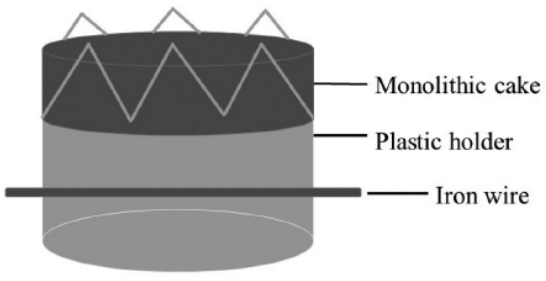


Figure 1. Set-up of the stir cake sorptive extraction technique.

The FPSE method allows direct extraction of analytes from a sample, hence eliminating any prior and post sample preparation procedures like filtration, centrifugation, solvent evaporation and sample reconstitution. Consequently, it prevents the risk of potential analyte loss, experimental errors and sample preparation costs. Therefore, FPSE has attracted interest in the extraction of several food contaminants, especially antibiotic residues from various food matrices of animal origin (Karageorgou et al. 2016; Samanidou et al. 2017; Zilfidou et al. 2018).

Advantages of FPSE include the reusability of the FPSE media without significant loss in efficiency, and a high primary contact surface area for rapid analyte-sorbent interaction resulting in fast and efficient extraction (Zilfidou et al. 2018). Long extraction times (up to 4 h) required to reach the extraction equilibrium is a major drawback of FPSE. New modifications of FPSE including ionic liquid immobilised FPSE, stir FPSE, stir-bar FPSE, dynamic FPSE and automated on-line FPSE have been developed to overcome this drawback (Lakade et al. 2016; Kazantzi and Anthemidis 2017). However, to the best of our knowledge, there are so far no studies reporting the application of these new modes of FPSE in the extraction of antibiotic residues from food samples.

Sorption mechanisms of antibiotic drugs on the sorptive phase

Adsorption of antibiotic drugs on sorptive material is mainly influenced by the diverse physicochemical properties of these analytes. The adsorption of these drugs on the sorbent is generally due to the formation of hydrogen bonds, π-π, electrostatic and hydrophobic interactions between the extractive phase and these analytes (Samanidou et al. 2017; Du et al. 2019; Khoobi et al. 2019). However, the sorption behaviour of antibiotics on an extractive phase is not only affected by the properties of these materials and analytes, but also by the chemistry of the sample solution. In sorptive extraction techniques, analyte extraction is based on partitioning between the sorbent and the matrix (Herrington et al. 2020). Therefore, it is critical to control parameters that may affect the extraction efficiency of these techniques such as sample pH, ionic strength, agitation and sample temperature. Possible mechanisms that favour the adsorption of antibiotic drugs on sorptive phases will be discussed briefly below.

Application of sorptive extraction techniques for the extraction of antibiotics from food samples

The hydrophilic affinity of the cellulose substrate for highly polar antibiotics and the high polarity of sol-gel PEG have made these two materials suitable for the preparation of efficient FPSE media for the extraction of antibiotic residues from milk samples. Karageorgou et al. (2016), Samanidou et al. (2015) and Samanidou et al. (2017) used cellulose coated with sol-gel PEG as FPSE media for the extraction of sulphonamides, amphenicols and penicillins, respectively, from milk samples without prior protein precipitation. In these studies, the impact of protein precipitation on the extraction recovery was also studied prior to the FPSE method which resulted in a significant loss of target analytes compared to whole milk that gave higher recoveries. This might be due to the fact that antibiotics bind to lipidic and proteinic material. Furthermore, sol-gel PEG was compared to other coatings such as sol-gel C_{18} and sol-gel PEG-PPG-PEG and all resulted in poor recovery values. However, the extractive phase could be reused several times without any significant loss in extraction efficiency.

FPSE is still far from being a complete sample preparation solution despite the elimination of solvent evaporation and sample reconstitution steps in this method, and thereby reducing analyte loss (Georgiadis et al. 2019). Furthermore, if the sample contains some particulates and insoluble matrix interferents, filtration is a necessary step in a FPSE procedure. Georgiadis et al. (2019) addressed these shortcomings of FPSE by using capsule phase micro-extraction for the extraction of four sulphonamide residues from milk samples. This technique could be considered as a miniaturisation of FPSE. The micro-extraction capsules consisted of a magnet, a cellulose fibre substrate coated with sol-gel hybrid organic-inorganic sorbent and a porous membrane. This technique combines filtration and the stirring mechanism into an extraction device, hence eliminating the filtration step prior to introducing the extraction device into the sample. Therefore, analyte loss might be reduced. In all the above studies, the hydrophilicity of the cellulose substrate favoured the adsorption of the antibiotics through hydrogen bonding, London dispersion and dipole–dipole interactions.

Another sorptive micro-extraction technique that has gained attention in recent years for the extraction and pre-concentration of antibiotic drugs from food samples is SPME. In contrast to FPSE, deproteinisation is a necessary step in the SPME procedure in order to prevent matrix interferences. The application of MOFs as a coating material in sorptive micro-extraction techniques for the extraction and pre-concentration of antibiotic residues from food samples has emerged in recent years due to their high surface areas, thermal stability and good adsorption affinity towards target analytes (Du et al. 2019; Mondal et al. 2019; Vardali et al. 2020). In a study done by Lirio et al. (2016), aluminium-based metal-organic framework (Al-MOF)-organic polymer monoliths were used as a sorbent in the SPME of penicillins from milk samples. This sorbent was synthesised via microwave-assisted polymerisation in a capillary tube. Despite the short synthesis time (5 min) of the Al-MOF-organic polymer monoliths, the preparation of the MOF took about 4 days. The adsorption of

penicillins was favoured by their hydrophilic nature and their ability to form π-π interactions between their aromatic rings and the MOF ligand. Moreover, the polymer skeleton could form hydrogen bonds with the N-H and O-H groups of the penicillins. The tremendous growth in the application of MOFs in sorptive micro-extraction in recent years can also be explained by the ease of functionalisation of these sorbents (Bagheri and Ghaedi 2020), resulting in their enhanced sorptive properties.

In a study done by Du et al. (2019), Fe_3O_4 @$Cu_3(btc)_2$ magnetic particles were embedded on a hydrophilic 2-ethylhexylacrylate/divinylbenzene/methyl methacrylate polyHIPE to form a Fe_3O_4@ $Cu_3(btc)_2$-1-polyHIPE monolithic cake. This was used as a sorbent in the magnetic stir cake sorptive extraction of tetracyclines from food samples. PolyHIPEs offer advantages including high specific surface areas, highly interconnected pore network and well-defined porosities (Sun et al. 2017). The basis of adsorption of tetracyclines on the MOF-polyHIPE cake was due to interactions including π-π interactions, hydrogen bonding and electrostatic interactions. It is noteworthy that this sorbent showed desirable durability (reused 150 times) compared to all the studies reviewed in this paper. This could be attributed to the configuration of this sorptive phase. Tetracycline and chlortetracycline were detected at levels above MRLs stipulated by the EU in chicken muscle and liver, respectively, indicating that there could be an overusage of these drugs for chicken production. Notwithstanding the unique properties of MOFs as coating materials for sorptive extraction methods, their use is still limited due to the loss of chemical stability in water or humid environment and the inconvenient retrieval from the sample matrix (Zhang et al. 2020). Mondal et al. (2019) used MIL-101(Cr)-NH_2 as coating material for SPME quartz fibre for the extraction of flumequine, nalidixic acid, sulfadimethoxine, sulfaphenazole, tilmicosin and trimethoprim from fish muscle. MIL-101(Cr)-NH_2 showed excellent chemical stability in water unlike most MOFs and this might be due to the formation of hydrogen bonds between the amine group of the coating material and water molecules. Furthermore, the amine groups on the surface of the MIL-101(Cr)-NH_2 pores could increase the adsorptive properties of this sorbent by attaching to the functional groups of the target analytes covalently (Wang et al. 2016). In vivo SPME was done in this study, and it offered advantages such as high sensitivity and better precision. Tang et al. (2017) also used in vivo SPME for the pre-concentration of fluoroquinolones from fish. However, in this study, that is very similar to the one above done by Mondal et al. (2019), the SPME fibre consisted of stainless steel wires coated with C_{18} particles. Polyacrylonitrile was used to glue a uniform layer of coating material onto the surface of the fibre in both these studies.

To overcome the non-selectivity of commonly used SPME coatings such as PDMS, the use of aptamers in detecting antibiotics in food samples has increased in recent years due to their low cost, higher affinity and specificity towards target analytes (Pavlov et al. 2005). Aptamers can fold into well-defined three-dimensional shape to selectively capture specific target analytes (Liu et al. 2017). Furthermore, aptamers have been incorporated into sorbents for solid phase based extraction methods such as SPME (Liu et al. 2017). A thiol functionalised aptamer was used as a coating for a 3-D SPME gold nanoparticle modified fibre array for the selective enrichment of chloramphenicol, thiamphenicol and florfenicol residues from milk samples by Liu et al. (2017). This method possessed advantages of high-throughput, high selectivity and adsorption capacity in one run.

The low extraction capacity resulting from low quantities of coating on the fibre is another disadvantage that limits the wide use of SPME. A study done by Chen and Huang (2016) addressed this disadvantage by employing a multiple porous monolithic fibre SPME based on poly(apronal-co-divinylbenzene/ethylenedimethacrylate) monoliths for the extraction of fluoroquinolones from milk and honey samples followed by analysis on the HPLC/MS-MS. The monolithic fibre was prepared by in situ polymerisation in a glass capillary and subsequently, a fibre bunch was assembled by binding together four monolithic fibres. Amino and phenyl functional groups in this sorbent could provide multi-interactions, including π-π, dipole-

dipole and hydrophobic interactions, and hydrogen-bond with fluoroquinolones through the polar piperazine and carboxyl groups and non-polar phenyl groups in the molecular structures of these analytes and hence facilitated extraction. In honey samples, trace levels of lomefloxacin, sarafloxacin and sparfloxacin were detected despite the use of fluoroquinolones being forbidden in honeybees. In milk samples, low contents of marbofloxacin and norfloxacin were found, which were below the respective MRLs.

To overcome the same problem, co-polymerisation can be done repeatedly on a fibre to achieve a desirable coating thickness that has more sensitivity. MIP coating for a SPME fibre was synthesised by multiple bulk co-polymerisation on the surface of silanised stainless steel wire and used for the selective extraction of tetracyclines from chicken, fish and milk by Lu et al. (2020). The adsorption performance of the MIP-SPME fibre in this study was improved by employing methacrylic acid and 2-hydroxyethyl methacrylate as bifunctional monomers. Moreover, 2-hydroxyethyl methacrylate endowed the MIP-SPME fibre with excellent hydrophilicity. Despite the high selectivity of this coating material, conventional SPME still suffer drawbacks such as easy breakage, low sorption capacity and poor durability (Queiroz et al. 2019).

Coating materials for sorptive micro-extraction techniques has become a major research subject in an attempt to improve the performance of these methods. Boronate affinity materials are among the coating materials that have received increasing attention in the extraction of antibiotic residues from food samples. This is due to their ability to form non-specific interaction with nitrogen-containing antibiotics such as fluoroquinolones and sulphonamides through boron-nitrogen (B-N) coordination (Espina-Benitez et al. 2018; Duan et al. 2020). Chi et al. (2019) synthesised a hydrophobic phenyl-boronic acid polymer monolith through initiator-free ring-opening polymerisation in a pipette tip for the extraction of sulfamethoxazole and trimethoprim from milk and egg samples. In a similar study done by Mei and Huang (2016), a boron-rich monolithic cake was synthesised from a vinylboronic anhydride pyridine complex and divinylbenzene as monomer and crosslinker, respectively. In contrast to the study done by Chi et al. (2019), radical polymerisation was used for the monolithic cake in the presence of azobisisobutyronitrile as an initiator. This sorbent was employed in the extraction of marbofloxacin, norfloxacin, ciprofloxacin, lomefloxacin, enrofloxacin, sarafloxacin and sparfloxacin from milk samples using SCSE. In both studies, adsorption of the target analytes was through B-N coordination between the sorbent and the antibiotics.

In attempts to address shortcomings of conventional SPME, several modified versions of SPME such as in-tube solid phase micro-extraction (Zhang et al. 2020) and pipette tip solid phase micro-extraction (Chi et al. 2019) have been developed. In contrast to conventional SPME, in-tube SPME enables direct SPME on-line coupling to HPLC systems and hence reduces analyte loss (Queiroz et al. 2019). In a more recent study done by Zhang et al. (2020), a fused-silica capillary with NH_2-MIL-53(Al) incorporated in poly (3-acrylamidophenylboronic acid/methacrylic acid-co-ethylene glycol dimethacrylate) coating on its inner surface synthesised by in situ polymerisation was used for the extraction of eight sulphonamides from fish and chicken samples. Most of these antibiotics were detected in both samples. However, their concentrations were below the MRL. The MOF-polymer monolithic column could be reused at least 100 times without any reduction in adsorption capacity, which shows better durability, compared to the results in the similar study conducted by Mondal et al. (2019) where a conventional SPME fibre was used. Moreover, the MOF-polymer monolithic column showed high extraction performance and water stability. Nonetheless, the preparation of the monolith column was time-consuming.

Automation of the SPME technique also promotes high throughput and hence it is a promising method that can be used for routine analysis like solvent extraction and Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) extraction that are already being used by the food industries. In a study done by Khaled et al. (2019), a matrix-compatible SPME coating material prepared from polyacrylonitrile/hydrophilic-lipophilic balance as an extractive phase in direct

immersion solid phase micro-extraction of more than 100 veterinary drugs including antibiotics from six different classes in chicken and beef tissue samples was employed. The polyacrylonitrile/hydrophilic-lipophilic balance coating material was deposited on the stainless-steel rod surfaces by dip coating. Unlike in conventional SPE bed systems, when matrix-compatible coatings are combined with the open-bed configuration of SPME, they facilitate multi-residual extraction from complex matrices without clogging.

The wide application of SBSE has been limited by the lack of stir bar coatings with high affinity for polar analytes such as antibiotic drugs. However, the applicability of this method to antibiotic drugs has increased in recent years due to new stir bar coatings such as MIPs, PEG and zeolitic imidazolate frameworks. To address the lack of coating materials for polar analytes, Rodríguez-Gómez et al. (2018) used PEG to coat stir bars for the extraction of seventeen quinolones from raw cows' milk. The efficient extraction of quinolones using this sorptive phase could be a result of the formation of hydrogen bonds with the carboxyl groups of these analytes. Extraction took approximately 15 h, which eliminates this method as a candidate for routine analysis by food industries. Some of the analysed milk samples showed the presence of ciprofloxacin, enrofloxacin and marbofloxacin which were below the regulated MRL (100 μg kg^{-1} for enrofloxacin and 75 μg kg^{-1} for ciprofloxacin).

Yao et al. (2015), who did similar work, employing immunoaffinity-SBSE and detection by HPLC-FLD, reported high sensitivity based on the extraction technique used. However, the preparation of the immunoaffinity-stir bar was also time consuming (119 h approximately) and expensive. The stir bars were prepared by binding monoclonal antibodies to glass bars through covalent linking using glutaraldehyde as the coupling reagent. Covalent linking increases the binding capacity and the stability of antibody on the glass bars (Lord et al. 2007). Monoclonal antibodies have homogeneous binding properties and only bind to a single binding site. Nevertheless, this might limit the use of this sorbent in multi-residual extraction. The extraction of quinolones by the immunoaffinity stir bar was through antibody–antigen interactions not adsorption on this sorbent.

Graphene oxide (GO) has also been proposed as a SBSE coating material. However, the irreversible aggregation and water solubility of GO resulting in the reduction of the sorption capacity and sorbent loss limits the direct use of this extractive phase. To combat these limitations, Fan et al. (2015), used a PEG/GO for the extraction of fluoroquinolones from chicken muscle and liver. This sorbent was prepared by solution co-blending. The rich oxygen-containing functional groups, ultra-high surface area, π-π and electrostatic stacking properties of GO provided numerous binding sites for fluoroquinolones through hydrogen bonding and π-π interactions.

Yang et al. (2017) employed a dual-template MIP coated stir bar based on difloxacin and ofloxacin for the extraction and pre-concentration of nine fluoroquinolone drugs and three other drugs from other classes from chicken, pork and fish samples prior to analysis on the HPLC-DAD. A glass tube filled with iron oxide powder was used as a stir bar. A dual template MIP was synthesised so as to increase the selectivity of this sorbent. The selectivity and affinity of this extractive phase towards target analytes was a result of MIP–analyte interactions, the shape and/or the size of template-imprinted cavities. The DMIP stir bar showed very low selectivity for furanzolidone, amoxycillin and tetracycline because they were not imprinted on the template. It is worth mentioning that among the three SBSE-based studies mentioned above, this is the only study where multi-residual analysis was done and not only achieved high enrichment factors and high sensitivities, but also avoided competitive adsorption. MIPs have high selectivity towards target analytes unlike other SBSE coatings made from non-selective materials that may lead to competitive adsorption when one sample contains different classes of analytes simultaneously.

In-situ growth of zeolite imidazolate frameworks (ZIFs) on the surface of layered double hydroxides (LDHs) for preparation of porous nanocomposites has also been explored in the design of potential sorbents for the extraction of antibiotic drug residues from food samples. The tunable nanoporosity that can be subject to functionalisation and the high surface area of ZIFs makes them attractive for the adsorption of organic compounds from complex matrices (Sutrisna et al. 2019). In a recent study

done by Khoobi et al. (2019), a nanoporous Zn-Al LDH/ZIF-8 composite was synthesised by the in-situ growth of ZIF-8 on the Zn-Al LDH surface. This nano-MOF was employed as a sorbent for extraction of benzylpenicillin from milk samples using SBSE. The aluminium oxide magnetic stir bar was prepared from an aluminium rod by the anodising process. The effective adsorption of benzylpenicillin on the anodised Al/Zn-Al-LDH/ZIF-8 coating could be due to hydrogen bonding, electrostatic interactions, π-π-stacking and hydrophobic interactions formed between the sorbent and the target analyte. Despite the attractiveness of this method, the sorbent required relatively long preparation time.

Generally, the most studied matrix is milk and this may be due to the fact that it is the most universally consumed food with great significance in human growth and health (Karageorgou et al. 2016). The commonly used FPSE coating in the extraction of antibiotic drug residues is sol-gel PEG due to the high polarity of these analytes. Compared to SBSE, SCSE and SPME, the recoveries obtained using the FPSE procedure are lower.

Challenges and future trends

Simultaneous determination of antibiotic residues is still a challenge due to varying chemistries of drugs from different classes (Moyo and Tavengwa 2019). Protein precipitation prior to FPSE protocol results in analyte loss, and hence decreased recovery values (Karageorgou et al. 2016; Samanidou et al. 2017). Moreover, the FPSE method gives lower recoveries compared to other extraction methods such as SPME and SBSE. This might be due to the insufficient amount of the coating material on the fabric substrate.

Despite the significant reduction of toxic and hazardous organic solvent consumption in sorptive micro-extraction techniques such as SPME compared to conventional extraction methods such as LLE, sorbents used in this technique suffer some major limitations. These limitations include limited number of commercially available sorbents, instability and swelling of the fibre in organic solvents and damage of the extractive phase coating material during operation hence resulting in reduced sensitivity (Liu et al. 2017; Zhang et al. 2020). Furthermore, low extraction capacity resulting from the low quantity of coating limits the use of SPME (Chen and Huang 2016; Du et al. 2019). Consequently, the growth of these green techniques has been impaired significantly; hence, conventional methods such as SPE are still widely used despite their drawbacks.

In order to meet the requirements of green analytical chemistry principles but also maintain the ease of use, high-throughput, high extraction capabilities for ultra-trace analysis and easy coupling to various separation techniques, modified versions of SPME such as in-tube SPME (Zhang et al. 2020) and pipette tip SPME (Chi et al. 2019) are increasingly evolving. Despite these promising innovations, the preparation of monoliths is time consuming. Therefore, there is still a demand for green sample clean-up technologies that are fast and can be used for onsite as well as routine analysis by food industries.

Poor recoveries of polar analytes from aqueous solutions using sorbent-based sorptive micro-extraction techniques are considered an insurmountable analytical problem (Spietelun et al. 2011). However, this limitation has been overcome by employing polar coatings based on different materials prepared by sol-gel technology, monolithic approach, graphene-oxide and MIPs which are highly selective towards target analytes (Cárdenas and Lucena 2017; Tang et al. 2017; Khaled et al. 2019).

he main drawback of SBSE-based methods is the long extraction time. However, this can be minimised by making multiple stirring positions available for the simultaneous extraction of many samples (Rodríguez-Gómez et al. 2018). Furthermore, the manual removal of a stir-bar from a sample, rinsing, drying, and in some cases, a need for an additional back extraction step in a proper solvent and extraction phase damage when stirring at high speeds are also limitations of this method (Telgheder et al. 2018; Du et al. 2019).

More studies involving automation of FPSE are more likely to be done in future since such studies are still limited.

Conclusion

Food contamination by antibiotic drug residues has become a concern in recent years due to the improper use of these drugs. Although regulatory bodies within the EU and FAO/WHO have set MRLs of these drugs in food samples, the development of fast, cheap, reliable and environmentally friendly extraction methods that can be used by food industries are required to ensure food safety (Rather et al. 2017). Most detected veterinary antibiotic drug residues in the studies that were reviewed in this paper are below the stipulated MRLs. However, in some studies these antibiotics were reported to be above MRLs (Chen and Huang 2016) and this might pose a health risk to consumers. The use of sorptive extraction techniques such as SPME, SBSE, SCSE and FPSE can be considered as a desirable alternative to conventional extraction methods, as these techniques reduce the consumption of and exposure to organic solvents, as well as the disposal cost and extraction time. Moreover, carryover effects that often affect the validity of results in sorbent-based extraction techniques are less pronounced in these sorptive methods than in conventional SPE cartridges that can only be used once. Reusability tests show that most of the sorptive extraction sorbents can be used for several times without loss in extraction efficiency. Nevertheless, great efforts still need to be done to address the shortcomings of these extraction methods.

Disclosure statement

The authors declare that they have no conflict of interest.

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2.B.3. Paper III

This paper "Recent applications of solid phase extraction in the pre-concentration of antibiotic residues from livestock and poultry manure" was submitted to the *Journal of Environmental Science and Health, Part B.* A critical overview of studies that have been conducted in recent years on the extraction of antibiotic residues from manure employing solid phase extraction with particular focus on Oasis HLB and Strata-X were discussed.

Recent applications of solid phase extraction in the pre-concentration of antibiotic residues from livestock and poultry manure

Babra Moyo[a,b], Nikita T Tavengwa[a,*]

[a]Department of Chemistry, School of Mathematical and Natural Sciences, University of Venda, Private Bag X5050, Thohoyandou, 0950

[b]Department of Food Science and Technology, School of Agriculture, University of Venda, Private Bag X5050, Thohoyandou, 0950

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

Abstract

The release of antibiotics into the environment from agriculture has received tremendous attention in recent years. Nonpoint source contamination of the terrestrial environment by these compounds can result from fertilization of agricultural soils with animal manure. The presence of antibiotics and their metabolites in manure may pose a threat to agro-ecosystems, plant growth, aquatic life and soil life, with antibiotic resistance increasingly becoming ubiquitous. Therefore, monitoring of manure for antibiotic residues is of vital importance in order to assess the risks of environmental pollution by these drugs. Several sample pre-treatment techniques have been developed for the extraction of antibiotic residues from complex matrices including manure over the years. Despite new developments in recent years in separation science where the common trend is miniaturization and green approaches, solid phase extraction is still the most widely used technique in the extraction of antibiotics from agricultural wastes such as manure. In view of this, the aim of this review is to give a critical overview of studies that have been conducted in the past 6 years on the extraction of antibiotic residues from manure employing solid phase extraction with particular focus on Oasis HLB and Strata-X. Increased knowledge on the levels of antibiotic residues in manure might be beneficial in the mitigation of the potential risks of these compounds to humans and the environment.

Keywords antibiotics, solid phase extraction, manure, environmental pollution, pre-concentration

Introduction

The presence of antibiotic residues in the environment is of increasing concern globally due to their potential in causing long-term adverse ecological effects [1, 2, 3]. Excretion of faeces and urine by commercially reared animals and the application of manure as a fertilizer to cropland are among the main routes of introduction of these compounds into the environment from agricultural industries [4, 5, 6, 7]. A sudden surge in the development and spread of antibiotic resistance that occurs as a result of overusage of these drugs has become a major concern worldwide due to the threat it poses to global health, agriculture and biosecurity [8, 9, 10]. As such, detection and quantification of these drugs in manure needs to be done in order to assess their impact to the environment and on humans [11]. Moreover, availability of information on the occurrence of these drugs in agricultural wastes might become an important tool in evaluating the development of antimicrobial resistance in animals and monitoring the usage trends of antibiotics in veterinary medicine [2, 12].

Despite significant technological advances in analytical chemistry over the years, sample pre-treatment is still inevitable for the extraction and isolation of analytes of interest from complex matrices prior analysis on analytical instrumentation. Furthermore, the occurrence of antibiobic residues at trace levels, the varying physico-chemical properties of these drugs and high complexity of manure samples are a substantial challenge in the analysis of this matrix to date. In order to mitigate undesirable effects of the presence of antibiotics in the environment, it is mandatory to develop efficient clean-up and pre-concentration techniques for antibiotics in agricultural waste.

Although several reviews have been published on the extraction of various antibiotic residues from different samples, most have focused on food matrices such as milk, honey and meat [13, 14, 15, 16] only a few have been published, if any on the application of solid phase extraction of antibiotic residues from manure. Meanwhile, Faraji et al. [17] reviewed the application of solid phase extraction (SPE) and miniaturized SPE based clean-up methods in the analysis of various organic and inorganic analytes including antibiotics food and water samples using novel sorbents, Płotka-Wasylka et al. [18] gave an overview of the applications of SPE in the extraction of various analytes including antibiotics in diverse biological and environmental samples such as water and human urine and plasma employing different new sorbents. In a review done by Pollard and Morra

[19], the fate of tetracyclines in dairy manure amended soils was discussed. Nonetheless, extraction techniques for antibiotic residues were not discussed, they might have been out of the scope of this review. Larivière et al. [20] reviewed the application of various extraction techniques in the extraction of various pharmaceuticals from environmental matrices including manure. However, most studies reviewed in this review were conducted before the year 2015. Christou et al. [21] reviewed recent knowledge on antibiotics in the agricultural environment as a result of the use of wastewater from for irrigation where the matrices of focus were soil and crops using various extraction methods such as pressurized liquid extraction, SPE and QuEChERS. In view of this, the aim of this review is to critically discuss recent applications of SPE in the extraction and concentration of antibiotics residues from livestock and poultry manure. Collating this information may help in the formulation of regulations regarding maximum residue limits in manure before application on farming land since none are available currently. Consequently, abate the dissemination of these drugs in the environment. The fate of antibiotics and SPE sorbents are also discussed briefly in this review.

Presence of antibiotics in livestock manure

Antibiotics are widely used to treat and prevent bacterial infections, as well as promote growth in commercially reared animals [22]. Although the latter use was banned within the European Union since 2006 (Regulation EC 1831/2003) due to the global threat of antibiotic resistance, in other countries, this practice is still employed [2, 22, 23, 25, 26]. Antibiotic use in veterinary medicine has increased substantially over the years due to the growth of human population and the subsequent high demand of food [27]. Notwithstanding their valuable attributes, antibiotics are gradually becoming recognized as potential environmental pollutants, which can result in the disruption of the ecological balance of the environment [28] and this poses a challenge to nutritionists and farmers.

Tetracyclines, sulfonamides, macrolides, flouroquinolones/quinolones and beta-lactams are the commonly used antibiotics in veterinary medicine [1, 10, 29, 30, 31]. It is estimated that over 60% of all antibiotics produced globally are used for veterinary purposes, currently [5, 32, 33, 34]. According to a recent report, antibiotic consumption increased by approximately 65% between the years 2000 and 2015, with the highest increases of 103%, 79% and 65% recorded in India, China

and Pakistan, respectively [35]. This trend is anticipated to grow by a further 65% by 2030 [36, 37, 38].

Application of livestock and poultry manure to cropland as a fertilizer is a common practice from the past, owing to the presence of substantial amounts of nitrogen, phosphorus and organic matter, hence, it is a good source of nutrients to crops [39, 40, 41]. However, manure from livestock farms is often applied directly to agricultural fields raw without any pre-treatment and resulting in high levels of antibiotic residues in the soil [6]. The removal of antibiotics from livestock waste before application to the soil has emerged in recent years in an attempt to reduce adverse ecological effects that may be caused by the occurrence of these compounds in the environment [39, 42]. Aerobic composting and anaerobic digestion are some of the approaches that have been assessed for the removal of antibiotic residues and antibiotic resistant genes from manure [43, 44, 45, 46]. However, these methods are not completely efficient, antibiotics such as tetracyclines partially degrade during these processes [39, 42, 46]. Consequently, the persistence of these compounds may result in the development of antibiotic resistant bacteria in manure and the environment [30, 47]. It has been reported that over a million people die from bacterial infections that cannot be treated each year globally due antibiotic resistance [48].

Fate of antibiotics in the environment

Many antibiotics used in the animal food producing industry are poorly adsorbed in the gut of animals due to their polar nature [22]. These compounds may have higher water solubility, lower sorption or less susceptible to degradation in manure than the parent drug due to the transformation that occurs within the animal's gut [49]. Depending on the pharmacokinetics of these compounds, a substantial fraction (30-90%) of these drugs are excreted mainly as the parent compounds or as metabolites via faeces or urine within hours of application [2, 21, 29]. Therefore, the soil serves as a sink for antibiotics through application of contaminated manure on agricultural land, from which these compounds can leach into groundwater, enter surface waters via runoffs, or persist in the soil [19, 50, 51, 52, 53, 54].

Some antibiotics such as tetracyclines and fluoroquinolones form complexes with soluble organics and multivalent cations and, hence, do not degrade easily during manure storage [10, 19, 55]. Therefore, when manure is applied to agriculture fields, only a fraction of these compounds become mobile with the flow of water in the soil and contaminate the surrounding environment

[11]. Moreover, complexation of these drugs with metals in the environment is growing as a new class of emerging contaminants since their interactions have shown increased antibiotic resistance [56]. Complexation occurs through the multiple electron-donor functional groups of TCs, such as acetamides, phenolic β-diketone moieties and dimethyl-ammonium groups. Subsequently, their extraction and detection using analytical techniques is quite challenging. Some metabolites are more potent than their parent compounds, while others such as acetic conjugates of sulfonamides can retrograde during manure storage [2, 57]. Moreover, the fate of antibiotics once they are in the environment is not only governed by their physico-chemical properties such as, water solubility, lipophilicity and sorption capacity but also by soil properties including pH, organic matter content and cation exchange capacity [42, 58]. Furthermore, climatic conditions such as soil temperature and moisture predominantly determine the environmental fate of antibiotics [5, 10]. Accordingly, the persistence of antibiotics in manure and the terrestrial environment ranges from less than one day to several months depending on these factors [31, 47, 59, 60].

Health and ecotoxicological effects of antibiotics

Manure is a major reservoir of antibiotic residues, antibiotic resistant bacteria and genes [11, 61]. Ecological disturbances and phyto-toxicological effects can occur as a result of these drugs in the environment. Crops and vegetables can take up and accumulate antibiotics, particularly, tetracyclines, fluoroquinolones, sulfonamides and amphenicols, hence, affecting sprouting and growth rates of these plants [2, 10, 62]. As a result, they can enter the food chain through this route and cause genetic selection of resistant bacteria that may pose a threat to humans, animals and, the environment [6, 29, 47]. Antibiotics may affect the human immune and metabolism systems [63]. In spite of the occurrence and persistence of the antibiotics in various environmental compartments, yet another class of pollutants posing serious concerns to human and animal health are antibiotic resistant genes [64]. Mounting evidence linking the use of antibiotics in veterinary medicine to the rapid increase of antibiotic resistance genes in the environment is increasingly developing [42, 65, 66, 67]. The presence of antibiotics in agro-ecosystems, even at subinhibitory levels, has been reported to induce population selection of antibiotic resistance genes [6, 51]. Furthermore, antibiotics have been reported to be lethal to aquatic organisms including freshwater algae, fishes and zooplankton [20, 68]. Also, antibiotic resistant genes can be transfered from gastrointestinal bacteria to soil bacteria [22, 69]. Apart from the widespread concern of the

development of antibiotic resistance, some antibiotics exhibit hormonal characteristics, thus they may act as endocrine disruptors on organisms [70].

Sample pre-treatment and extraction techniques for antibiotics from manure

The main challenges in the analysis of antibiotics in manure are the presence of these drugs in minute concentrations (as low as ng kg^{-1}) and the high complexity of this matrix [71]. Therefore, purification steps need to be carried with great diligence in order to reduce matrix interferences. Pre-treatment of manure normally involves sample homogenization, which includes, lyophilization, grinding, blending and sieving steps [21]. Generally, extraction of antibiotics from manure is based on ultrasound sonication assisted liquid partitioning using a suitable solvent [6, 41, 72] followed by centrifugation and clean-up on a SPE cartridge (Figure 1). Protein precipitation can be done using solvents such as lead acetate [29] and hexane [1, 53, 54] to prevent clogging of the cartridges. Antibiotics such as tetracyclines and flouroquinolones can strongly bind to manure samples and this may affect the accurate quantification of these compounds [20, 73]. Consequently, extraction solvents containing chelating agents including ethylenediaminetetraacetic acid, citric acid and oxalic acid are commonly used [30, 74, 75]. Chelating agents reduce the tendency of antibiotic drugs from forming complexes with cations in manure and hence improve analyte-sorbent interactions. Moreover, matrix components of the manure may interact with the sorbent reducing adsorption sites available for the retention of analytes. Furthermore, addition of these agents improves peak shape during chromatographic analysis [76].

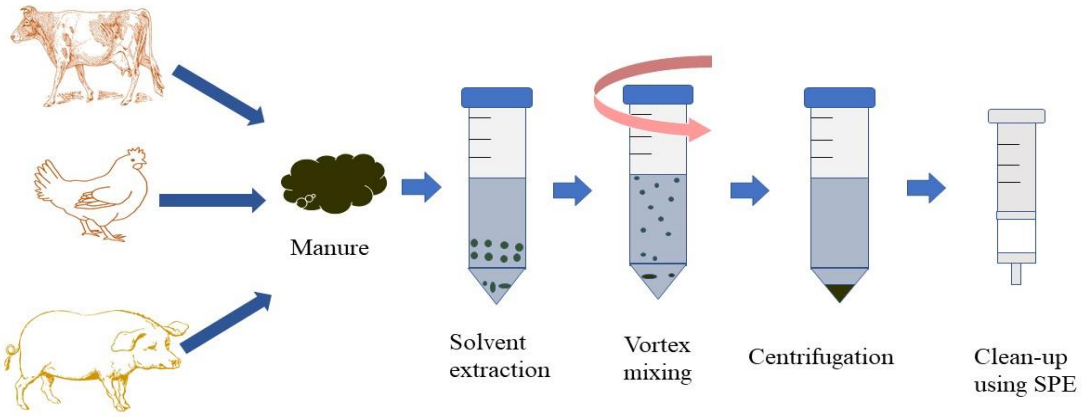

Figure 1 Sample pre-treatment steps for manure

Several sample preparation techniques have been used for the extraction of antibiotics from manure samples, such as solvent extraction [60], solid phase extraction [2, 6, 29, 74], QuEChERS [3] and dSPE [64]. However, SPE is one of the most widely used techniques for the extraction of antibiotic residues from manure [5, 12, 31, 54]. SPE and liquid-liquid extraction (LLE) are considered the oldest and most commonly used methods of sample preparation of trace amounts of most analytes including antibiotics. To this day, some extraction official standard methods (dSPE and QuEChERS) used in the analysis of food and environmental samples are based on these techniques. Nonetheless, LLE is time consuming and uses large amounts of potentially toxic organic solvents. In contrast, SPE consumes less of organic solvents and can be coupled to different detection techniques either in an on-line or offline mode [13, 61]. Another important advantage of SPE over LLE, is that the former can extract a broad range of analytes (from unpolar to very polar) from a wide variety of matrices [18, 77]. Moreover, SPE offers the possibility of automation, resulting in high sample throughput, as well as higher precision and accuracy of the results. Nevertheless, this mode suffers memory effects and systematic mistakes.

Solid phase extraction

Tremendous progress has been made since the initial introduction of SPE in 1951 resulting in miniaturization and automation of this technique. Additionally, new sorbents with a broad range

of morphologies and chemistries have evolved over the years [17, 18]. Consequently, solid phase based extraction methods such as solid phase micro-extraction, micro extraction by packed sorbent, miniaturised pipette tip extraction and dispersive solid phase extraction have been born from this technique. Nonetheless, these techniques are not within the scope of this review hence they will not be discussed further as they have been extensively discussed elsewhere [13, 16, 78, 79, 80]. Moreover, the application of these developments in SPE for the extraction of antibiotics from manure are still limited [64]. SPE can be operated in online and offline modes, with specific benefits and disadvantages of each approach. Contrary to offline SPE, the online mode offers advantages such as better enrichment factors, sensitivity and recoveries and, low consumption of solvent volumes of thereby complying to some of the principles of green analytical chemistry [80]. Cartridges are by far the mostly used format of SPE for the extraction and enrichment of various analytes from different matrices by separation scientists due to their wide commercial availability [18]. However, they suffer drawbacks such as channeling, analyte loss and pluggling. Despite these short comings associated with cartridges, they are still widely used in the extraction of antibiotics from manure samples. In an attempt to address these challenges associated with cartridges extraction disks has been introduced in recent years. In contrast to cartridges, disks have a longer life span. However, disks are more expensive [17] and their application in the clean-up of manure samples is still limited.

SPE sorbents

Sorbents play a critical role in the overall SPE procedure, particularly, the selectivity of the sorptive material has a remarkable influence on the accuracy of results. The efficiency of an SPE clean-up technique is dependent on the affinity that the sorbent has for target analytes [81]. Additionally, the pH of the sample solution also notably influences the interaction between the analyte and the sorbent [76]. Sorbents are mainly classified into three main groups, i.e., inorganic, organic and organic-inorganic hybrid materials [81]. Examples of inorganic sorbents include silica and titanium dioxide. Organic sorbents include molecularly imprinted polymers and chitosan whereas organic-inorganic hybrid materials include chitosan modified silica and metal organic frameworks.

Polystyrene-divinylbenzene copolymers (PS-DVB), silica-based sorbents and those modified with C_{18}, C_8, or NH_2 were the first to be applied to SPE [82]. However, these sorbents are

susceptible to interferences by impurities and suffer drawbacks such as activity of residual silanols (silica based sorbents), competitive adsorption in multi-residue extraction and, they cannot be reused. Additionally, silica dissolves in high pH mediums and can only be used within the pH range of 2 to 8 [83]. Over the years, significant progress has been made in the extraction of polar compounds including antibiotics by the introduction of more polar groups to PS-DVB [84] and hence resulting in an increase of commercially available SPE cartridges. One of the key advances in SPE over recent decades, has been polymer-based sorbents, with continuous progression due to its ability to adsorb compounds in a wide range of polarities [76]. In recent years, however, other developments in sorbent technology apart from those involving polymer-based materials have also taken place in the extraction of antibiotics. These novel materials include metal-organic frameworks [85, 86, 87], carbon nanomaterials [88, 89, 90] and molecularly imprinted polymers [91, 92, 93], among others. Nonetheless, there are few studies that have explored the use these in the extraction of antibiotic residues from manure samples. In view of this, only polymeric sorbents (i.e, Oasis HLB and Strata-X) are reviewed in this paper.

Oasis hydrophilic lipophilic balance

Despite continuous developments of SPE new sorbents, Oasis hydrophilic lipophilic balance (HLB) ((poly(N-vinylpyrrolidone-divinylbenzene)) is the widely used sorbent in SPE for the extraction of antibiotic residues from manure as shown on Table 1. Owing to the presence of non-polar benzene and aliphatic chains as well as the polar pyrrolidone regions, this sorptive material exhibits good water wettability and an enhanced capability of interacting with both polar and non-polar organic molecules [76]. Meanwhile, the pyrrolidone region of this polymer provide dipole-dipole and hydrogen bonding interaction sites for antibiotics through carbonyl, hydroxyl and amine groups (Figure 2), the benzene rings enhance partitioning to Oasis HLB through π-π interactions thus improving the retention of these analytes [82, 94]. Oasis HLB is stable across whole pH range on the contrary to silica based sorbents. Furthermore, water-wettability allows the elimination of conditioning and equilibration steps.

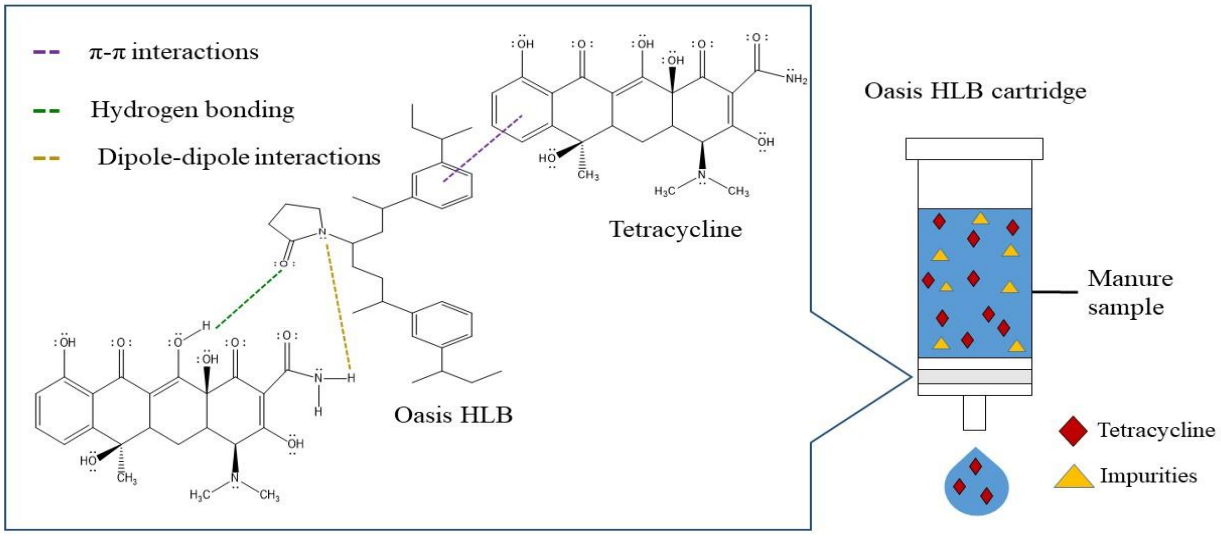

Figure 2 Adsorption mechanisms of antibiotics on Oasis HLB

Strata-X

Strata-X is a surface modified polymer of styrene with N-vinylpyrrolidone functional groups hence it has hydrophilic properties. It is nearly a universal SPE material for acidic, basic and neutral analytes [95]. This sorbent addresses common limitations associated with conventional SPE sorbents including swelling, shrinkage, low recoveries and reproducibility [96]. Antibiotics are retained on this sorptive material by different mechanisms such as hydrophilic, hydrophobic and π-π interactions as shown of Figure 3.

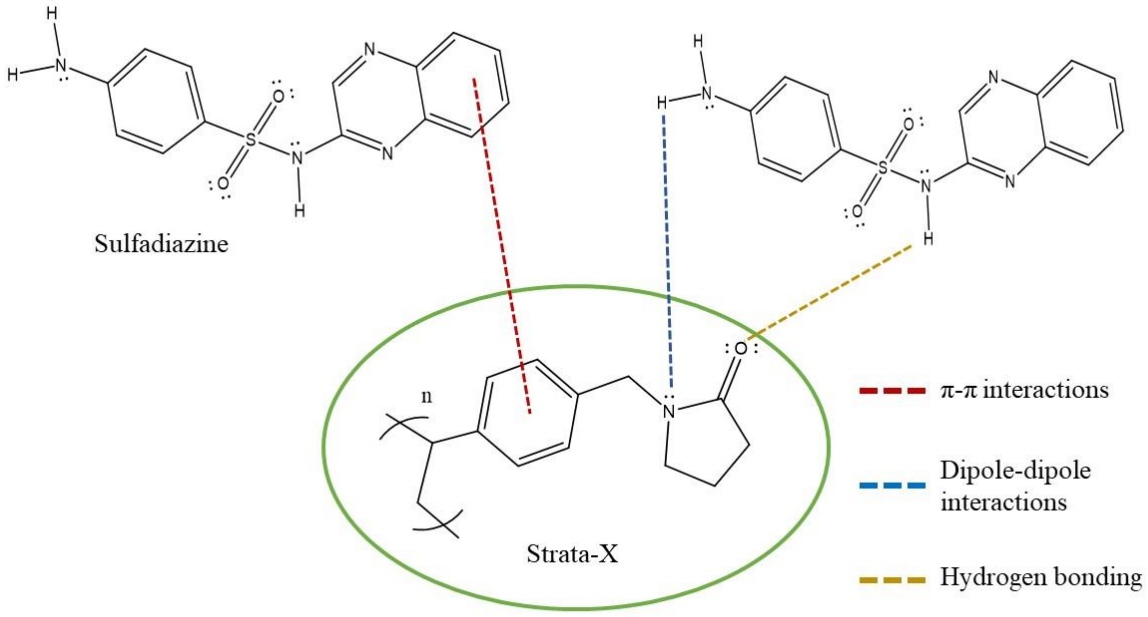

Figure 3 Adsorption mechanisms of antibiotics on Strata-X

2. Application of SPE in the clean-up of antibiotics from manure

Generally, in majority of the studies reviewed in this paper, commercial Oasis HLB in the off-line SPE mode was employed as a sorbent for the extraction of antibiotics from manure samples as shown on Table 1 [1, 4, 5, 10, 22, 27, 31, 54, 77, 74, 75, 97, 98, 99]. Lipophilic divinylbenzene provides the reversed-phase retention necessary to retain analytes with distinct physico-chemical properties [4, 100] through the formation of π-π interactions between the aromatic rings of the analytes and the sorbent, whereas the polar vinylpyrrolidone allow for hydrogen bonding. Interestingly, in most of these studies, conditioning of the SPE cartridges was done using methanol followed by water. Water was employed as a washing solvent and finally target analytes were eluted using methanol or methanol containing solvents. Unsurprisingly, most of these studies have reported the presence antibiotics from different classes in manure since a large fraction of these compounds are poorly absorbed in the animal's gut. However, this is concerning given that these drugs may pose a threat to humans and the environment. Therefore, the presence of antibiotics in manure should be actively monitored. Tetracyclines are the most predominant compounds detected

in these studies. This might be due to that they are the commonly used in veterinary medicine and they are persistent in manure.

The complexity of manure poses a challenge in the extraction of antibiotics due to presence of organic matter. Therefore, anionic exchange cartridges such as SAX and NH_2 are normally set up in tandem with HLB cartridges to reduce matrix interferences. Negatively charged organic matter can be retained on these sorbents, thereby improving retention of target compounds on Oasis HLB and detection limits [76]. Guo et al. [75] employed SAX cartridges for the removal of negatively charged humic and fulvic acids in manure from livestock farms prior further clean-up and pre-concentration on a HLB cartridge. Employing the same approach, Hou et al. [1], extracted sulfonamides, tetracyclines, fluoroquinolones, macrolides and nitrofurans from cattle, chicken and swine manure. In an attempt to reduce organic materials that could accumulate in the liquid chromatography column and electrospray source resulting in signal suppression, Patyra et al. [41], employed dispersive solid phase extraction as a pre-purification step prior clean-up of pig, chicken manure and digestates on Strata-X-CW-SPE cartridges. Doxycycline was detected with the highest concentrations in this study. In a study done by Wallace and Aga [51], NH_2 cartridges were used in tandem to HLB cartridges for the clean-up of tetracyclines, sulfonamides and macrolides from dairy manure. The weak anion exchanger sorbents retained the weakly anionic carboxylate and polarizable phenolic residues in the extract before loading on the HLB sorbent. Macrolides were analysed with a separate method using a different mobile phase because of their comparatively poor response in wrong-way-round ionization. However, analysis of antibiotics from different classes was done separately in this study and in other studies covered in this review [2, 6, 27] and this is not cost effective. This is evident that multi-residue analysis of these drugs is still an insurmountable challenge due to their varying chemistries.

High proportion of antibiotic drugs can be excreted at high concentrations in manure, even after the end of a therapy course, hence, it is imperative to treat manure before application on agricultural fields to curb the dissemination of residues of these compounds in the environment and uptake by plants. Yévenes et al. [2] assessed and compared concentrations of florfenicol, florfenicol amine, chlortetracycline, 4-epi-chlortetracycline and sulfachloropyridazine in broiler chicken droppings after the administration therapeutic doses of pharmaceutical formulations of these drugs experimentally. This study showed that florfenicol and florfenicol amine concentrations were detected in chicken droppings up to 10 days after ceasing treatment, while

chlortetracycline and 4-epi-chlortetracycline were detected up to 25 days. Sulfachloropyridazine residues were detected up to 21 days. Extraction and clean-up was done using Oasis HLB SPE for tetracyclines and amphenicols while SCX was employed for sulfachloropyridazine followed by analysis on the LC-MS/MS. It is worth mentioning that extractions for antibiotics from each class were done separately and analysis done differently on different instruments which can be time-consuming and expensive. In a similar study done by Cornejo et al. [47], Oasis HLB was used for the clean-up of broiler droppings. It was observed that excretion of chicken droppings containing residues of chlortetracycline and its degradation product, 4-epi-chlortetracycline persisted for several days after stopping the therapy course. In view of concerns surrounding bacterial resistance, strict regulations on the treatment of raw manure should be considered.

Peng et al. [27] investigated withdrawal periods for amoxicillin, ciprofloxacin, and doxycycline in layer hens manure. Neither amoxicillin nor doxycycline were detected 3 and 10 days, respectively, after withdrawal in the manure. The non-detection of amoxicillin could be due to that penicillins are unstable in the faeces matrix [101]. However, doxycycline results are not aligning to the findings of the rest of the studies in this review. Yet again, in this study, extraction and analysis were done separately.

Due to excretion of antibiotics or their metabolites in considerable amounts in manure, the removal of antibiotics from livestock waste before their application to soil has emerged in the past years as a way of mitigating the presence of these compounds in the environment and antibiotic resistance. Nonetheless, currently available methods are not completely efficient hence further scientific research focusing on more effective antibiotic removal techniques from manure need to be done. Concentrations of antibiotics after degradation is dependent on the time of storage of the agricultural waste in manure pits. Carballo et al. [31] reported the presence of tetracyclines in pig slurry that was sampled from ponds where it was stored for at least three months under anaerobic conditions. Doxycycline was detected in digestate samples in concentration ranges of 1.3-10.5 mg kg^{-1} by Widyasari-Mehta et al. [97], hence, confirming that anaerobic digestion was not effective in the removal of these antibiotics. In a study conducted by Rasschaert et al. [6], aminoglycosides and colistin were extracted from pig slurry that was collected from manure pits where Oasis HLB and WP-CBX SPE were employed as sorbents, respectively. It is noteworthy that three different extraction methods (one was not SPE based) were used in this study. Additionally, extraction and

detection of aminoglycosides proved to be considerably challenging due to their high polarity. These analytes lack chromophore or fluorophore characteristics and tend to strongly bind to proteinic material in the matrix. As such, compared to other classes of antibiotics, aminoglycosides are infrequently investigated [102].

Another technique that has been explored for the removal of antibiotics is composting and it is environmentally friendly [99]. Ravindran and Mnkeni [39] investigated a combination of thermophilic composting for 20 days and vermicomposting for 7 weeks as an approach for reducing the concentrations of oxytetracycline and its metabolites (4-epi-oxytetracycline, α-apo-oxytetracycline and β-apooxytetracycline) in chicken manure. Clean-up was done using a C_{18} which is another common conventional sorbent used in SPE. The concentration of oxytetracycline at the beginning of the thermophilic composting was in the range of 123.3-35.2 mg kg^{-1}, which reduced to 44-25.3 mg kg^{-1} after this process, and further reduced to the least concentration of 9.8 mg kg^{-1} at the end of the vermicomposting stage. This shows that composting can significantly reduce the concentrations of antibiotics. Similar findings were reported by Zhang et al. [99] where fluoroquinolones showed high persistence in the compost samples. Nonetheless, like anaerobic digestion it is not effective considering that even at low antibiotic concentrations, bacterial resistance can occur.

Strata-X is another commonly used sorbent in the SPE of antibiotics from manure. Strata-X was used in the extraction of 44 antibiotic compounds including tetracyclines, quinolones, macrolides and sulfonamides in swine and cattle manure sampled directly from animal's gut by Berendsen et al. [101]. Oxytetracycline and doxycycline had the highest concentration in cattle and swine manure, respectively. In yet another study by Berendsen et al. [12], using the same sorbent, the fate of multiresidues of 46 antibiotics during manure storage of different livestock animals (calves, pigs, broilers) was investigated over a period of 24 days. In this study it was observed that, generally, tetracyclines, quinolones, macrolides, lincosamides and pleuromutilins were much more persistent, in particular, lincomycin, pirlimycin, tiamulin, enrofloxacin, difloxacin, flumequine and sarafloxacin. Dissipation occurred mostly through abiotic processes.

Antibiotics are challenging analytes due to their diverse physico-chemical properties and their tendency to bind to constituents of manure may result in non-extractable residues, hence, affecting accurate quantification of these compounds. Consequently, it is necessary to investigate the extent to which non-extractable residues influence the extraction procedure and accurate analysis of these

analytes [20, 103]. This phenomenon is prevalent in multi-residue analysis where a single solvent extraction is used. Jansen et al. [29] investigated non-extractable residues of 48 antibiotics from 6 different classes in manure. Sample clean-up was done using Strata-X cartridges. Tetracyclines and quinolones were observed to have a large amount of non-extractables as expected, in this study. Antibiotics that were detected with the highest concentrations include, oxytetracycline, doxycycline, tilmicosin, flumequine and sulfadimidine.

In another study by Jansen et al. [104], vertical transmission of enrofloxacin, doxycycline and sulfachlorpyridazine from the parent hen to hatched broilers was investigated by extracting chick droppings using Strata-X as an SPE sorbent. Interestingly, both doxycycline and enrofloxacin were detected up to 30 days in chick droppings after hatching.

Although multi-residue analysis is still a challenge, it is noteworthy that incredible progress in being made to address this challenge. A multi-residue method was developed by Gao et al. [53] for detecting and quantifying 92 antibiotics from eight classes (β-lactams, quinolones, sulfonamides, tetracyclines, lincomycins, macrolides, chloramphenicols and pleuromutilins) in livestock excreta from farms by liquid chromatography with tandem mass spectrometry. Faecal samples were extracted by ultrasound-assisted extraction followed by a clean-up using SPE based on NH_2 cartridge. It is noteworthy that all these 92 antibiotics were analysed simultaneously

Table 1 Extraction and pre-concentration of antibiotic residues from manure using SPE

Target analyte	Matrix	Sample pre-treatment/ extraction solvent	SPE sorbent	Method of detection	Concentration detected	Recoveries (%)	LOD	Reference
7 Sulfonamides, 4 tetracyclines, 6 fluoroquinolones and 6 macrolides	Cattle, pig and chicken manure	Acetonitrile, phosphate buffer (pH = 3) and Na_2EDTA. Defatting with hexane.	Commercial SAX-Oasis HLB	UPLC-MS/MS	0.4-5775.6 µg kg^{-1}	50.0-121.9	0.1-0.3 ng L^{-1}	[75]
5 Sulfonamides, 4 tetracyclines, 3 fluoroquinolones, 3 macrolides, 1 nitrofuran and trimethoprim	Cattle, chicken and swine manure	Na_2EDTA-McIlvaine buffer (pH 4.0) followed by vortexing, sonication and centrifugation. Methanol-acetonitrile-acetone (2:2:1 v/v/v) mixture was used for further extraction. Hexane was used for defatting	Commercial SAX - Oasis HLB	UPLC-MS/MS	2.6-2133.2 µg kg^{-1}	42.3-90.0	0.1-2 µg kg^{-1}	[1]
2 Fluoroquinolones, 4 tetracyclines, lincomycin, tylosin, tiamulin and trimethoprim	Pig and poultry feaces, liquid manures and digestate	McIlvaine-Na_2EDTA buffer solution	Commercial Strata-X-CW	LC-MS/MS	200-175 400 µg kg^{-1} (doxycycline), 220-1450 µg kg^{-1} (oxytetracycline), 1320-1710 µg kg^{-1} (tetracycline) and 340-17 700µg kg^{-1} (chlortetracycline)	63-93	15.5-92.0 µg kg^{-1}	[41]
7 Tetracyclines, 2 sulfonamides and 3 macrolides	Dairy manure	Sonication in acetonitrile, methanol, and 0.1 M EDTA-McIlvaine buffer	Commercial Oasis HLB	LC-MS/MS	1.9-2100 µg kg^{-1}	62-94.3	0.5-7.9 µg kg^{-1}	[51]
2 Tetracyclines, 1 sulfonamides and 2 amphenicols	Chicken droppings	Chloramphenicols were extracted in water and acetone followed by extraction in dichloromethane while tetracyclines were extracted in	Commercial Oasis HLB and SCX	HPLC-MS/MS	-	-	-	[2]

		EDTA-McIlvaine buffer solution and acetonitrile						
Chlortetracycline	Broiler droppings	EDTA-McIlvaine buffer solution and acetonitrile	Commercial Oasis HLB	LC-MS/MS	179.5-665.8 µg kg^{-1}	-	20 µg kg^{-1}	[47]
Amoxicillin and doxycycline	Layer chicken manure	0.1 M potassium dihydrogen 0.2 phosphate (pH = 4.5) and 75% trichloroacetic acid (amoxicillin) McIlvaine-Na2EDTA buffer (doxycycline)	Commercial Oasis HLB	HPLC-UV and HPLC-FD	ND	60.54-95.16	0.025 µg g^{-1}	[27]
3 Tetracyclines	Chicken manure and pig slurry	Extraction in methanol and 0.003 M EDTA	Commercial Oasis HLB	HPLC-MS	<0.01- 1.38 mg kg^{-1}	73-83	0.01-0.3 mg kg^{-1}	[31]
Sulfonamides, diaminopyrimidines, tetracyclines, fluoroquinolones, macrolides and pleuromutilines	Pig and bovine liquid manure, chicken manure, and digestates	Pre-treatment with EDTA-McIlvaine buffer solution followed by extraction using methanol/ethyl acetate mixture	Commercial Oasis HLB	LC-MS/MS	0.2-300 mg kg^{-1} (liquid manure from breeding farms) 0.7-381 mg kg^{-1} (liquid manure from breeding farms with biogas plants) 0.2-10.5 mg kg^{-1} digestates	-	-	[97]
12 Aminoglycosides and 1 polymyxin	Pig slurry	Dissolution in 20% trichloroacetic acid followed by centrifugation	Commercial Oasis HLB	LC-MS/MS	72 and 97 µg kg^{-1} (aminoglycosides) 116.1 µg kg^{-1} (colistin)	-	10-394 µg kg^{-1}	[6]
Oxytetracycline and 3 of its metabolites	Chicken manure	McIlvaine-Na_2EDTA buffer (pH 4.0) and acetonitrile	C_{18}	LC-MS/MS	19.3-166.6 mg kg^{-1} (prior composting)	-	-	[39]

		followed by vortexing and centrifugation			9.8 -35.4 mg kg^{-1} (after composting)			
4 Tetracyclines, 8 Sulfonamides, 4 fluoroquinolones and 1 macrolide	Chickens, ducks, pigs and cattle manure	EDTA- sodium phosphate buffer with acetonitrile/$Mg(NO_3)_2$-NH_3•H_2O	Commercial Oasis HLB	LC-MS/MS	2.1-416 750 µg kg^{-1} (manure) 2.0-10 400 µg kg^{-1} (compost)	68.8-136.0	0.09-3.16 µg L^{-1}	[99]
4 tetracyclines, 18 sulfonamides, 14 macrolides and 10 quinolones	Cattle and swine manure	McIlvaine-EDTA buffer, ACN and lead acetate	Commercial Strata-X	LC-MS/MS	1-21 000 µg kg^{-1} (cattle manure) 1-95 000 µg kg^{-1} (swine manure)	83.7-147	5-50 µg kg^{-1}	[101]
4 Tetracyclines, 18 sulfonamides, 10 (fluoro)quinolones, 10 macrolides, 2 lincosamides and 2 pleuromutilins	Calves, pigs, broilers manure	McIlvaine-EDTA buffer, 0.125% TFA in ACN and lead acetate	Commercial Strata X	LC-MS/MS	-	-	-	[12]
3 Tetracyclines, quinolones, macrolides, lincosamides and sulphonamides	Calf manure	0.125% trifluoroacetic acid in acetonitrile in combination with McIlvaine-EDTA buffer	Commercial Strata X	LC-MS/MS	1-7000 µg kg^{-1}	-	1-50 µg kg^{-1}	[29]
Enrofloxacin, doxycycline and sulfachlorpyridazine	Chick droppings	McIlvain-EDTA buffer, ACN and lead acetate solution (droppings)	Commercial Strata X	LC-MS/MS	2.0 mg kg^{-1} sulfachlorpyridazine, 2.4 mg kg^{-1} doxycycline and 3.0 mg kg^{-1} enrofloxacin (first day)	-	-	[104]

21 β-lactams, 20 quinolones, 24 sulfonamides, 7 tetracyclines, 3 lincomycins, 12 macrolides, 3 chloramphenicols, and 2 pleuromutilins	Livestock Manure	Acetonitrile/water (80:20, v/v) and Na_2EDTA followed by addition of hexane (manure)	NH_2 column	LC-MS/MS	0.1-56.8 µg L^{-1} /kg^{-1}	75-99	0.1-0.3 ng L^{-1}	[53]
2 Tetracyclines, 3 quinolones, 4 sulfonamides, chloramphenicol and tylosin	Cow, pig and chicken manure	Extraction in EDTA-Mcllvaine buffer pH=4 followed by centrifugation. Hexane was used for defatting.	Commercial Oasis HLB	HPLC-PDA	0.1- 62 852.8 µg kg^{-1}	62.65-99.16	0.1-1.9 µg kg^{-1}	[4]
4 Tetracyclines and 4 sulfonamides	Pig and chicken manure	0.1 M EDTA-McIlvaine buffer solution	Commercial Oasis HLB	LC-MS/MS	0.02-143.97 µg kg^{-1}	-	5-15 µg kg^{-1}	[5]
2 Tetracyclines	Chicken and cattle manure	Extraction in McIlvaine-EDTA buffer, sonication and centrifugation	Commercial Oasis HLB	HPLC-UV	0.047-13.77 mg kg^{-1}	64-113	0.011 and0.01 mg kg^{-1}	[7]
Sulfonamide, ciprofloxacin and tetracycline	Cattle, chicken and swine manure	Na_2EDTA-McIlvaine buffer (pH 4.0) followed by defatting using hexane	Commercial Oasis HLB	ELISA	12.16-2083.26 µg kg^{-1}	45.0-115.0	26.96-78.43 µg kg^{-1}	[54]
5 Sulfonamides, 4 tetracyclines, 4 fluoroquinolones, 3 chloramphenicols and trimethoprim	Cattle ,pig and chicken manure	Acetonitrile:Na_2EDTA-McIlvaine (pH = 4.0) (sulfonamides) and pH = 7.0 for fluoroquinolones) Na_2EDTA, acetonitrile: phosphate buffer (pH = 3) at a ratio of 1:1 (v/v) for tetracyclines	Commercial Oasis HLB (for sulfonamides and flouroquinolones) and SAX-HLB (tetracyclines)	LC-MS/MS	3.1-16280 µg kg^{-1}	-	-	[30]

2 Sulfonamides, 1 tetracycline, 4 fluoroquinolones, 2 amphenicols and 1 nitrofuran	Pig manure	Na_2EDTA, citric buffer (pH 3) and acetonitrile (50:50, v/v) mixture	Commercial SAX-Oasis HLB	LC-MS/MS	0.33-1057.6 µg kg^{-1}	-	-	[10]
2 Tetracyclines	Pig manure	20 % CCl_3COOH	Commercial Oasis HLB	LC-MS/MS	2.99-10 mg kg^{-1}	-	11.5-34.4 µg kg^{-1}	[98]
2 Sulfonamides, 2 cephalosporins, 1 carbapenems, 1 monobactam, 1 tetracycline, 1 macrolide, 2 fluoroquinolones and 1 glycopeptide	Cattle, chicken and swine manure	Aqueous solution of citric acid, pH 4 and acetonitrile	Commercial Oasis HLB	HPLC-DAD/MS	6.36-295.13 mg kg^{-1}	51.57-92.64	-	[72]

- Data not available

Challenges and future trends

Despite the wide application of Oasis HLB and Strata-X in the extraction of antibiotics from manure, these sorbents are susceptible to competitive adsorption especially in multi-residue analysis. Therefore, there is considerable need to explore selective sorbents for extracting and isolating these compounds from such matrices such as molecularly imprinted polymers. Moreover, since the current trends in analytical chemistry are focusing on green solvents, miniaturization and automation of SPE, future studies should focus on employing these new technologies in the isolation of antibiotics from manure.

Another challenge in the extraction of antibiotics from manure are matrix interferences due to the high amount of organic matter in these samples. Organic matter can significantly reduce the extraction efficiency of a clean-up technique and affect the detection of analytes of interest especially when using electrospray ionization mass spectrometry where the signal intensity can be markedly suppressed [105]. It is interesting that, in most studies reviewed in this paper, matrix effects were not investigated. However, matrix matched calibration curves and internal standards have been used for quantification in an attempt to counter matrix interferences.

Moreover, tetracycliness are well known to strongly adsorb to organic and inorganic matter in manure through complexation with di- and trivalent cations [51] and this can affect the efficient extraction of these analytes. However, to minimize the occurrence of these interactions, a complexing agent can be added to the extraction solvent. Citric acid and ethylenediaminetetraacetic acid are the widely used chelating agents.

Due to the persistence of tetracyclines and fluroquinolones in manure for long periods of time, penicillins may be used in place of these drugs since their degradation in manure is fast and occurs as a result of the hydrolysis of the chemically unstable beta-lactam ring [12]. However, the fast degradation of these antibiotics does not eliminate their potential in promoting antibiotic resistance bacteria [6]. Moreover, their use in veterinary medicine will also depend on their effectiveness in treating bacterial infections. In light of the undesirable effects associated with the occurrence of the currently available antibiotics in the environment, future research should focus on the development of alternative antimicrobial drugs that can minimize these effects.

Conclusion

Oasis HLB and Strata-X are still the commonly used sorbents in the extraction of antibiotic residues from manure using SPE despite the continuous development of new sorbents. Moreover, the application of miniaturized versions of SPE methods for these particular analytes in manure are still limited. Despite the good analytical performance of SPE in the clean-up of manure, there is need to explore cheap, fast and green analytical chemistry compliant extraction techniques. Moreover, since the use of antibiotics is continually increasing due to demand, it is imperative to develop extraction methods that can be used for routine analysis of these compounds in manure by agricultural industries.

Application of manure as a fertilizer on agricultural fields causes unintended dissemination of antibiotic residues and resistant bacteria into the environment, thus posing a risk to humans, animals, soil organisms and aquatic life. Therefore, regulations on the use of antibiotics in veterinary medicine have become more stringent in some countries. Currently available manure management practices employed in the extraction of antibiotic residues are not effective hence efficient measures that can be employed in the removal of these drugs from agricultural waste are needed in order to contain the emerging problem of the spread of resistance bacteria and genes. Moreover, the antibiotics pollution in livestock farms that occurs as result of antibiotics should be given more attention in terms policies regarding manure management practices.

Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 3

Materials and methods

In this chapter, general approaches of the carrier mediated HFLPME and m-MIP-PTE techniques for the pre-concentration of antibiotic drugs in honey and cattle manure, respectively, are outlined.

3.1 Chemicals and reagents

Chemicals and reagents used in the two techniques for the extraction of antibiotic residues under this study are mentioned in **paper IV** and **IV**, respectively.

3.2. General research approach: HFLPME technique

The HFLPME technique was used to pre-concentrate tetracyclines in honey samples. The general approach during HFLPME is given in Figure 4.

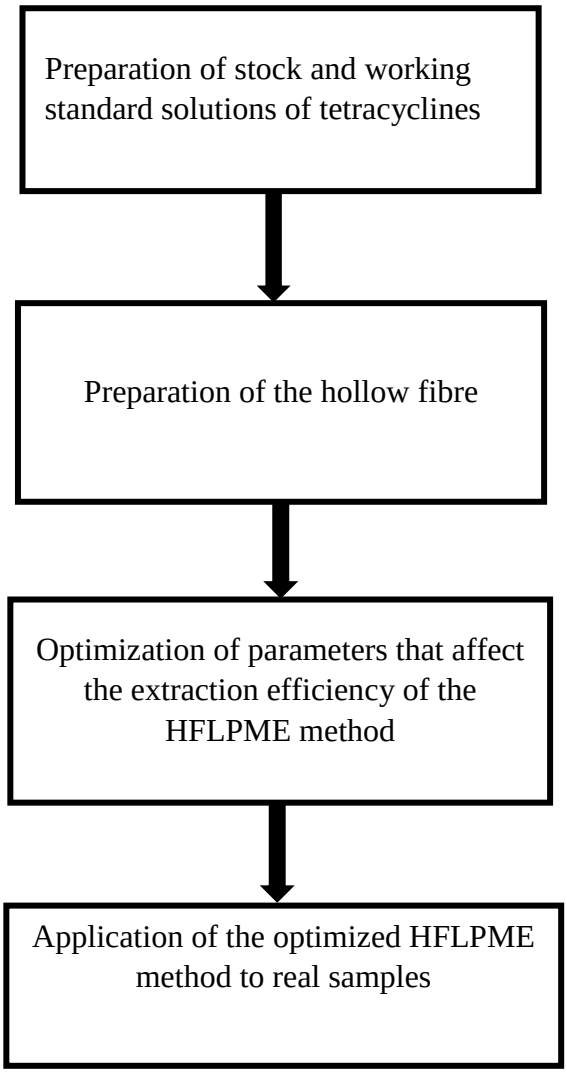

Figure 4 Schematic diagram for the general research approach: HFLPME

3.3. General research approach: m-MIP-PTE technique

Extraction and pre-concentration of sulfamethoxazole was performed using the m-MIP-PTE technique. Figure 5 gives the general approach of this method.

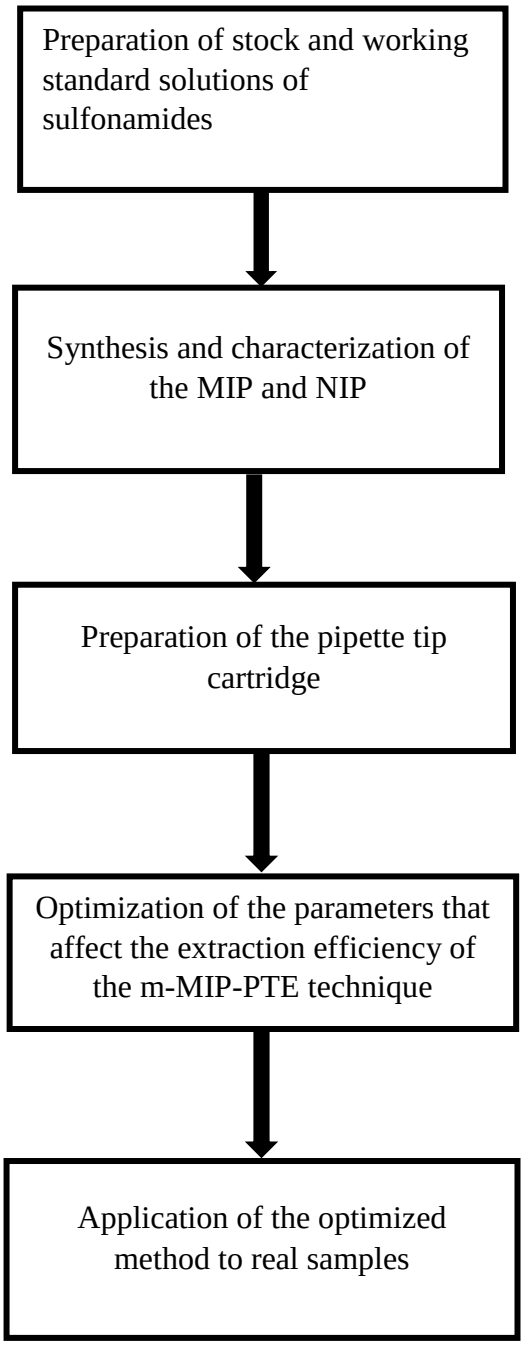

Figure 5 Schematic diagram for the general research approach: m-MIP-PTE

Chapter 4

Prepared manuscripts

This chapter gives the manuscripts that were written during the MSc program.

4.1. Paper IV

This paper "Enrichment of tetracycline residues from honey samples using carrier mediated hollow fibre liquid phase micro-extraction" was submitted *to Journal of Food Analytical Methods.* The presence of tetracycline residues from commercial honey was investigated.

Enrichment of tetracycline residues from honey samples using carrier mediated hollow fibre liquid phase micro-extraction and quantification on the LC-Q-TOF/MS

Babra Moyo[a,b], Nikita T Tavengwa[a,*]

[a]Department of Chemistry, School of Mathematical and Natural Sciences, University of Venda, Private Bag X5050, Thohoyandou, South Africa, 0950

[b]Department of Food Science and Technology, School of Agriculture, University of Venda, Private Bag X5050, Thohoyandou, South Africa, 0950

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

Abstract

In this study, development and validation of a simple, miniaturised and, environmentally friendly carrier mediated three phase hollow fibre liquid phase micro-extraction technique was done for the enrichment of tetracycline residues in honey samples. The extracts were analyzed using UV/Vis spectrophotometry and the LC-Q-TOF/MS. Parameters affecting the extraction efficiency of HFLPME such as pH of the donor and acceptor solutions, salt addition, agitation speed and extraction time were optimized. The calibration curves showed good linearity, in the range of 20-500 µg L^{-1} with correlation coefficients ranging between 0.9918 and 0.9998, under the optimized conditions. The recoveries of blank honey samples at three spiking levels (75, 200 and 300 µg L^{-1}) ranged from 56.1% to 120.8% with RSDs between 0.37% and 11.21%. LODs and LOQs were in the ranges of 0.042 to 0.661 µg kg^{-1} and 0.127 to 2.002 µg kg^{-1}, respectively. Finally, the proposed method was successfully applied for the extraction of five tetracyclines from honey samples. Doxycycline residues were detected in a commercial honey sample at a concentration of 0.20 µg kg^{-1}. Due to the advantages offered by HFLPME, it can be concluded that this method can be employed as an alternative to conventional extraction techniques.

Keywords: tetracyclines, hollow fibre micro-extraction, honey, food safety, enrichment

1. Introduction

The domestication of honeybees in artificial hives for the production of honey began several centuries ago (Moretti et al. 2017; Reybroeck et al. 2012). Honey is a valuable food commodity worldwide that is used as a natural sweetener for food. Furthermore, since ancient times, it has been promoted around the world for its nutritional and medicinal benefits (Fonte et al. 2018; Mattonai et al. 2016). Bees, like any other living organisms, can suffer from certain diseases, for example, foulbrood caused by *Paenibacillus larvae* and *Melissococcus pluton* (Dluhošová et al. 2018). As a result, beekeepers use antibiotics to treat foulbrood in bee colonies (Arabsorkhi and Sereshti 2018), with tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DXC) being the most commonly used in apiculture (Liu et al. 2007; Singh et al. 2015). However, the improper use of these drugs may result in a carry-over of residues into honey (Moyo et al. 2020). Liver damage, drug resistance and gastro-intestinal disturbance in humans are some of the health effects caused by the presence of tetracyclines in food products (Singh et al. 2015). Furthermore, antibiotic residues may reduce the natural protective properties of the honey, and hence may affect its antimicrobial activity (Barganska et al. 2011; Kim et al. 2014). Therefore, the determination of residues of these drugs in honey samples is imperative. Although, the EU regulation has set the maximum residue limits (MRLs) of 100 μL^{-1} (kg^{-1}) for tetracyclines in milk and meat, and 300 μkg^{-1} in liver, there is no specific reference for antibiotics in honey (EU 2010; Zhang et al. 2019).

Tremendous research on the presence of antibiotics in honey has been done and more is currently underway. Also, detection methods are continuously improving due to the occurrence of these compounds in trace amounts (Jia et al. 2017; Kivrak et al. 2016). Conventional clean-up techniques such as liquid-liquid extraction (LLE) (Dluhošová et al. 2018; Kivrak et al. 2016; Louppis et al. 2017) and solid phase extraction (SPE) (Xu et al. 2016; Singh et al. 2015; Tu et al. 2019) are the widely used and have been used for the extraction of tetracyclines from honey samples. Despite the good analytical performance of both these methods, LLE consume large volumes of organic solvents which is undesirable. On the other hand, although, SPE uses lower volumes, it is expensive and time consuming (Moyo et al. 2020). Therefore, there is a need to explore extraction techniques that overcome these drawbacks such as hollow-fibre liquid phase micro-extraction (HFLPME). Owing to its good enrichment, excellent sample clean-up and low consumption of

organic solvents, HFLPME can be an efficient alternative to conventional extraction techniques for the pre-concentration of antibiotic residues (Moyo and Tavengwa 2019; Khan et al. 2020).

Nevertheless, there is little information available on the use of HFLPME in the extraction and pre-concentration of antibiotics from food samples at the moment hence a need to do more research on this technique. Therefore, the aim of this study was to investigate the occurrence of tetracycline residues in honey samples from local vendors and supermarkets. A three-phase carrier mediated HFLPME method was employed prior to analysis of the extracts on the UV/Vis spectrophotometer and the LC-Q-TOF/MS. Method validation and applicability was done on five tetracyclines (tetracycline, chlortetracycline, oxytetracycline, doxycycline and methacycline).

Chemicals and reagants

Tetracycline (> 98%), oxytetracycline (96%), chlortetracycline (> 97%), doxycline HCl (> 98%), minocycline (98.1%), methacycline (95.3%), tri-caprylil methyl ammonium chloride (Aliquat-336, $R_3NCH_3^+Cl^-$) were purchased from Sigma-Aldrich (Johannesburg, South Africa). Sodium hydrogen phosphate dibasic, 1-octanol, HPLC grade methanol and acetonitrile were purchased from Merck (Johannesburg, South Africa). LC-MS grade methanol, acetonitrile and ultrapure water were purchased from Monitoring and Control Laboratories (Johannesburg, South Africa). Q 3/2 Accurel 200/600 Accurel® PP polypropylene hollow fibres with a wall thickness of 200 µm, 600 µm inner diameter and pore size of 0.2 µm were purchased from Membrana GmbH (Wuppertal, Germany). A 100 µL Hamilton syringe was purchased from the Hamilton Company (Nevada, USA). Distilled water was obtained from the Milli-Q water purification system (Guyancourt, France).

Instrumentation

All mass spectral measurements were done using an LC-Q-TOF/MS 9030 mass spectrometer (Shimadzu, Japan) with an electrospray interface (ESI) operating in positive mode. The mass range was 100-1000 m/z and Table 1 shows the optimum ESI parameters for individual tetracyclines. Quantification of each tetracycline was done by selecting the most abundant pre-cursor to product ion transitions in multi reaction monitoring mode. High-purity nitrogen was used as a nebulizing and drying gas. The following parameters were used: drying gas flow, 10 L min^{-1} and drying gas

temperature, 250°C. For the chromatographic separation, a Shimadzu 9030 LC instrument consisting of an autosampler, thermostated column compartment and a binary pump (Shimadzu, Japan) was employed. Tetracyclines were separated using a Shimpack C_{18}, 2.1 x 100 mm, 2.7 µm column from Shimadzu (Honeydew, South Africa). Data acquisition was done using the Lab solutions software. The absorption spectra and absorbances for the optimization experiment of the proposed extraction method were obtained using a Biowave II UV/Visible spectrophotometer (Biochem, England) with 2 cm quartz cuvettes. Spectra was acquired between 250 and 500 nm. All pH measurements were done using a Basic 20 pH meter (Crison, Germany).

Table 1 The optimized MS compound dependent parameters of six tetracycline compounds

Analyte	Molecular mass (g mol^{-1})	Precursor ion (m/z)	Product ion (m/z)	Collision energy (volts)
Tetracycline	444.43	445.16	427.15	15
Chlortetracycline	478.80	479.13	462.09	15
Doxycycline	444.43	445.16	428.13	15
Oxytetracycline	460.43	461.16	443.14	15
Methacycline	442.40	443.14	426.11	20
Minocycline	457.50	458.19	441.16	15

Preparation of standard solutions

The tetracyclines stock solutions were prepared in methanol at a concentration of 1 mg mL^{-1} and were stored at 4°C until ready for analysis. Six series of the working standard solutions at the concentration values of 0.01 to 2 mg L^{-1} were prepared from the tetracycline stock standard solution by diluting with 50% (v/v) methanol for HFLPME optimization. For method validation and application, matrix-matched standards were prepared in blank honey samples at five spiking levels from 20 to 500 µg L^{-1}, to compensate for matrix effects. The internal standard (minocycline) was added to standards and samples for the method validation, and application steps. The final concentration of the internal standard was 100 µg L^{-1}.

Extraction and clean-up procedure

Modified methods by Shariati et al. (2009) and Ncube et al. (2016) were used. Briefly, 11 cm long hollow fibres with an internal volume of 31.1 µL were cut using a scalpel and heat-sealed on one end. To remove any contaminants, the hollow fibres were sonicated in acetone for 5 min and allowed to air dry. Under optimized conditions, for each experiment, 15 mL of the sample diluted in 0.05 M Na_2HPO_4 (pH 9.5) was poured into a 20 mL vial. A 100 µL Hamilton syringe was used for injecting the acceptor phase solution (0.1 M H_3PO_4, 1.0 M NaCl with pH = 1.0) into the lumen of the hollow fibre. The hollow fibre pores were impregnated with an organic solvent by immersing them in 10% (w/v) of Aliquat-336 in 1-octanol for 10 s followed by immersion in distilled water for 10 s for the removal of the excess organic solvent from the surface of the hollow fibre. In a U-shape configuration, the hollow fibre was introduced into the sample and the vial was placed on a magnetic stirrer and, extraction was done for 45 min. 20 µL of the acceptor phase was drawn into the microsyringe after extraction, diluted to 2 mL in an Eppendorf tube and analysed on the UV/Vis spectrophotometer at the wavelength 286 nm or the LC-Q-TOF/MS. Standards and samples and were filtered through 0.22 µm Nylon filters before they were injected into the LC-Q-TOF/MS. Enrichment factors were used for the evaluation of the method efficiency. Various parameters that could affect the extraction efficiency of the proposed method were then optimized. 10% (w/v) Aliquat-336 in octanol was employed as a supported liquid membrane. A standard solution of tetracycline with a concentration of 500 µg L^{-1} was used for the optimization experiments and extractions were done in triplicates. The enrichment factor (EF) was expressed as a ratio of the concentration of the analyte in the acceptor phase (C_A) to its concentration in the donor phase (C_D) using equation (1).

$$EF = \frac{C_A}{C_D} \qquad (1)$$

Chromatographic conditions

Five tetracyclines and minocycline (internal standard) were separated at a flow rate of 0.4 mL min^{-1}. The injection volume was 5 µL and the column was maintained at 40ºC. 0.1% (v/v) formic acid in ultrahigh purity water was mobile phase A and mobile phase B was 0.1% (v/v) formic acid in

acetonitrile. The mobile phase composition was 90% of mobile phase A at 0-2 min and it was held at this composition for 1 min. At 3 min, mobile phase A decreased to 10% and was maintained at this composition for 1 min. Mobile phase A was then returned to the initial composition at 5 min and held for 1 min until the next injection.

Analysis of real samples

Honey samples were obtained from local vendors and supermarkets around Thohoyandou, Limpopo, South Africa. All the samples were kept at 4°C until analysis. Samples were diluted in the ratio 1:5 (w/v) with 0.05 M Na_2HPO_4 (pH 9.5) and extracted according to the proposed method under optimum conditions.

2. Results and discussion

Optimization of parameters affecting extraction efficiency

Effect of the donor phase pH

In this study, the effect of the donor phase pH on the extraction efficiency of HFLPME was investigated using a solution of 0.05 M Na_2HPO_4 with a pH range of 7.5 to 11.5 and the findings are shown on Figure 1. The best extraction efficiency was observed at pH 9.5. Above this value, a decrease in enrichment factors was observed. Some percentage of TC exist in the form of TC^- at the pH range of 7 to 11 according to its pK_a values (Figure 2). Although analytes should exist in the neutral form in the donor phase so that they can diffuse through the organic supported liquid membrane into the acceptor solution (Sharifi et al. 2016; Bahrami et al. 2017), in this study, the cationic carrier (Aliquat-336) was used to facilitate mass transfer of TC in anionic form (TC^-) from the donor to the acceptor solution. This mechanisms is schematically shown on Figure 3. The decrease in extraction efficiency at pH values above 9.5 could be due to the formation of TC^{2-} at this pH range. These findings corresponded to those found by Xu et al. (2017), who did similar work in milk samples, where the highest transport of tetracyclines was observed at pH 9.0 using hollow fibres coupled to dynamic liquid-liquid micro-extraction.

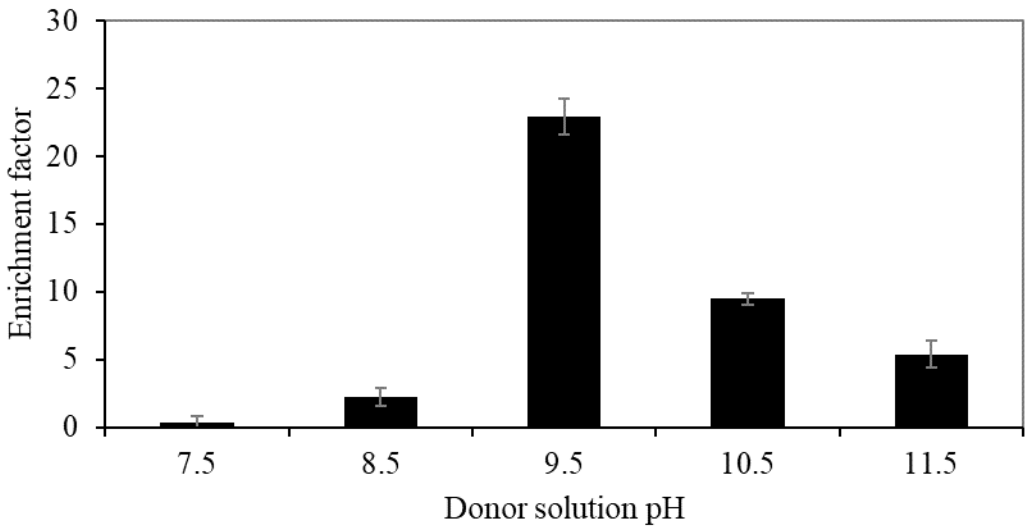

Figure 1 Effect of the donor phase pH on the extraction efficiency of tetracycline (n = 3, RSD). Extraction conditions: 15 mL of 500 µg L^{-1} TC in 0.05 M Na_2HPO_4 solution as the donor phase solution, acceptor phase solution: 0.1 M H_3PO_4 (pH 1.0), stirring rate: 400 rpm, extraction time: 15 min.

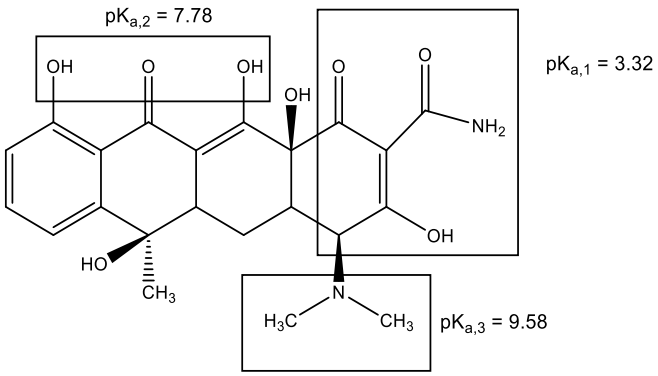

Figure 2 The chemical structure and pK_a values of tetracycline

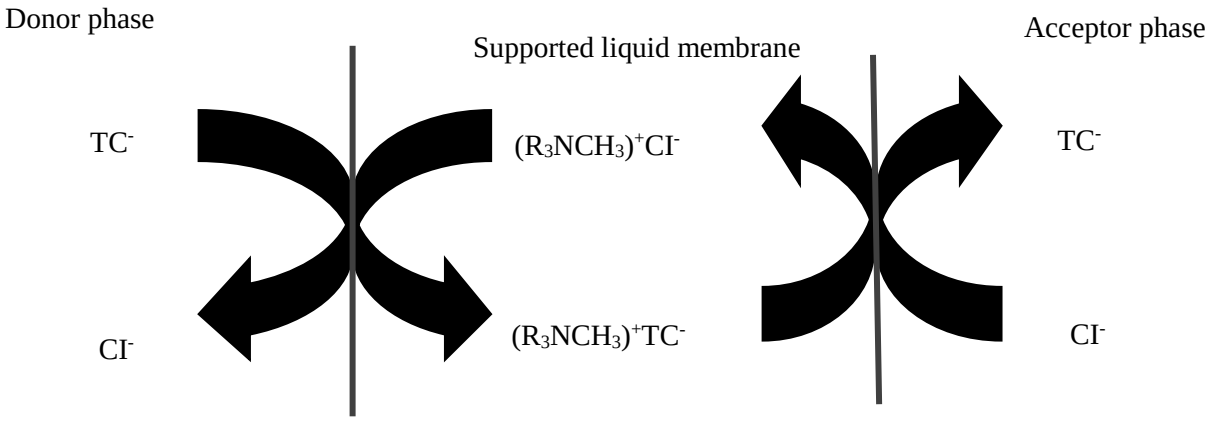

Figure 3 Extraction mechanisms of TC using carrier mediated three phase HFLPME

Effect of acceptor phase pH

H_3PO_4 (0.1 M) solutions with pH ranges 1 to 3 were used for the investigation of the effect of the acceptor phase solution pH on the extraction efficiency of HFLPME. The enrichment factors of TC decreased gradually above pH 1 as shown in Fig. 4. The pH of the acceptor phase should be at a level that guarantees the ionization of analytes (Sharifi et al. 2016; Kaynaker et al. 2018). The trend of the findings in this study might be due to the fact that TCs exist in protonated forms (TC^+) under these conditions. These ionic forms remain in aqueous phase during the extraction process because of their solubility in water (Lebedinets et al. 2019). Therefore, 0.1 M H_3PO_4 with pH 1 was optimum for efficient extraction and was used in subsequent experiments. These findings align with those by Tajabadi et al. (2016) where tetracyclines and quinolones were extracted employing HFLPME into 0.1 M HNO_3 and NaCl with pH 1 from various food samples.

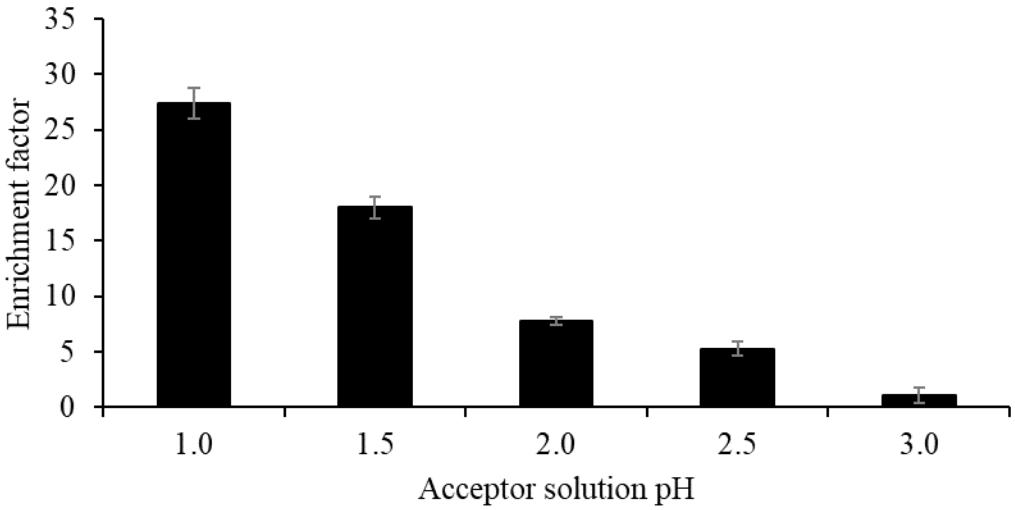

Figure 5 Effect of the acceptor phase pH on the extraction efficiency of tetracycline (n = 3, RSD). Extraction conditions: 15 mL of 500 µg L^{-1} TC in 0.05 M Na_2HPO_4 solution (pH 9.5) as the donor phase solution, acceptor phase solution: 0.1 M H_3PO_4, stirring rate: 400 rpm, extraction time: 15 min.

Effect of salt addition

To study the effect of salt addition on the extraction efficiency of TC, solutions of 0.1 M H_3PO_4 (pH 1.0) containing concentrations of NaCl ranging from 0 to 2 M were used as the acceptor solution. Fig. 6 shows that the enrichment factors increased with the increase in NaCl concentrations. Maximal enrichment factors were observed at NaCl concentrations of 1.0 M and above. As such, the addition of salt could have resulted in an increase in the ionic strength of the solution and contributing to the decrease of the solubility of tetracycline in the donor phase. Consequently, partitioning of the analytes into the organic phase (supporting liquid membrane) might be enhanced, and hence their migration towards the acceptor phase was facilitated (Bahrami et al. 2017; Jia et al. 2017). Therefore, in subsequent experiments sample solutions of 0.1 M H_3PO_4 (pH 1) containing 1.0 M NaCl were used as the acceptor phase solution. Du et al. (2014) reported similar findings, where the enrichment factor and relative recoveries increased as the concentration of NaCl increased from 0% to 30% (w/v) in the extraction of methomyl from water samples using PT-MIM-µ-SPE.

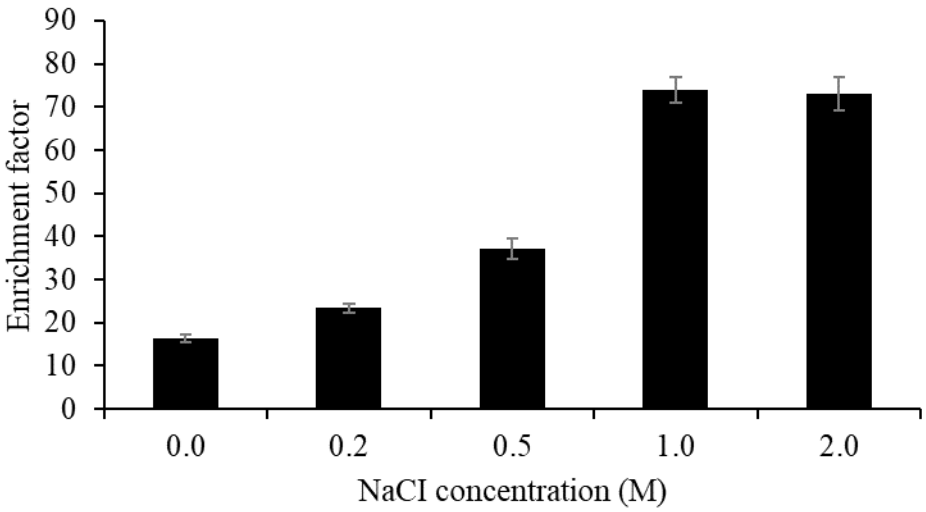

Fig 6 Effect of salt addition on the extraction efficiency of tetracycline (n = 3, RSD). Extraction conditions: 15 mL of 500 µg L^{-1} TC in 0.05 M Na_2HPO_4 solution (pH 9.5) as the donor phase solution, acceptor phase solution: 0.1 M H_3PO_4 (pH 1.0), stirring rate: 400 rpm, extraction time: 15 min.

Effect of agitation speed

The study of the effect of agitation speed was carried out from 100 to 900 rpm. As shown on Fig. 7, it was observed that an increase in agitation speed from 100 to 700 rpm resulted in an increase in enrichment factors. As agitation speed increases, mass transfer between the donor and the acceptor phase increase due the increase in the kinetic energy of the analytes (Ncube et al. 2016). However, very high stirring rates can result in the production of excessive air bubbles and solvent loss, and hence affecting enrichment factors (Shariati et al. 2009). This might be the explanation for the decrease in enrichment factors at the agitation speed of 900 rpm. Therefore, the agitation speed of 700 rpm was used in subsequent experiments. Mondal et al. (2019) reported similar results, where 600 rpm was considered an optimum agitation speed for the solid phase micro-extraction of antibiotic multiresidues from fish muscle.

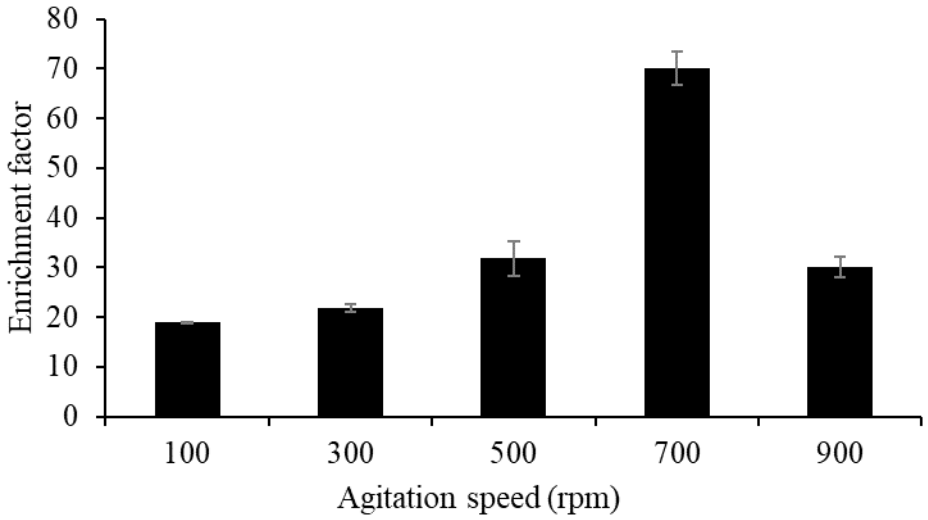

Fig 7 Effect of the agitation speed on the extraction efficiency of tetracycline (n = 3, RSD). (Extraction conditions: 15 mL of 500 µg L^{-1} TC in 0.05 M Na_2HPO_4 solution (pH 9.5) as the donor phase solution, acceptor phase solution: 0.1 M H_3PO_4,1M NaCl (pH 1.0), extraction time: 15 min.

Effect of extraction time

The effect of extraction time on the extraction efficiency of the proposed method was investigated in the range of 15 - 90 min. It was observed that the enrichment factors were increasing as the extraction time increased from 15 min to 45 min (Fig. 8). The enrichment factors decreased at extraction times higher than 45 min. This trend could be due to that at equilibrium time, analytes in the acceptor phase are in a steady state and remain at their maximum concentration (Bahrami et al. 2017). A decrease in the enrichment factors at longer extraction times could be due to loss of the organic solvent in pores of hollow-fibre (Esrafili et al. 2012). Similar results were reported by Xu et al. (2017) where 40 min was reported as the optimum extraction time for the extraction of tetracyclines from milk samples.

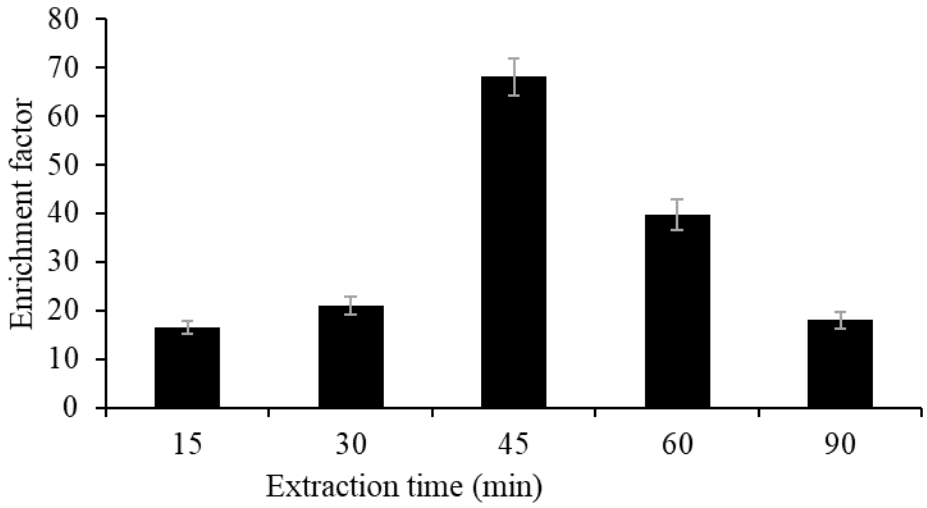

Fig 8 Effect of the extraction time on the extraction efficiency of tetracycline (n = 3, RSD). (Extraction conditions: 15 mL of 500 µg L^{-1} TC in 0.05 M Na_2HPO_4 solution (pH 9.5) as aqueous sample, acceptor solution: 0.1 M H_3PO_4, 1M NaCl (pH 1.0), stirring rate: 700 rpm.

Method validation and method applicability to honey samples

Method performance of the proposed three-phase HFLPME technique for the five tetracyclines (tetracycline, chlortetracycline, oxytetracycline, doxycycline and methacycline) was evaluated under the optimized conditions as described above (donor phase: 15 mL of 0.05 M Na_2HPO_4 (pH of 9.5), acceptor phase: 0.1 M H_3PO_4 and 1.0 M NaCl (pH 1.0), stirring rate: 700 rpm and extraction time: 45 min). Linearity, limit of detection (LOD), limit of quantification (LOQ), dynamic linear range and the recoveries were determined. Table 2 summarises these determined figures of merit. The ratio of each tetracycline peak area to the internal standard peak area was used for the quantification of these analytes.

Linearity was evaluated from matrix-matched calibration curves that were prepared by spiking blank honey samples with five tetracyclines. Linearity in the range of concentrations 20 - 500 µg L^{-1} was obtained with correlation coefficients ranging from 0.9918 to 0.9998 as indicated in Table 2. An average enrichment factor at each calibrator spiking level was estimated as the overall enrichment factor for each tetracycline. The LOD and LOQ were calculated from the matrix

matched calibration curves and they ranged from 0.042 to 0.661 µg kg^{-1} and, from 0.127 to 2.002 µg kg^{-1}, respectively.

Mean recoveries of spiked blank honey samples at three levels (75, 200 and 300 µg L^{-1}) ranged from 56.1% to 120.8% as shown on Table 3. The method showed good repeatability with RSDs ranging between 0.37% and 11.2%. Oxytetracycline showed the lowest recoveries. Figure 9 shows the total ion chromatogram of spiked honey samples at a concentration of 100 µg L^{-1}. The applicability of the proposed HFLPME technique was investigated by analyzing two commercial and one wild honey samples. Only doxycycline residues were detected in one of the commercial honey sample at a concentration level of 0.20 µg kg^{-1} which was lower than the MRL set by the European Union in other food products such as meat and liver from food producing animals. Therefore, the honey was suitable for human consumption.

Table 2 Summary of method validation parameters (five spiking concentrations each at n = 3)

Analyte	Matrix based regression equation	R^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Dynamic linear range	Enrichment factor
Tetracycline	y = 0.184x - 0.0184	0.9998	0.092	0.278	20 - 500	65
Chlortetracycline	y = 0.770x + 0.1259	0.9982	0.308	0.934	20 - 500	86
Doxycycline	y = 0.990x + 0.1621	0.9918	0.661	2.002	20 - 500	105
Oxytetracycline	y = 0.255x - 0.0017	0.9997	0.042	0.127	20 - 500	58
Methacycline	y = 0.759x + 0.1566	0.9939	0.150	0.453	20 - 500	104

Table 3 Recoveries of TCs determined in spiked honey samples (n = 3)

Analyte	75 µg kg^{-1}		200 µg kg^{-1}		300 µg kg^{-1}	
	Mean Recovery (%)	RSD (%)	Mean Recovery (%)	RSD (%)	Mean Recovery (%)	RSD (%)
Tetracycline	65.3	5.61	65.4	9.06	63.3	6.73
Chlortetracycline	105.2	11.21	89.1	5.75	63.9	10.19
Doxycycline	110.0	1.86	120.8	2.41	85.5	10.45
Oxytetracycline	56.1	8.96	61.0	7.95	57.0	6.41
Methacycline	112.7	2.76	101.7	5.05	97.9	0.37

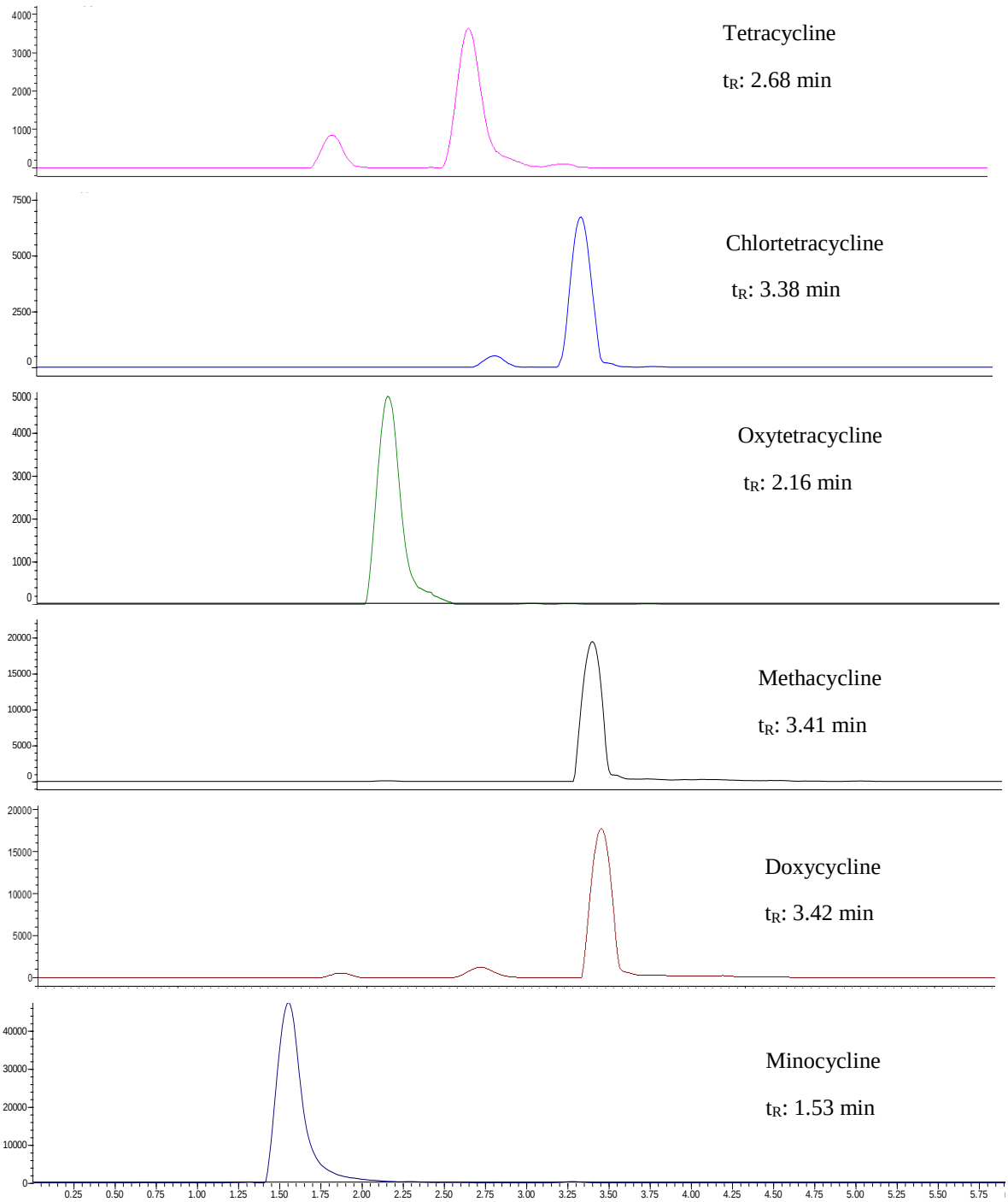

Figure 9 Chromatograms of spiked blank honey sample at a concentration of 100 µg L^{-1} for each tetracycline

Comparison of the proposed method with other methods developed

A comparison of other published extraction methods for the analysis of tetracycline antibiotics from different food samples was evaluated in terms of the LODs and the enrichment factors to establish the efficiency of the proposed three phase HFLPME. Several extraction techniques for the clean-up and pre-concentration of tetracyclines in various food samples of animal origin have been developed over the years. Table 4 shows the summary of comparative studies. It was noted that a comparison between the LODs of the proposed method and those of its liquid phase extraction methods counterparts in literature, showed higher sensitivity and good enrichment factors for this method. The obtained LODs of the proposed HFLPME method are lower than those obtained in studies done by Rodríguez et al. (2016) and Santana et al. (2018) where DLLME was employed for the extraction of TCs from food samples. This could be attributed by the methods of detection used in these studies. Enrichment factors (58 to 105) obtained in this study are comparable to those obtained in studies presented on Table 3 except for those in a study conducted by Saei et al. (2020) where two extraction methods were coupled, hence resulting in much higher enrichment factors.

Table 4 Comparison of the proposed method with other methods developed

Tetracyclines	Matrix	Extraction method	Detection method	LOD	Enrichment factor	Reference
5 Tetracyclines	Honey	HFLPME	LC-Q-TOF/MS	0.042-0.661 µg kg^{-1}	58-105	This study
OTC	Sausage	SI-LLHE-SFO-DLLME	Ion mobility spectrometer	1.52-2.73 ng g^{-1}	1260-1720	Saei et al. (2020)
TC, CTC, MTC and DXC	Honey	DLLME	LC-MS/MS	0.12-0.45 µg L^{-1}	25-98	Kaynaker et al. (2018)
TC, CTC, OTC, and DXC	Honey	DLLME	UFLC-DAD	6.3-6.8 µg kg^{-1}	100-128	Santana et al. (2018)
DMC, DXC, CTC, MTC, TC and OTC	Beef	DLLME	LC-MS/MS	2.2-3.6 µg kg^{-1}	-	Mookantsa et al. (2016)
TC	Honey	LLE	LC-MS/MS	0.1-9.2 µg kg^{-1}	-	Louppis et al. 2017
TCs	Honey	LLE	UPLC-ESI-MS	0.15-0.54 µg kg^{-1}	-	Kivrak et al. (2016)
TC, OTC, DXC and CTC	Egg supplements	US-DLLME	Flow injection analysis	6.4-11.1 µg L^{-1}	-	Rodríguez et al. (2016)
TC, DXC, MTC, OTC and CTC	Milk, eggs and honey	SUPRAS-LPME	HPLC-UV	0.7-3.4 µg L^{-1}	48-198	Gissawong et al. (2019)

-Enrichment factor not given

3. Conclusion

The proposed method involved a simple, sensitive, rapid and low cost pre-concentration technique for the determination of tetracycline residues in honey using the LC-Q-TOF/MS. Doxycline was the only tetracycline detected in limits safe for human consumption in one of the honey samples. The carrier mediated three phase HFLPME showed satisfactory enrichment factors which ranged from 58 to 105 and the limits of detection were lower than the MRLs of TCs in foods, under the optimum conditions. In contrast to conventional methods, this method exhibited higher enrichment factors. Moreover, low volumes of organic solvents were used, hence to some extent, this method was environmentally friendly. Therefore, it can be concluded from the obtained results that the developed HFLPME-LC-Q-TOF/MS method could be utilized as a favourable alternative to conventional methods for the analysis of antibiotics in food samples.

Conflict of interest

Authors declare no interest of conflict.

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4.2. Paper V

This paper "Pre-concentration of sulfonamides from manure samples using molecularly imprinted polymer miniaturized pipette-tip extraction" will be submitted to *Journal of Chromatographia*. The presence of sulfonamide residues in manure samples from a cattle farm was investigated in this paper.

Pre-concentration of sulfonamides from manure samples using molecularly imprinted polymer miniaturized pipette-tip extraction

Babra Moyo [a,b] and Nikita T. Tavengwa [a,*]

[a]Department of Chemistry, School of Mathematical and Natural Sciences, University of Venda, Private Bag X5050, Thohoyandou, South Africa, 0950

[b]Department of Food Science and Technology, School of Agriculture, University of Venda, Private Bag X5050, Thohoyandou, South Africa, 0950

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

Abstract

The presence of antibiotics in animal manure is a concern worldwide because residues of these drugs may be released into the environment and result in the development of antibiotic-resistant pathogens. Therefore, it is necessary to monitor the occurence of these drugs in manure in order to mitigate the dissemination of these drugs into the environment. The aim of this study was to develop, optimize and validate a selective analytical method employing molecularly imprinted polymer based miniaturized pipette-tip extraction of sulfonamide residues from manure samples prior to analysis on the LC-Q-TOF/MS. Molecularly imprinted polymers were synthesized by precipitation polymerization using sulfamethoxazole as a template and characterized using FTIR. Parameters that could affect the extraction efficiency of the proposed extraction method including number of aspirating and desorption cycles, sample pH, sorbent amount, salt addition and type of elution solvent were optimized. The developed method was validated and linearity in the range of 20-400 µg L^{-1} was obtained with regression coefficients ranging between 0.9881 and 0.9990, under the optimized conditions. The recoveries of blank manure samples at three spiking levels (100, 200 and 300 µg L^{-1}) ranged from 67.73% to 120.48% with RSDs between 0.08% and 15.3%. LODs and LOQs were in the ranges of 0.111 to 0.319 µg kg^{-1} and 0.336 to 0.966 µg kg^{-1}, respectively. Finally, the proposed method was successfully applied for the extraction of five sulfonamides from cattle manure samples. Sulfamethoxazole and sulfamethoxypyridazine were detected at concentration levels of 0.1083 and 0.065 µg kg^{-1}, respectively.

Keywords: Miniaturized pipette-tip extraction, sulfonamides, molecularly imprinted polymers, manure, pre-concentration, selectivity

Introduction

Sulfonamides is one of the common classes of antibiotics that is widely used for the treatment of various bacterial infections in humans and veterinary medicine (Mehdi, 2015; Nunes et al., 2018). Moreover, sulfonamides are used as growth promotants in animal husbandry (Qiu et al., 2016; Dmitrienko et al., 2014). A substantial fraction (30-90%) of these drugs are excreted mainly as the parent compounds or as metabolites via faeces or urine, and hence residues of these compounds may enter the environment either by application of manure to agricultural fields as a fertilizer or in the form of sludge after manure collection and storage (Jiang and Wang, 2017; Quaik et al., 2020; Qian et al., 2016). Consequently, these drugs may either accumulate or be readily available for transport into surface and groundwater through leaching or water run-off when they reach the upper soil layer (Pan and Chu, 2017; Mohameda et al., 2017). Furthermore, antibiotic residues may be taken up by plants, hence, resulting in potential growth risks, seed germination suppression and also affect terrestrial fauna dependent on these plants (Kumar et al., 2005; Hanna et al., 2018; Carter et al., 2014). Previous studies have shown that degradation of antibiotics occur to a certain extent during composting and anaerobic fermentation (Sun et al., 2016; Oliver et al., 2020; Ravindran and Mnkeni, 2017), therefore, these waste management methods are inefficient in the removal of these compounds from manure. Subsequently, if these antibiotic residues are not completely degraded they may result in development of antibiotic resistant microbial populations in the environment (Andersson and Hughes, 2014; Heuer et al., 2011; Beyene, 2016).

Therefore, the contribution of manure as a carrier of antibiotic residues and antibiotic resistant bacteria and genes has become a concern globally (Jansen et al., 2019; Oliver et al., 2020). However, the accurate determination of antibiotics in manure samples is a challenge due to the complexity of this matrix and the varying physico-chemical properties of antibiotics from different classes (Wu et al., 2015; Qian et al., 2016). Therefore, developing efficient extraction and pre-concentration techniques for antibiotic residues is imperative. Several extraction methods such as dispersive solid phase extraction (Rashid et al., 2020), solid phase extraction (Patyra et al., 2020; Guo et al., 2016; Jansen et al., 2019) and QuEChERS (Wang et al., 2020) have been used for the

pre-concentration of these drugs in manure. Miniaturized pipette-tip extraction (m-PTE) is one of the most promising and simplest techniques of miniaturized SPE (Du et al., 2014; da Silva et al., 2017). It offers advantages such as small amounts of the sorbent, small volumes of the samples and the elution solvent and reduced extraction time hence proving to be compliant with principles of green analytical chemistry (Tavengwa et al., 2016; Yan et al., 2014; Brigante et al., 2017; Corazza et al., 2017).

Decreased hydrophobicity and the low degrees of extraction of sulfonamides usually affects the extraction and pre-concentration of these drugs (Dmitrienko et al., 2014). As a result, the efficiency and selectivity of sulfonamides extraction can be improved by using molecularly imprinted polymers (MIPs). MIPs are ideal for the selective extraction of compounds at trace levels, particularly when the sample is complex due to the strong interaction between this sorbent and target analytes (Zhao et al., 2018). The use of MIPs as sorbents in the selective extraction of antibiotics from various complex matrices has grown significantly in the past few years (Moreno-Gonzalez et al., 2017; Kechagia., 2018; Zhao et al., 2018). However, the application of this sorbent in the extraction of antibiotic residues in manure is still limited.

The application of m-PTE to the clean-up of sulfonamides from food samples has emerged in the past years (Yan et al., 2014). However, to the best of our knowledge, this technique has not been applied to the analysis of sulfonamides in manure samples so far. Manure being a substantial source of pollution in the environment by pharmaceutical drugs, is currently not actively monitored. There are few reports, if any, of monitoring antibiotic residues in animal waste or in the environment at the moment. Due to this gap in knowledge worldwide, the aim of this study is to investigate sulfonamide residue levels in dairy cows' manure. Extraction and pre-concentration of sulfonamides was done using m-MIP-PTE followed by quantification on the UV/Vis spectrophotometer and LC-Q-TOF/MS.

Materials and methods

Chemicals and reagants

Sulfamethoxazole (≥98%), sulfadiazine (99%), sulfamethoxypyridazine (99.8%), sulfachloropyridazine (≥ 98%), sulfaquinoxaline (99.6%), oxytetracycline (96%), methacrylic acid, azo-bis-(isobutyronitrile) and ethylene glycol dimethacrylate were purchased from Sigma-

Aldrich (Johannesburg, South Africa). Methanol, acetonitrile, hydrochloric acid, sodium hydroxide and acetic acid were purchased from Merck (Johannesburg, South Africa). All chemicals were of analytical reagent grade. LC-MS grade methanol, acetonitrile and ultrapure water were supplied by Monitoring and Control Laboratories (Johannesburg, South Africa).

Instrumentation

An LC-Q-TOF/MS 9030 mass spectrometer (Shimadzu, Japan) with an electrospray interface (ESI) operating in positive mode was used for all mass spectral measurements. The mass range was 100-1000 m/z and Table 1 shows the optimum ESI parameters for each sulfonamide. Quantification of the sulfonamides was carried out based on the most abundant pre-cursor to product ion transitions in the multi reaction monitoring mode. High-purity nitrogen was used as a nebulizing and drying gas with the following parameters used: drying gas flow, 10 L min^{-1} and drying gas temperature, 250°C. A Shimadzu 9030 LC instrument consisting of an autosampler, thermostated column compartment and a binary pump (Shimadzu, Japan) was employed for chromatographic separation. Sulfonamides were separated using a Shimpack C_{18}, 2.1 x 100 mm, 2.7 µm column from Shimadzu (Honeydew, South Africa). Lab solutions software was used for data acquisition. The absorption spectra and absorbance for the optimization experiment of the extraction method in this study were obtained by using a Biowave II UV/Visible spectrophotometer (Biochem, England) with 2 cm quartz cuvettes. All pH measurements were done using a Basic 20 pH meter (Crison, Germany). A V-1 plus vortex mixer (Bueco, Germany), an ultrasonic bath (Labotec, South Africa) and a Universal 320R centrifuge (Hettich, Germany) were used for sample preparation. Structural information of the synthesized MIP and NIP was obtained by Fourier transform infrared spectroscopy (FTIR), using a Bruker spectrometer (Billerica, USA).

Table 1 Optimum MRM parameters of six sulfonamide drugs

Analyte	Molecular mass (g mol^{-1})	Precursor ion (m/z)	Product ion (m/z)	Collision energy (volts)	t_R (min)
Sulfachlorpyridazine	284.70	285.02	156.01	15	2.66
Sulfadiazine	250.30	251.06	156.01	15	2.36
Sulfamethoxypyridazine	280.30	281.07	156.01	15	2.63
Sulfamethoxazole	253.30	254.06	156.01	15	2.67
Sulfaquinoxaline	300.30	301.07	156.01	20	2.79
Sulfabenzamide	276.31	277.06	108.04	20	2.72

Synthesis of the MIP and NIP

A modified method by Baeza Fonte et al. (2018) was used for the synthesis of the polymers. Briefly, an amount of 0.5 mmol of the template (sulfamethoxazole) was weighed into a round bottom flask where 70 mL of methanol (porogen) and 0.422 mL of methacrylic acid (monomer) were added and the mixture was stirred for 10 min. Approximately, 1.9 mL of ethylene glycol dimethylacrylate (cross-linker) was added, and the mixture was purged with nitrogen gas for 2 min. Approximately, 50 mg of 2,2-azobis-(isobutylronitrile) was added and the mixture was purged for another 2 min, and the flask was closed and the mixture was heated in an oil bath at 80°C for 4 h. The final product was filtered through Whatman filter paper no. 4. For comparison, the non-imprinted polymer (NIP) was also synthesized as described above but without the sulfamethoxazole template. NIP and MIP particles were dried in a drying oven overnight at 55°C. The template was eluted from the obtained MIP particles using a column for 72 h using methanol/acetic acid (9:1, v/ v) as an elution solvent. Finally, the MIP and NIP particles were dried at 110°C for 2 h before use.

Assembling of the m-MIP-PTE

For assembling the pipette-tip cartridge, 100 µL and 2.0 mL pipette-tips were employed as shown on Figure 1. Glass wool was used on one end of the cartridge to avoid MIP loss before packing approximately, 3.0 mg of the MIP or NIP into the 100 µL pipette tip followed by a connection to the cut 2.0 mL tip. The cartridge was conditioned succesively with methanol (1.0 mL) and water (1.0 mL) prior extraction.

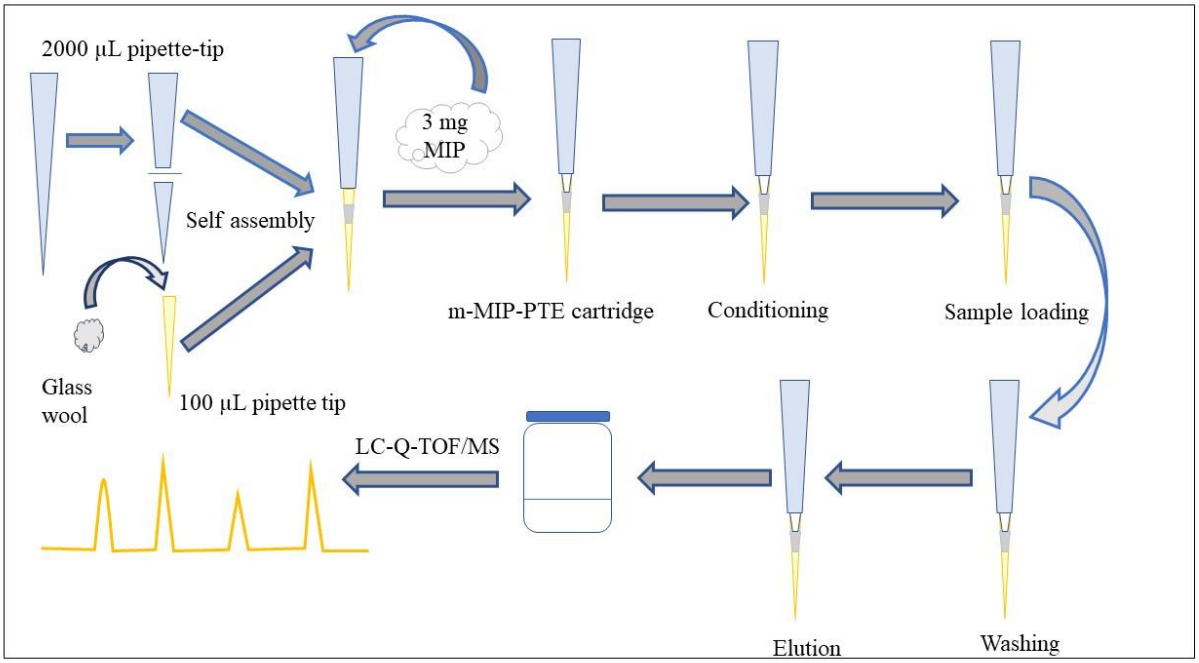

Figure 1 Schematic illustration of the m-MIP-PTE set-up and procedure

Stock and working standard solutions preparation

Sulfonamide stock solutions were prepared in methanol at a concentration of 1 mg mL^{-1} and were stored at 4°C until ready for analysis. Twelve series of the working standard solutions at the concentration values of 0.01 to 2 mg L^{-1} were prepared from the sulfamethoxazole stock standard solutions by diluting with 50% (v/v) methanol for the optimization of m-MIP-PTE method. For method validation and application, matrix-matched of five sulfonamides standards (sulfachlorpyridazine, sulfadiazine, sulfamethoxypyridazine, sulfamethoxazole and sulfaquinoxaline) were prepared in blank manure samples at six spiking levels from 20 to 400 µg L^{-1}, to compensate for matrix effects. Sulfabenzamide was employed as an internal standard and was added to standards and samples for the method validation, and application steps. The final concentration of the internal standard was 100 µg L^{-1}.

Sampling of manure, extraction and clean-up

A sample of manure was collected from a local commercial cattle farm after stirring manually in the manure pits for a minimum of 30 min. About 500 mL of the manure was sampled into plastic

containers and transported to the laboratory where they were homogenized by stirring manually before dry matter was determined by air drying until constant mass was reached.

A modified method by Li et al. (2019) was used for sample preparation. Approximately, 2 g of manure samples were weighed into 50 mL PTFE centrifuge tubes, 10 mL methanol/acetic acid 9:1 (v/v) was added and the samples were immediately homogenized for 2 min using a vortex mixer. After sonicating in an ultrasonic bath for 15 min at 30°C, the mixtures were shaken again for another 2 min and centrifuged for 15 min at 5000 rpm. The supernatants were decanted, and the remaining residue was extracted using the same procedure two more times. Finally, all supernatants were collected and filtered. The pH of the sample was adjusted with 0.5 M HCl or 0.5 M NaOH to 6.0 before extraction. A volume of 1 mL of the obtained supernatant was aspirated into a conditioned cartridge, washed with water (1 mL) and eluted with 1 mL of methanol/acetic acid (9:1, v/v). The sample was filtered through 0.22 μm Nylon filters before analysis on the LC-Q-TOF/MS.

Results and discussion

Synthesis of the MIP and elution of the template

There should be interactions between the template molecule and methacrylic acid, a functional monomer, during MIP synthesis. However, interactions including electrostatic binding and hydrogen bonding between the amino group of the template and the acid groups of the monomer can be disrupted at elevated temperatures (Arabi et al., 2017), therefore, MIPs were synthesized by precipitation polymerization at 80°C in this study. A white precipitate started forming after 15 min of heating the reaction mixture and increased gradually with time. White powder was obtained after drying the MIP and NIP.

The template was eluted using a mixture of methanol/acetic acid (9:1, v/v) in a column and aliquots of the eluate were collected every 10 min and read at 285 nm on the UV/Vis spectrophotometer until sulfamethoxazole could not be detected. Figure 2 shows the set-up during elution. The concentration of the aliquots at each wash was calculated using the equation of the calibration curve ($y = 0.3715x + 0.0232$), where $R^2 = 0.9986$ was obtained. A cyclic decay curve (Figure 3) was obtained as the washings were done successively on different days. As the MIP

was left overnight soaking in the elution solvent, swelling of the network could have been induced and, at the same time, favouring the dissolution of the drug hence sulfamethoxazole could be detected in successive washings of the following day, despite, non-detection of the drug on the last wash of the previous day.

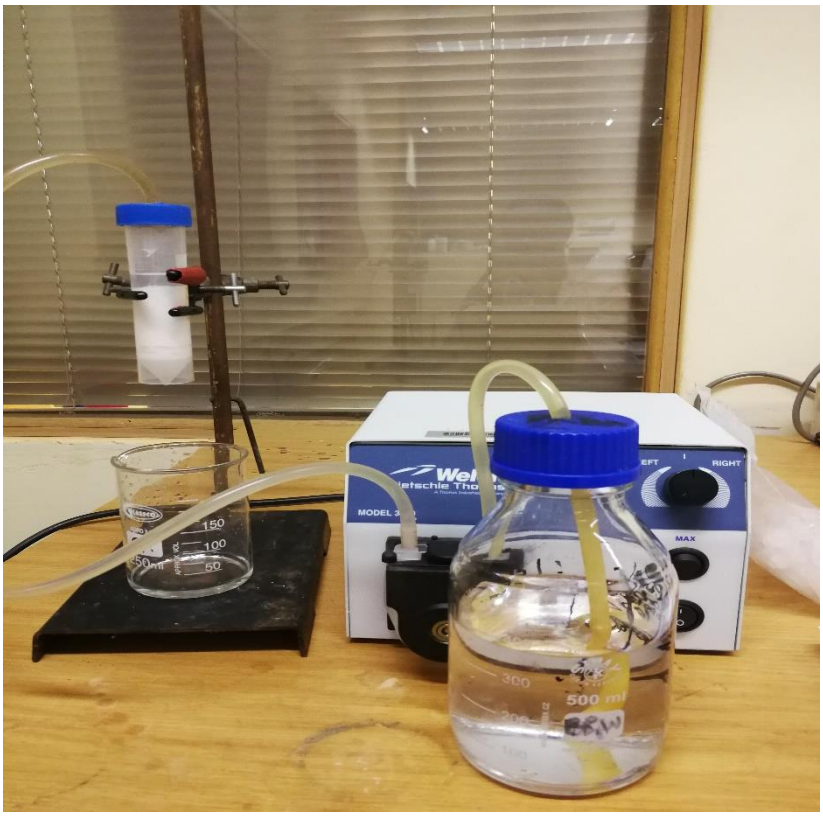

Figure 2 Elution of the template set-up

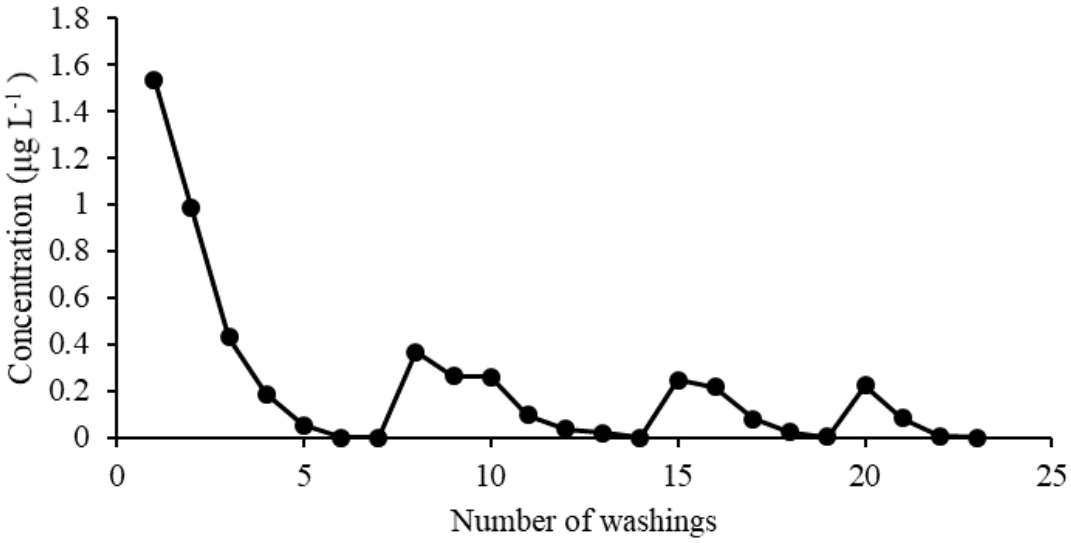

Figure 3 Sulfamethoxazole elution from the MIP

Characterization of the MIP and the NIP

Functional group analysis

Figure 4 shows the FTIR spectra of the MIP before and after elution against the NIP and sulfamethoxazole. Sulfamethoxazole showed characteristic absorption bands at 1594.94 cm^{-1} for the N-H of the primary amine group, 1616.94 cm^{-1} for C=O connected to the secondary amide group, 3142.90 cm^{-1} for C-H of the aromatic ring and 3376.27 cm^{-1} for N-H of the sulfonamide group. As shown on the spectrum, no N-H and aromatic rings absorption bands were observed for the NIP since it did not contain the template. The FTIR spectra of the MIP after elution was almost similar to that of the NIP, which indicated that the template was successfully eluted from the polymer matrix. The MIP prior template elution showed comparable absorption bands at 3389.44 cm^{-1}, 1621.33 cm^{-1}, and 1594.94 cm^{-1} with sulfamethoxazole, however, these characteristics weakened or totally disappeared after elution. Asymmetric stretching of C-O-C, asymmetric deformation peak of C-H and symmetric stretching of C=O were observed, respectively, at about 1147.38 cm^{-1}, 1453.96 cm^{-1} and 1716.77 cm^{-1}, exhibiting structural characteristics of EGDMA on the NIP. Similar peaks were observed on the MIP before and after elution. The presence of the

above characteristic peaks demonstrated that the MIP was successfully synthesized and the template was also efficiently eluted.

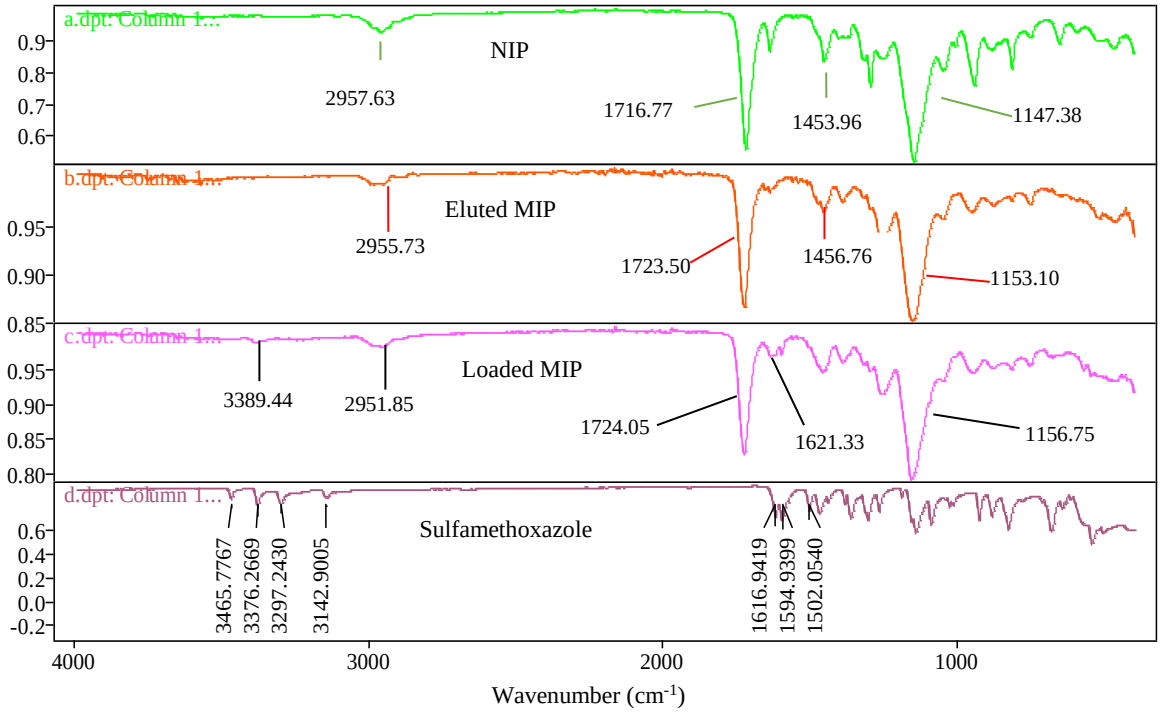

Figure 4 Fourier Transform Infrared (FT-IR) spectra of (a) NIP (b) MIP after sulfamethoxazole elution (c) MIP before sulfamethoxazole elution and (d) sulfamethoxazole

Optimization of parameters affecting the extraction efficiency of the m-MIP-PTE method

The effect of different parameters that can affect the extraction efficiency of the m-MIP-PTE method were investigated. Number of loading/desorption cycles, sample pH (adjusted using 0.5 M HCl and 0.5 M NaOH), mass of the MIP, salt addition and the type of elution solvent were the optimized parameters. A solution of 500 µg L^{-1} sulfamethoxazole (1 mL) was used in all the optimization experiments. The desorption step was performed using 1 mL of the elution solvent. All experiments were done in triplicate and the recovery (%) was calculated according to the following equation:

$$\text{Recovery (\%)} = \frac{C_e}{C_o} \text{ x } 100 \qquad (1)$$

where C_0 is the concentration of the original sulfamethoxazole solution and C_e is the concentration of sulfamethoxazole desorbed from the MIP.

Effect of aspirating cycles

In this study the effect of aspirating cycles on the extraction efficiency of the proposed method was investigated by aspirating the sulfamethoxazole standard at different loading cycles (10 to 150). As shown on Figure 5, it was observed that increasing the number of loading cycles also increased the recoveries of sulfamethoxazole. However, there was no significant increase in sulfamethoxazole recoveries beyond 25 aspirating cycles. This might be due to the fact that the adsorption sites on the MIP were saturated with sulfamethoxazole after 25 aspirating cycles. As the recoveries beyond 25 aspirating cycles exhibited no significant increase, this value was taken as the optimum for use in subsequent experiments. Similar results were obtained by Tavengwa et al. (2016) where the studied nitroaromatic compounds were maximally recovered by the MIP after 20 aspirating/dispensing cycles of the sample.

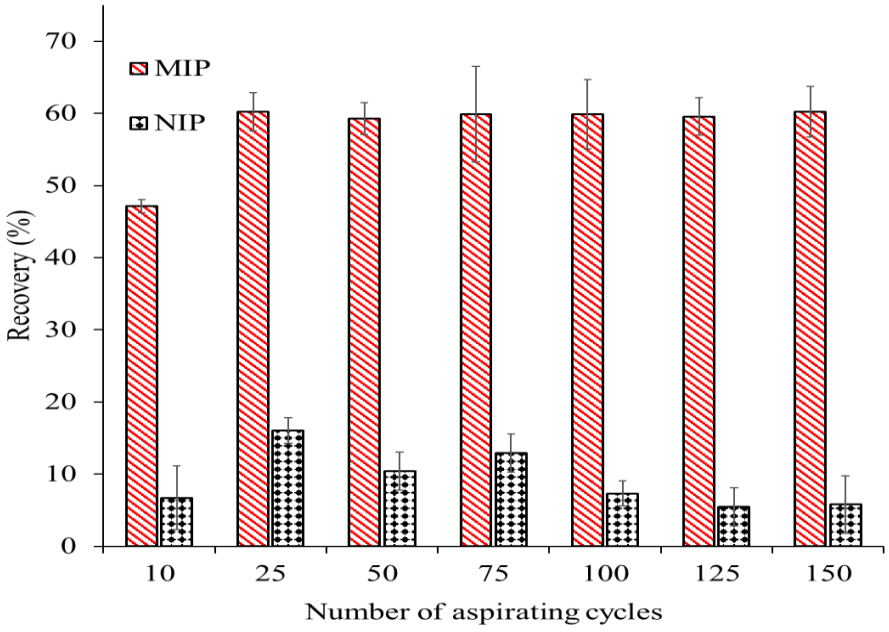

Figure 5 Effect of loading cycles on extraction efficiency (n = 3, RSD). Extraction conditions: [sulfamethoxazole] = 500 µg L^{-1}, number of desorption cycles = 15, sample pH = 3, sorbent mass = 3 mg, no NaCl addition, methanol/acetic acid 9:1 (v/v) as the eluent.

Effect of desorption cycles

The desorption experiment was investigated from 5 to 75 cycles and it was observed that 25 cycles resulted in the highest recoveries of sulfamethoxazole as shown on Figure 6. Therefore, 25 desorption cycles were applied in subsequent experiments. The re-absorption of sulfamethoxazole by the MIP (An et al., 2018) might have resulted in the decrease in recoveries as the number of desorption cycles increased. Similar results were reported by Corazzo et al. (2017) where increasing the number of extraction cycles also increased the mass of extracted analyte in the determination of phenolic endocrine-disrupting compounds from environmental water samples.

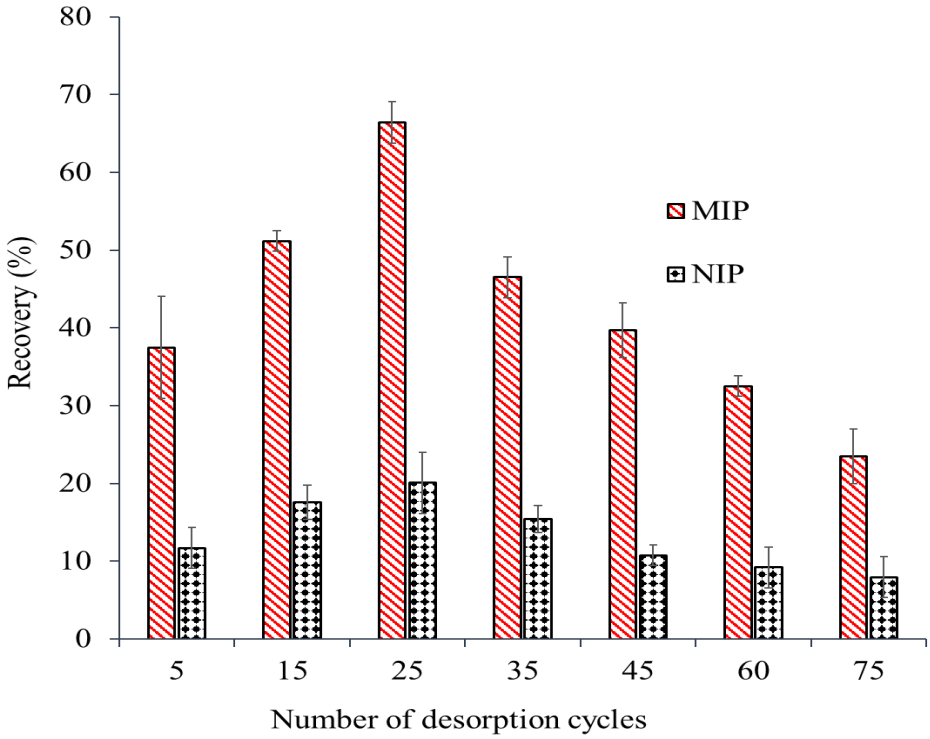

Figure 6 Effect of desorption cycles on extraction efficiency (n = 3, RSD). Extraction conditions: [sulfamethoxazole] = 500 µg L^{-1}, number of aspirating cycles = 25, sample pH = 3, sorbent mass = 3 mg, no NaCl addition, methanol/acetic acid 9:1 v/v as the eluent.

Effect of sample pH

The influence of the sample pH on the extraction efficiency of the proposed was evaluated using solutions of 500 μg L^{-1} sulfamethoxazole at pH values ranging from 3 to 11. The recoveries of the sulfamethoxazole were close to 76% at pH 6.0 and decreased at pH values above this (Figure 7). According to the pK_a value of sulfamethoxazole (pK_a = 5.81), this might be due to that this analyte was in a molecular state under these conditions, which could increase its adsorption capacity. The binding capacity of sulfamethoxazole under alkaline conditions (pH > 7) decreases because most sulfonamides exist in an ionic state at high pH ranges (Zhao et al., 2018). Consequently, a sample pH of 6.0 was selected for further study. Similar results were obtained by Zhao et al. (2018) who extracted 6 sulfonamides using MIP-SPE from animal derived foods at pH 6.

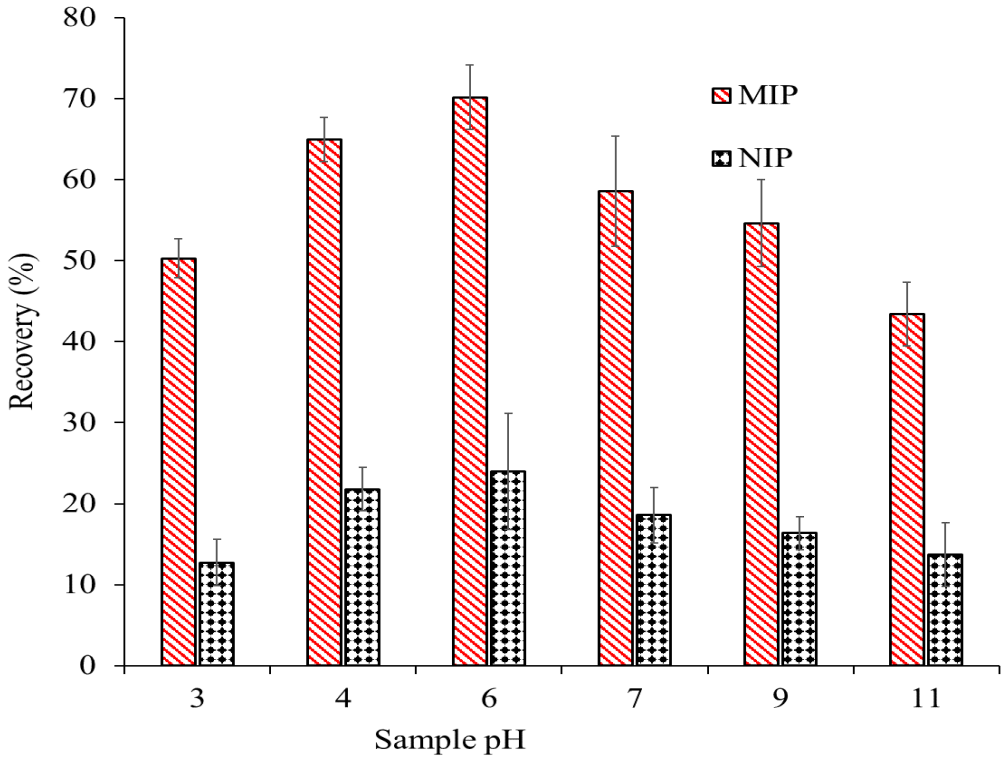

Figure 7 Effect of sample pH on extraction efficiency (n = 3, RSD). Extraction conditions: [sulfamethoxazole] = 500 μg L^{-1}, number of aspirating cycles = 25, number of desorption cycles = 25, sorbent mass = 3 mg, no NaCl addition, methanol/acetic acid 9:1 v/v as the eluent.

Effect of sorbent mass

Amounts of the MIP/NIP ranging from 1.0 to 12.0 mg were used in the investigation of the effect of sorbent mass on the extraction efficiency of the extraction technique under this study. Figure 8 shows that satisfactory recoveries were obtained for the MIP amounts of up to 3.0 mg and further

increases in the sorbent mass had no obvious influence on the analyte recoveries. An increase in sorbent mass may result in higher recoveries, however, additional packing material can result in clogging of the cartridge (Corazza et al., 2017). Therefore, 3.0 mg was selected as the optimum sorbent loading amount. These findings correspond to those of Yan et al. (2014) where a graphene mass of 3 mg was used for the extraction of sulfonamides from bovine milk samples.

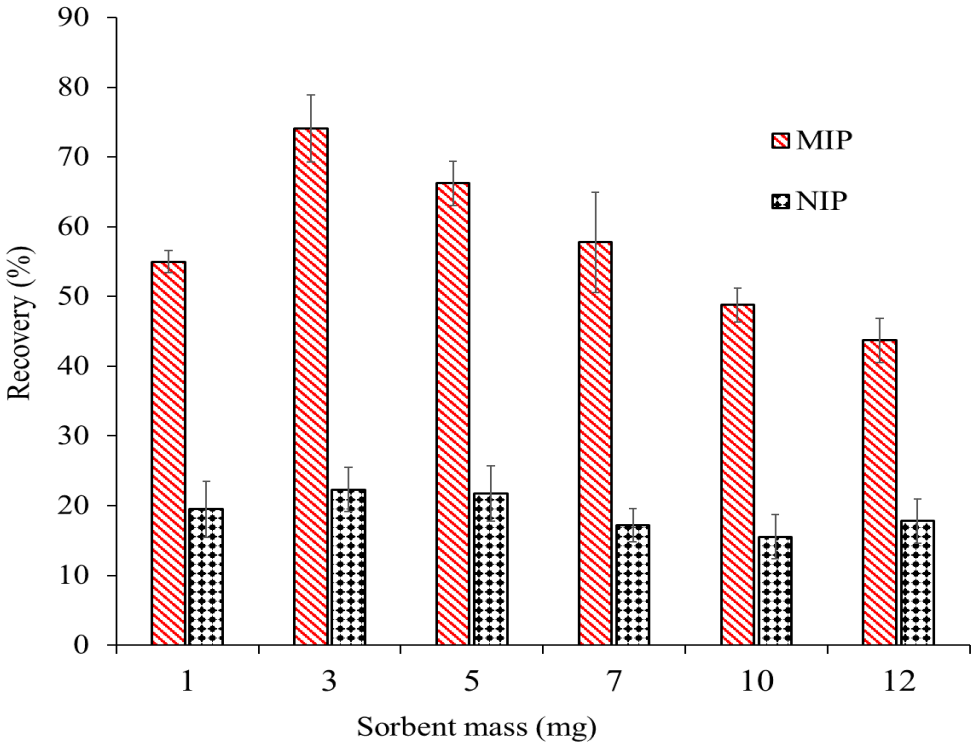

Figure 8 Effect of sorbent mass on extraction efficiency (n = 3, RSD). Extraction conditions: [sulfamethoxazole] = 500 μg L^{-1}, number of aspirating cycles = 25, number of desorption cycles = 25, sample pH = 6, no NaCl addition, methanol/acetic acid 9:1 v/v as the eluent.

Effect of salt addition

In this study, concentrations of 0, 0.2, 0.5, 1 and 2M of NaCl were evaluated for their influence on the extraction efficiency of m-MIP-PTE and it was found that the recoveries of all analytes gradually declined with the increase of NaCl concentration as shown on Fig 9. This could be due to the fact that the addition of salt increased the viscosity and the density of the sample matrix, hence, hindering the mass transfer of the analytes to the sorbent (Jia et al., 2017). In view of this,

no salt addition was done in subsequent experiments. These findings correspond to those by Jia et al. (2017) where salt addition resulted in lower recoveries in the dispersive micro-solid phase extraction of sulfonamides from milk samples using metal organic frameworks.

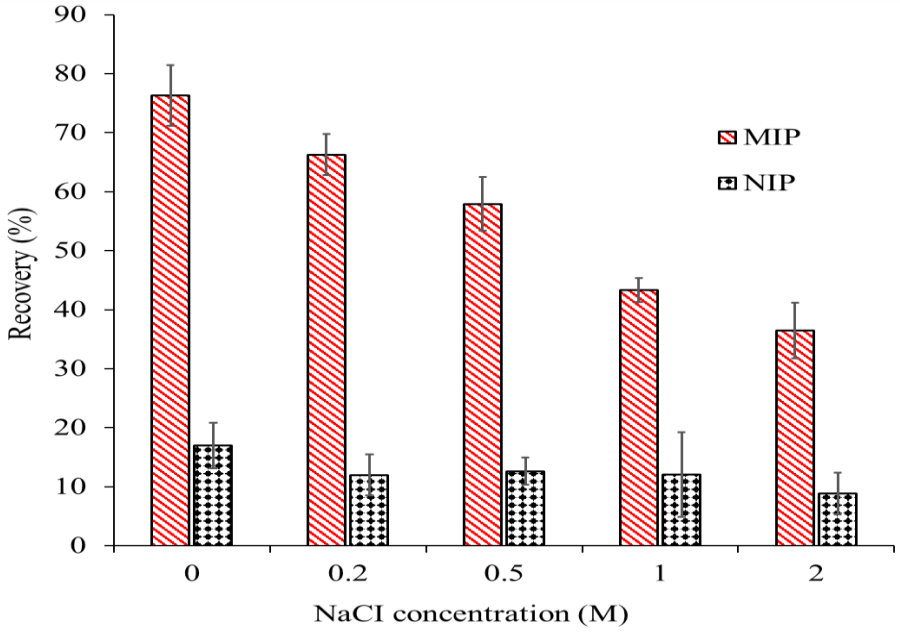

Figure 9 Effect of salt addition on extraction efficiency (n = 3, RSD). Extraction conditions: [sulfamethoxazole] = 500 μg L^{-1}, number of aspirating cycles = 25, number of desorption cycles = 25, sample pH = 6, sorbent mass = 3 mg, methanol/acetic acid 9:1 v/v as the eluent.

Effect of the type of elution solvent

Various elution solvents including methanol, methanol/acetic acid 9:1 v/v; methanol/ammonia 9:1 v/v, acetonitrile/acetic acid 9:1 v/v and acetonitrile/ammonia 9:1 v/v were studied in this experiment. As it can be seen on Figure 10, the recoveries obtained using methanol/acetic acid 9:1 v/v exhibited higher recoveries, and hence this solvent was used for further experiments. Compared to methanol elution alone, methanol systems modified by ammonia or acetic acid increased the elution ability. This might be due to that alkaline and acidic conditions are favorable for the ionization of sulfamethoxazole, thus reducing the affinity of this analyte for the MIP and facilitating elution (Arabi et al., 2017; Yan et al., 2014). Methanol/acetic acid (9:1, v/v) resulted in the highest recovery and this might be due to that acetic acid is more effective in breaking hydrogen bonding between sulfamethoxazole and the MIP and thus leading to its easier removal (Arabi et al., 2017). The elution ability of acetonitrile with modifiers was inferior to methanol.

Therefore, methanol/acetic acid 9:1 v/v was used as the optimal elution solvent. Chen et al. (2009) reported similar findings where magnetic MIPs were employed as a sorbent in extraction of sulfonamides from honey and acidified methanol was used as an elution solvent.

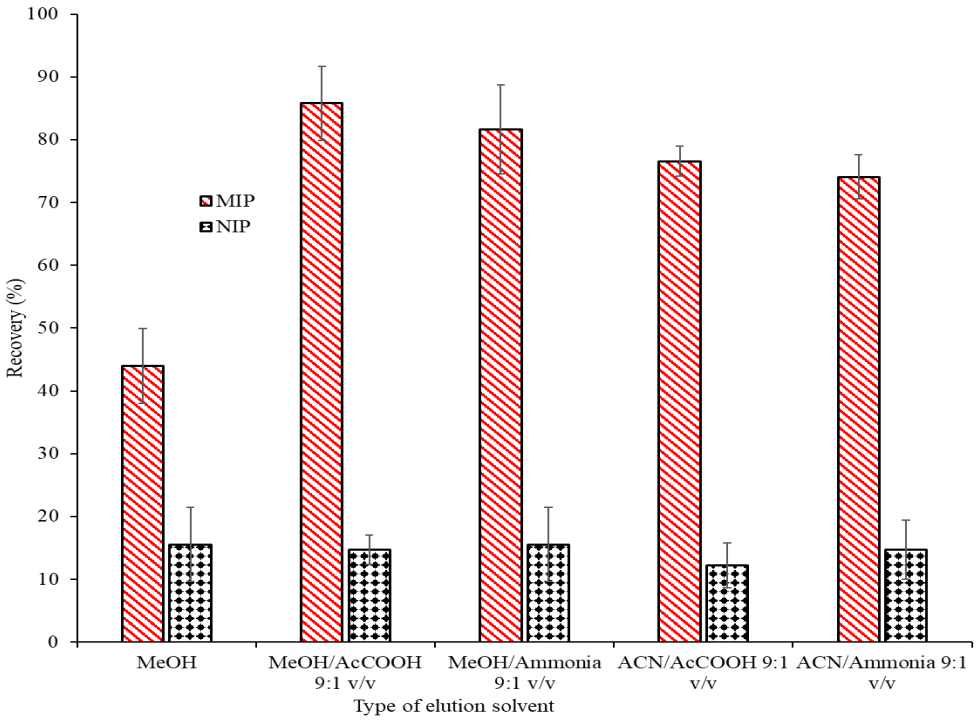

Figure 10 Effect of type of eluent solvent on extraction efficiency (n = 3, RSD). Extraction conditions: [sulfamethoxazole] = 500 µg L^{-1}, number of aspirating cycles = 25, number of desorption cycles = 25, sample pH = 6, sorbent mass = 3 mg, no NaCl addition.

Static binding capacity

The static binding capacity was measured by aspirating 1 mL sulfamethoxazole solutions in methanol with concentrations (100, 250, 400, 500, 750 and 1000 µg L^{-1}) and the binding capacity of the MIP or NIP was calculated by Equation (2). The binding capacity increased with increasing sulfamethoxazole initial concentrations, as depicted on the adsorption isotherm curve (Figure 11). Moreover, it was observed that the binding capacities of the MIP was higher than that of the NIP, where the maximum adsorption capacity of 83.89 µg g^{-1} of the MIP was close to 1.62 times that of the NIP (51.67 µg g^{-1}) at the initial concentration of 1000 µg L^{-1}. The binding mechanisms of MIP could be attributed to the selective and specific adsorption of the sulfamethoxazole molecules

at the imprinted recognition sites on the sorbent surface, whereas the adsorption pattern of the NIP could be due to nonspecific binding properties of this polymer.

$$Q = \frac{(C_i - C_f)}{m} \text{ x } V \qquad (2)$$

where Q is the adsorption capacity (µg L^{-1}), C_i is the initial concentration of sulfamethoxazole (µg L^{-1}), C_f is the final concentration of sulfamethoxazole, V is the volume of the initial sulfamethoxazole solution (L) and m is the mass of the MIP or the NIP (g).

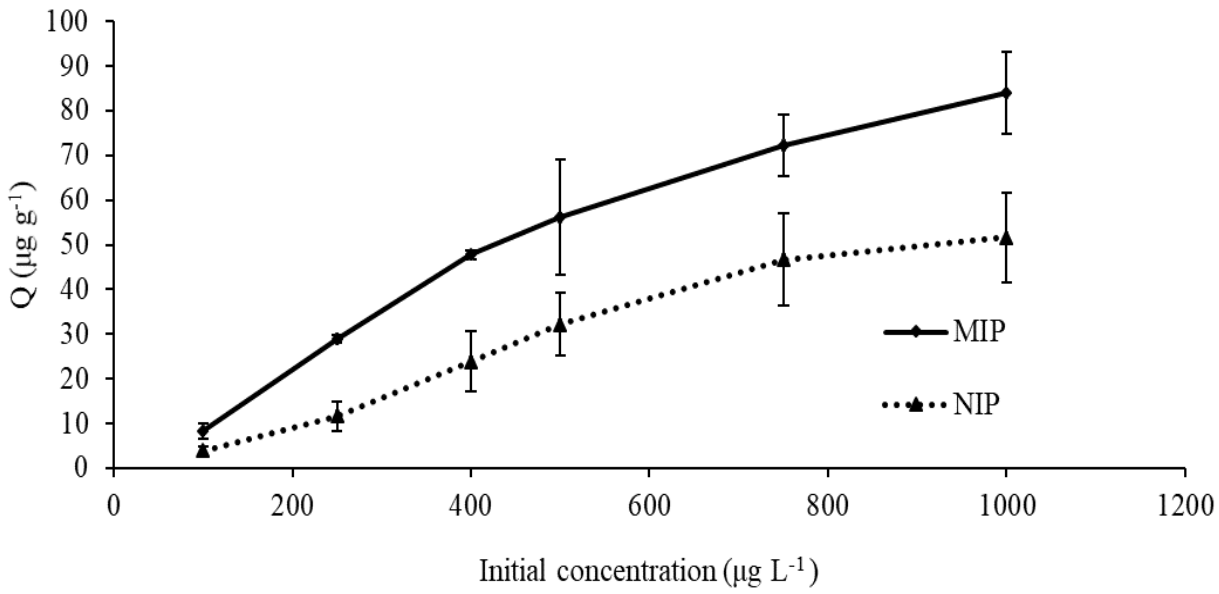

Figure 11 Effect of initial concentration on the binding capacity of the MIP and NIP (n = 3, RSD). Experimental conditions: sample volume = 1 mL, desorption solvent volume = 1 mL, sample pH = 6 and sorbent mass = 3 mg.

Adsorption selectivity of the MIP

For this experiment, the cartridge was loaded with 1 mL of a mixed substrate methanol solution (containing 250 µg L^{-1} of sulfamethoxazole, sulfadiazine, sulfamethoxypyridazine, sulfachloropyridazine, sulfaquinoxaline and oxytetracycline) and desorption of the analytes was

done with 1 mL methanol/acetic acid 9:1 (v/v). The resulting solution was filtered through 0.22 µm syringe filter prior to analysis on the LC-Q-TOF/MS. The selectivity of the MIP was evaluated by the obtained recoveries. Figure 12 clearly shows that the recoveries of the five sulfonamides on the MIP are much higher (75-115%) than that of oxytetracycline (20%). These findings might be due to the existence of tailor-made cavities on which sulfamethoxazole and it's structural analogs can selectively bind, hence the synthesized sorbent can be applied for the extraction and enrichment of these five sulfonamides.

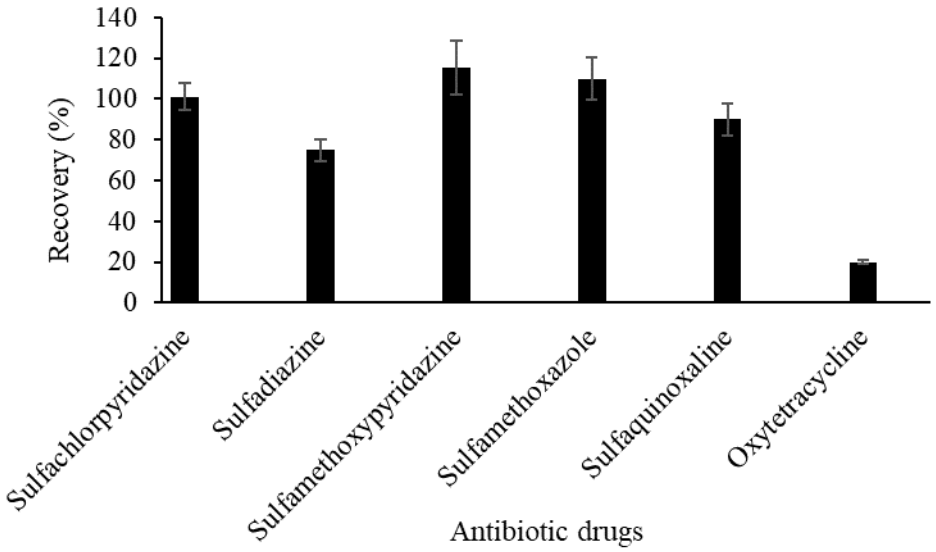

Figure 12 Selectivity adsorption test of the MIP (n = 3, RSD). Experimental conditions: [sulfonamides] = 250 µg L^{-1}, sample volume = 1 mL, desorption solvent volume = 1 mL, sample pH = 6 and sorbent mass = 3 mg.

MIP reusability

MIPs were used to rebind the blank sample spiked with a mixture of the five sulfonamides at a concentration of 250 µg kg^{-1} in five consecutive adsorption-desorption cycles to evaluate the reusability of this sorbent. No significant reduction in recoveries was observed after five times repeated adsorption-desorption operations as shown on Figure 13. The slight loss in recoveries of sulfadiazine could be due to a decrease in binding sites that occurs as a result of repeated loading and elution cycles (Zhao et al., 2018). Generally, the obtained results indicated that the synthesized MIPs can be suitable for reuse in at least five cycles in real applications.

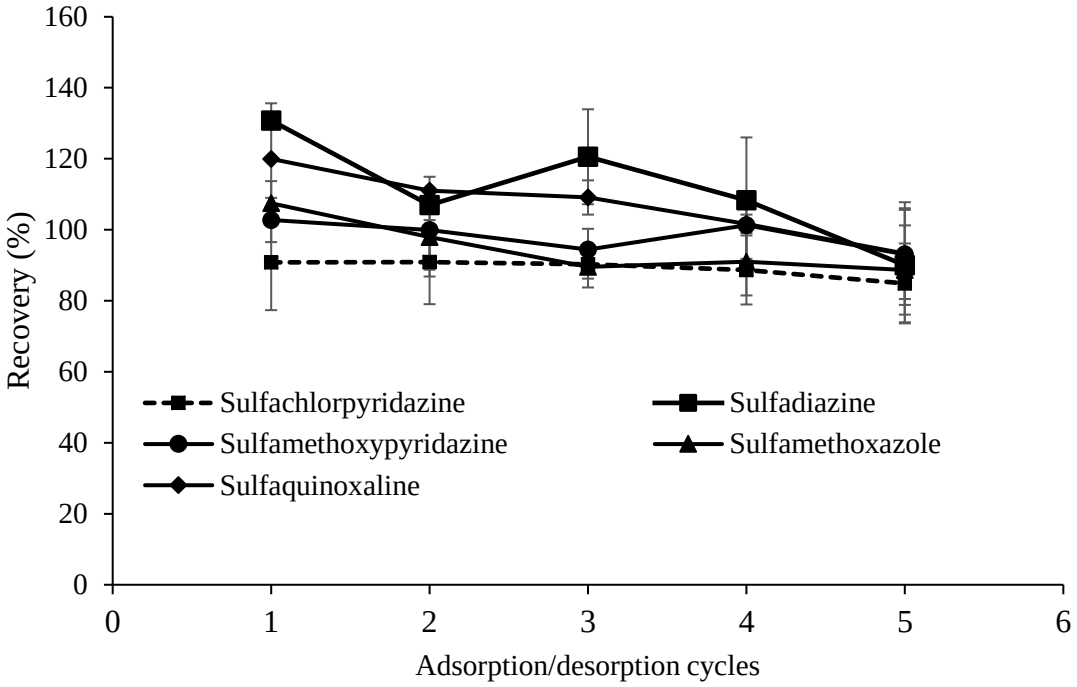

Figure 13 Recoveries of five sulfonamides of five adsorption-desorption cycles. Experimental conditions: [sulfonamides] = 250 µg L^{-1}, sample volume = 1 mL, desorption solvent volume = 1 mL, sample pH = 6 and sorbent mass = 3 mg.

Method validation and applicability to real samples

Method performance of the proposed extraction technique was evaluated based on linearity, limit of detection (LOD), limit of quantification (LOQ), linear range and recoveries under the optimized conditions. Table 2 summarises the calculated figures of merit. Quantification of the analytes in

this study, was calculated from the ratio of each sulfonamide peak area to the internal standard peak area.

Linearity was evaluated from matrix-matched calibration curves that were prepared by spiking blank manure samples with sulfonamides. Linearity in the range of concentrations, 20 - 400 µg L^{-1} was obtained with correlation coefficients ranging from 0.9881 to 0.9990 as indicated in Table 2. The LODs and LOQs were calculated from the matrix matched calibration curves and they ranged from 0.111 to 0.319 µg kg^{-1} and, from 0.336 to 0.966 µg kg^{-1}, respectively. Mean recoveries of spiked blank manure samples at three levels (100, 200 and 300 µg L^{-1}) ranged from 67.73% to 120.83% as shown on Table 3 and the method showed good repeatability with RSDs ranging between 0.08% and 15.35%. Figure 14 shows the total ion chromatogram of spiked manure samples at a concentration of 100 µg L^{-1}. The applicability of the proposed m-MIP-PTE technique was investigated by analyzing a manure sample from a local cattle commercial farm. Sulfamethoxazole and sulfamethoxypyridazine residues were detected in the manure samples at concentration levels of 0.1083 µg kg^{-1} and 0.065 µg kg^{-1}, respectively.

Table 2 Summary of validation parameters of the five sulfonamides.

Analyte	Matrix based regression equation	R^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Linear range
Sulfachloropyridazine	y = 0.6881x + 0.0489	0.9990	0.1107	0.3355	20 - 400
Sulfadiazine	y = 0.4820x - 0.0981	0.9881	0.2752	0.8339	20 - 400
Sulfamethoxypyridazine	y = 1.6833x - 0.0910	0.9986	0.3188	0.9661	20 - 400
Sulfamethoxazole	y = 0.4762x + 0.1421	0.9970	0.3150	0.4091	20 - 400
Sulfaquinoxaline	y = 0.4189x + 0.1547	0.9882	0.2379	0.7209	20 - 400

Table 3 Recoveries of spiked manure samples by the m-MIP-PTE method (n = 3)

Analyte	100 µg kg^{-1}		200 µg kg^{-1}		300 µg kg^{-1}	
	Mean Recovery (%)	RSD (%)	Mean Recovery (%)	RSD (%)	Mean Recovery (%)	RSD (%)
Sulfachlorpyridazine	90.06	2.27	101.27	2.72	89.99	4.25
Sulfadiazine	67.73	12.34	75.16	5.82	69.93	15.35
Sulfamethoxypyridazine	77.19	1.44	85.20	2.10	76.13	11.07
Sulfamethoxazole	91.91	10.44	109.21	0.08	95.49	7.46
Sulfaquinoxaline	118.57	2.11	120.83	9.70	101.03	1.59

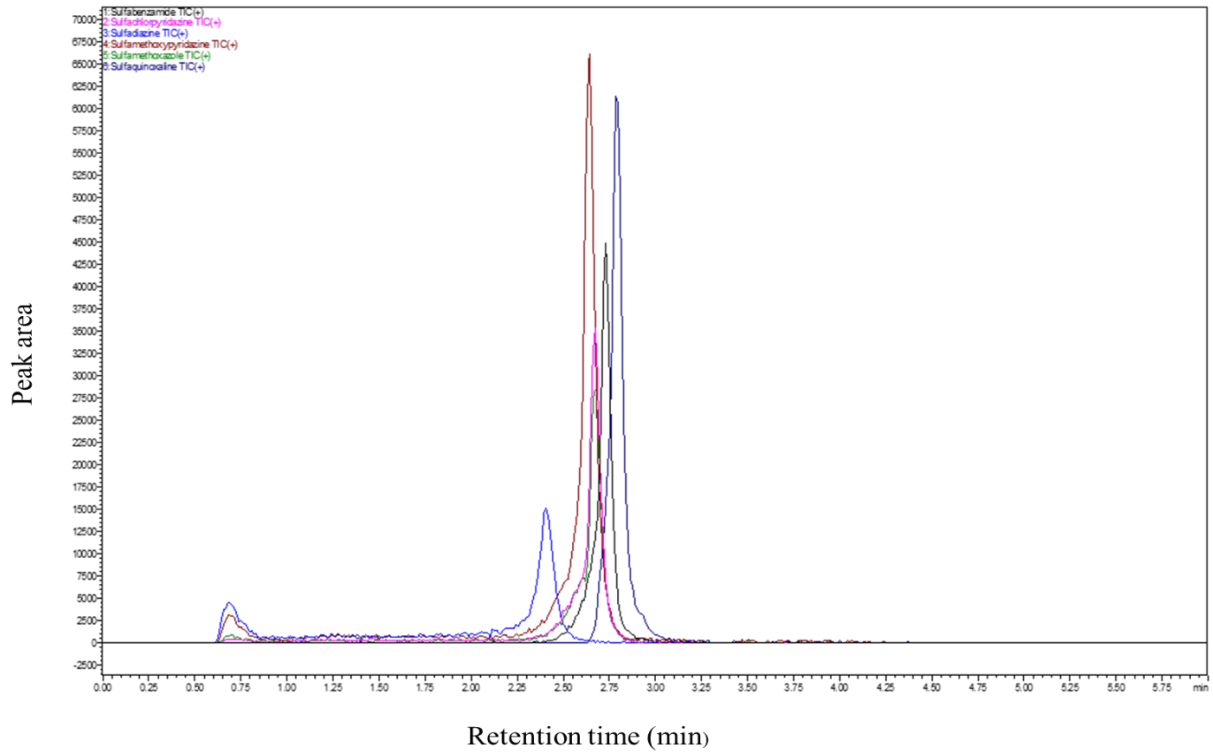

Figure 14 Chromatogram of spiked blank manure sample at a concentration of 100 µg L^{-1} for each sulfonamide

Comparison of the proposed method with other methods developed

A comparison of published SPE methods for the extraction of sulfonamides from manure samples was performed in terms of the LODs and recoveries to further evaluate the efficiency of the proposed m-MIP-PTE technique. SPE is the most widely used clean-up technique for manure samples in literature where commercial conventional sorbents such as Oasis HLB and Strata-X are the commonly used. Table 4 shows a summary of comparative studies. It was noted that a comparison between the LODs of the proposed method and those of SPE methods in literature, showed higher selectivity. Recoveries obtained in this study are comparable to those obtained in studies presented on Table 4.

Table 3 Comparison of the proposed method with other reported SPE based methods for the detection of sulfonamides in cattle manure

Sulfonamide	Extraction method	Sorbent	Detection method	LOD	Recovery (%)	Reference
5 sulfonamides	m-PTE	MIPs	LC-Q-TOF/MS	0.111-0.319 μg kg^{-1}	67.73-120.83	This study
5 sulfonamides	SPE	Commercial Strata-X-CW	LC-MS/MS	15.5-92.0 μg kg^{-1}	63-93	Patyra et al. (2020)
18 sulfonamides	SPE	Commercial Strata X	LC-MS/MS	5-50 μg kg^{-1}	83.7-147	Berendsen et al. (2015)
4 sulfonamides	SPE	Commercial Oasis HLB	HPLC-PDA	0.1-1.9 μg kg^{-1}	62.65-99.16	Feng et al. (2016)
2 Sulfanomides	SPE	Commercial Oasis HLB	HPLC-DAD/MS	-	51.57-92.64	Opriş et al. (2017)
1 sulfonamide	SPE	Commercial Oasis HLB	ELISA	26.96-78.43 μg kg^{-1}	45.0-115.0	Mohameda et al. (2017)
7 Sulfonamides	SPE	Commercial SAX/Oasis HLB	UPLC-MS/MS	0.1-0.3 ng L^{-1}	50.0-121.9	Guo et al. (2016)
5 Sulfonamides	SPE	Commercial SAX - Oasis HLB	UPLC-MS/MS	0.1-2 μg kg^{-1}	42.3-90.0	Hou et al. (2015)

Conclusion

The proposed method based on m-MIP-PTE followed by analysis on the LC-Q-TOF/MS using sulfamethoxazole as template in the synthesis of a selective adsorbent material for the extraction of five sulfonamides from manure samples showed good recoveries and exhibited adequate sensitivity and reliability. Furthermore, this technique consumes low volumes of solvents, it was fast, cheap and easy to execute. Sulfonamides were detected in concentration levels of 0.065 and 0.1083 μg kg^{-1} for sulfamethoxypyridazine and sulfamethoxazole, respectively. Finally, the comparative study showed that this extraction method has comparable analytical performance as established clean-up techniques such as SPE. Therefore, proving to be a potential alternative to conventional extraction methods that are the widely used currently.

Conflict of interest

The authors declared no conflict of interest.

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Chapter 5

General conclusions and future work

This chapter gives general conclusions based on the research findings of this work. The recommended future work is outlined also in this chapter.

5.1. Conclusions

Food contamination by antibiotic drug residues has become a concern in recent years due to the improper use of these drugs. Although, regulatory bodies such as the EU and FAO/WHO have set maximum residue limits of these drugs in food samples, the development of fast, cheap, reliable and environmentally friendly extraction methods that can be used by food industries is imperative to ensure food safety (**Paper I-V**). Despite the increasing developments in extraction methods in an attempt to comply with the principles of green analytical chemistry, most of these techniques still have some limitations. Therefore, great efforts still need to be done address shortcomings associated with most extraction that have been developed thus far.

Application of animal manure in agricultural land is not a new practice, and it has been carried out for a very long time. However, the extensive use of antibiotics in animals that produce food and the possibility of these drugs polluting the environment has raised concern worldwide due to the risks that they may pose to human health, particularly the occurrence of antibiotic resistance bacteria. Nonetheless, manure being the source of a significant part of the veterinary drug pollution in the environment, is currently not actively monitored (Paper **III** and **V**).

The two techniques that were developed in this work, were fast, cheap, easy to execute and consumed low volumes of solvents hence they are potential alternatives to conventional extraction methods that are the widely used currently. Moreover, comparative studies showed that their analytical performance is comparable to established clean-up techniques such as SPE. Doxycline was detected in honey samples at concentrations below the stipulated MRL. In cattle manure samples, sulfamethoxypyridazine and sulfamethoxazole were detected.

5.2. Future work

The commonly used methods of detection of antibiotics are chromatographic techniques, due to automation, accurate quantification, simultaneous detection and the high specificity based on the structural information of the analytes. Despite the good performance of these well-established methods, the equipment is very costly and well-trained technical staff is required to operate such instruments (Luan et al., 2020). Moreover, sample preparation prior analysis on chromatographic instruments is laborious. Therefore, biosensors have emerged in recent years. Compared to other detection techniques, biosensors are capable of performing residue screening relatively fast and accurately without the need for a specialist user. Moreover, they have strong specificities and high sensitivities, and are simple, small and, portable, hence, they can be used for on-site analysis. In contrast to most well-established extraction methods such as SPE and LLE, biosensors require minimal sample pre-treatment (Mishra et al., 2018). Moreover, conventional sample pre-treatment techniques are time consuming, expensive and some consume large volumes of organic solvent. In future work, MIPs will be employed as biosensors for the detection of antibiotic drugs from various food samples. Compared to conventional bioreceptors, MIPs offer advantages such as high robustness and stability under a wide range of conditions, ease of synthesis and low production costs. More interestingly, different active molecules can be incorporated during synthesis hence multi-residual detection can be done. At the moment, most biosensors can only detect one analyte.

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Appendix

Supplementary data

This section gives supplementary data for the manuscripts in this dissertation and a supplementary paper

Appendix A: Supplementary data

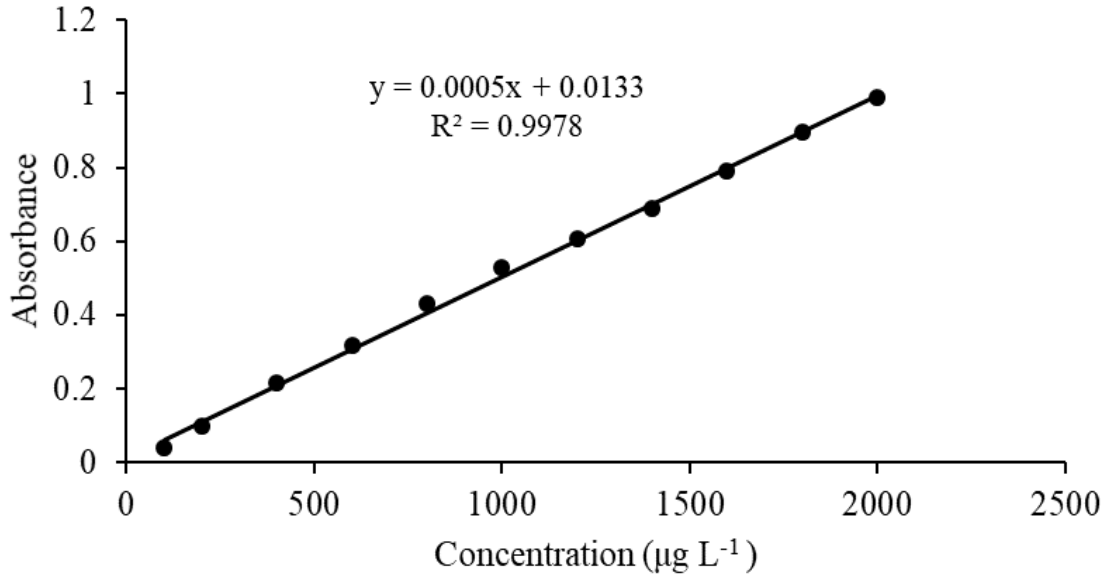

Fig. A1: Calibration curve for tetracycline

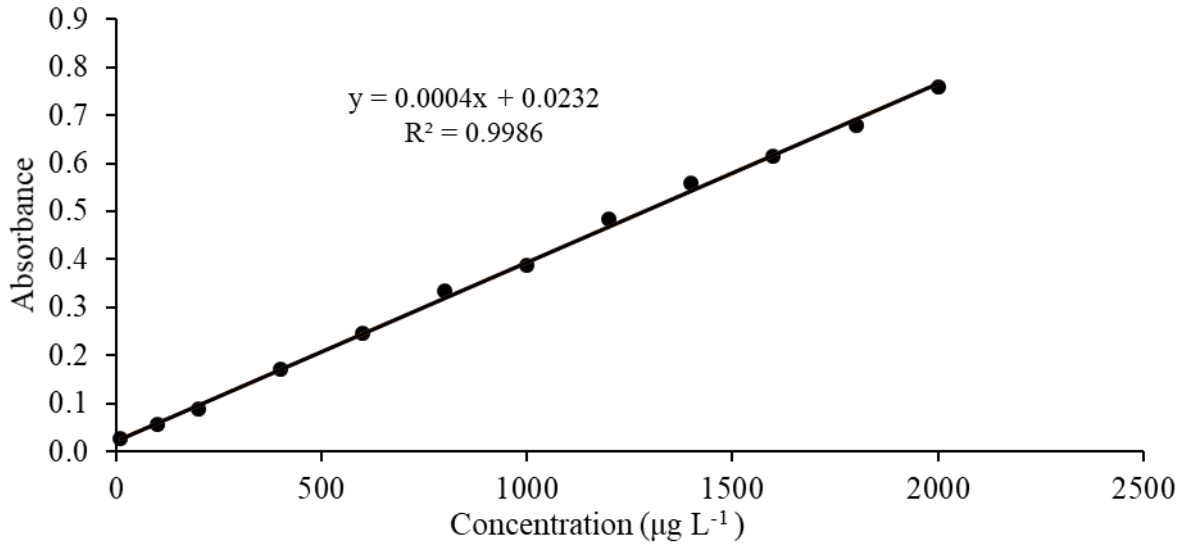

Fig. A2: Calibration curve for sulfamethoxazole

Fig. A3: Synthesis of the MIP and NIP set-up

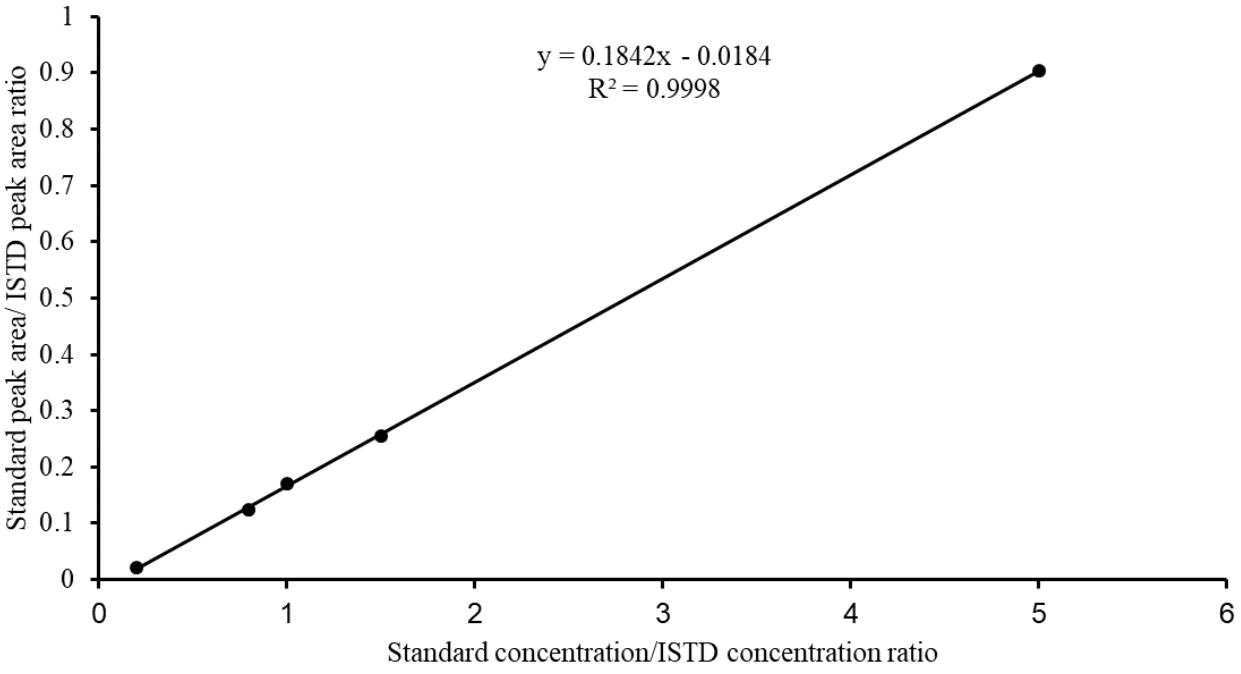

Fig. A4: Matrix matched calibration curve for tetracycline

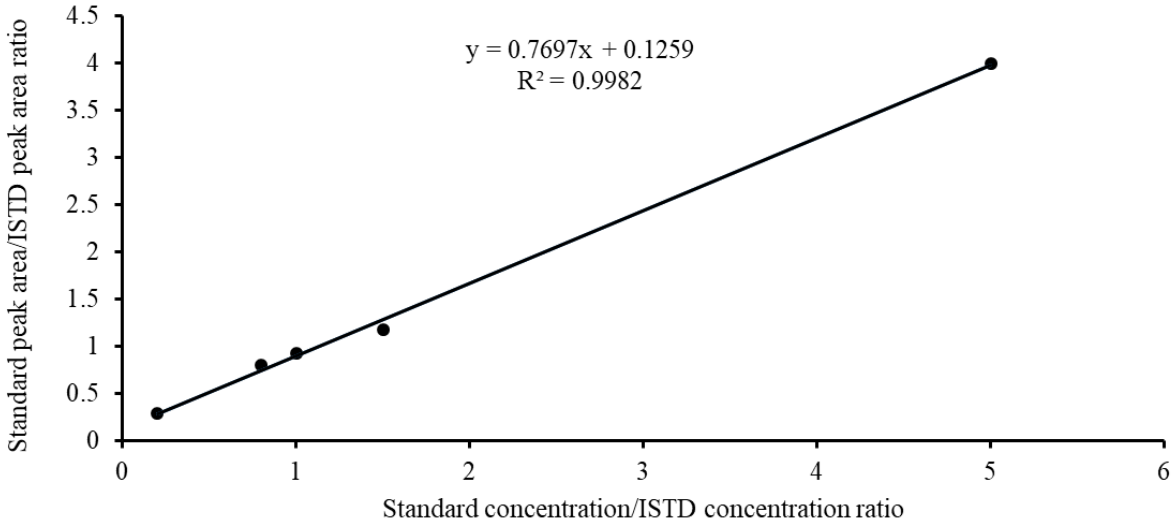

Fig. A5: Matrix matched calibration curve for chlortetracycline

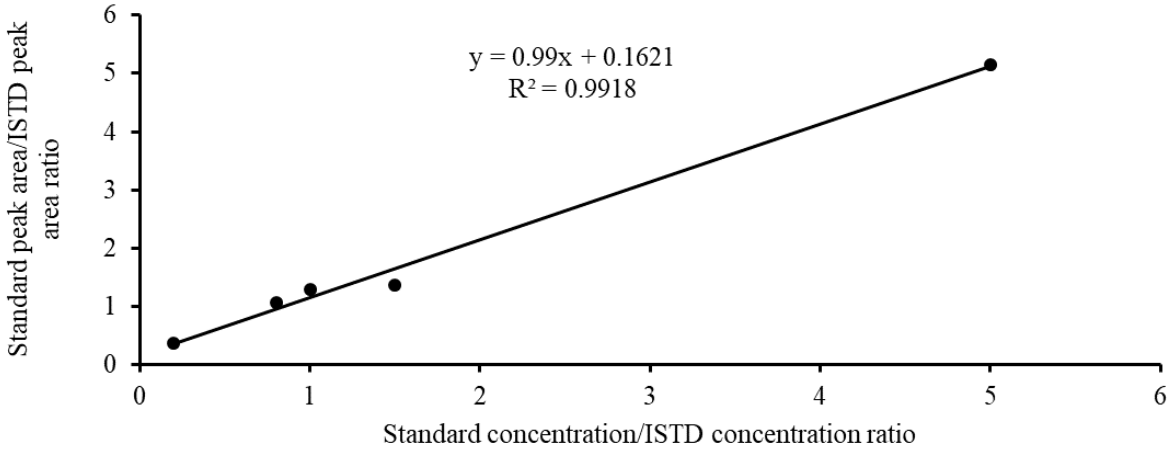

Fig. A6: Matrix matched calibration curve for doxycycline

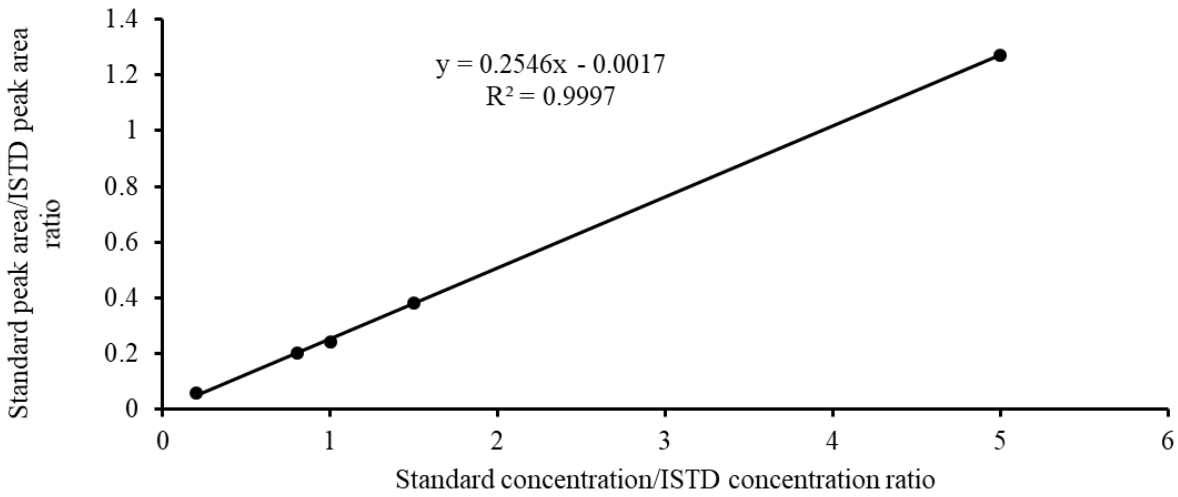

Fig. A7: Matrix matched calibration curve for oxytetracycline

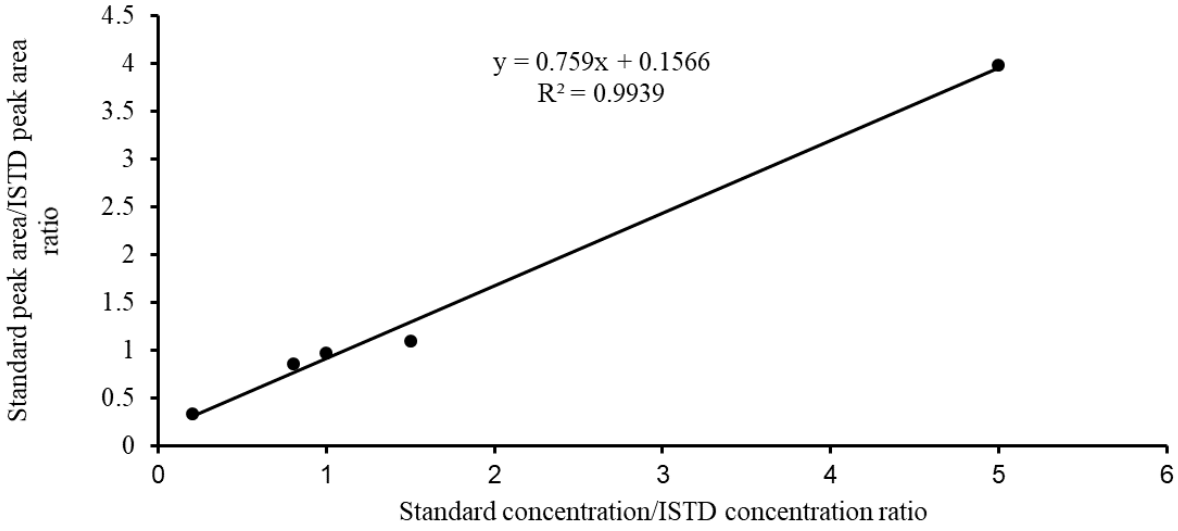

Fig. A8: Matrix matched calibration curve for methacycline

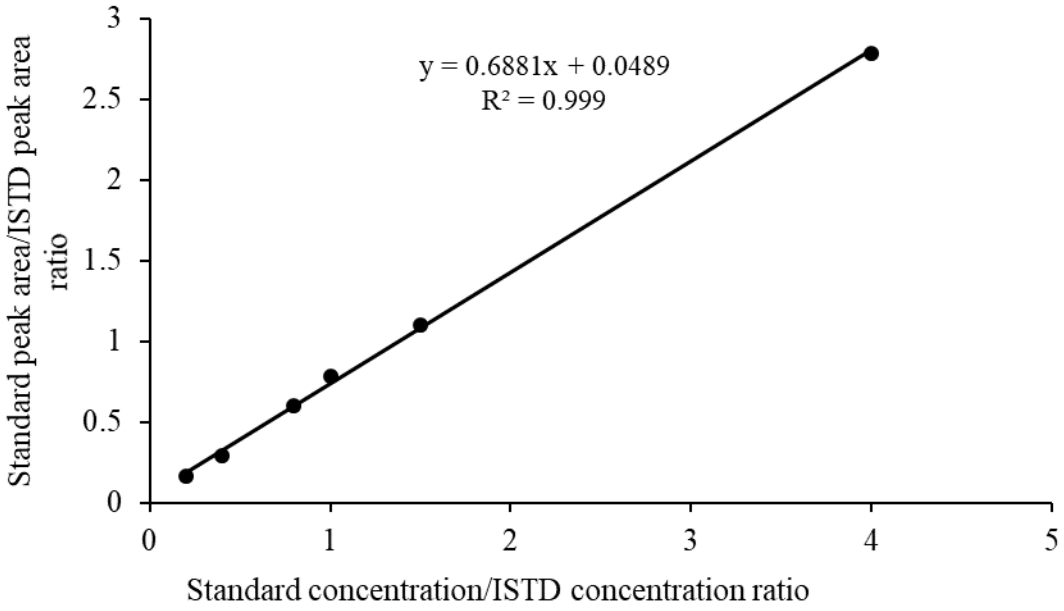

Fig. A9: Matrix matched calibration curve for sulfachlorpyridazine

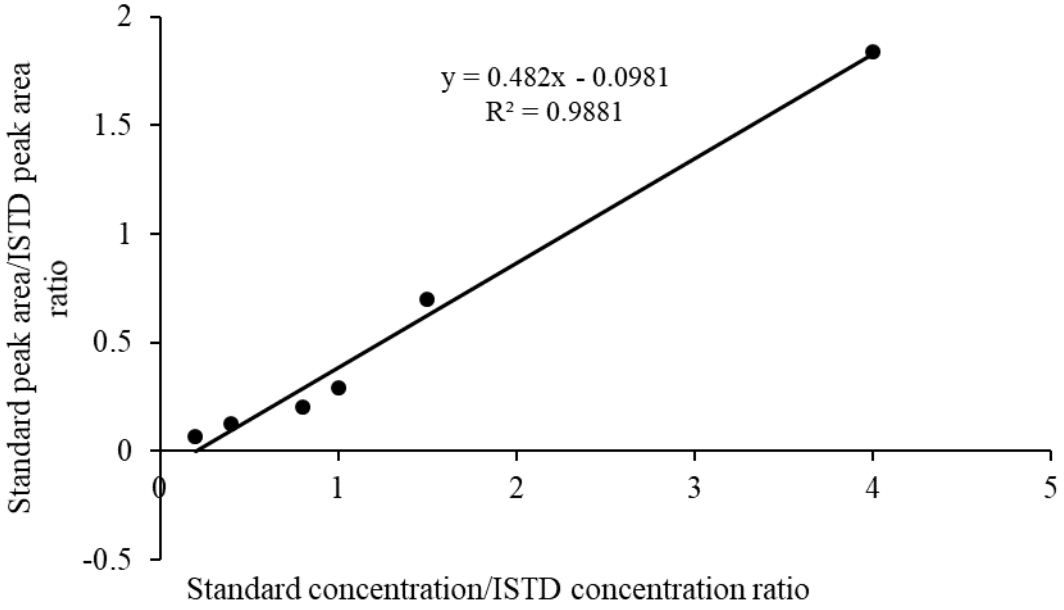

Fig. A10: Matrix matched calibration curve for sulfadiazine

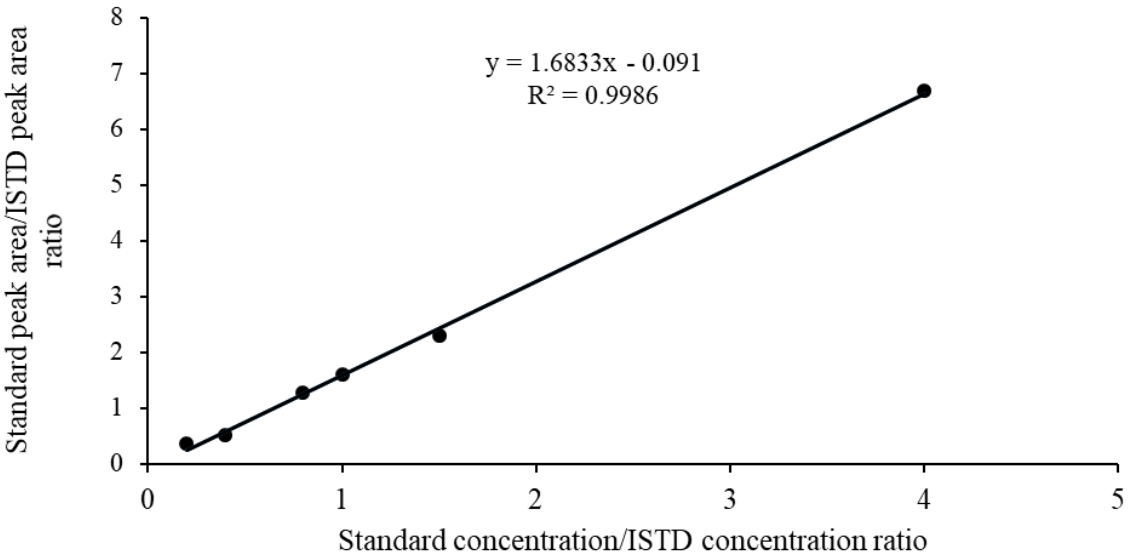

Fig. A11: Matrix matched calibration curve for sulfamethoxypyridazine

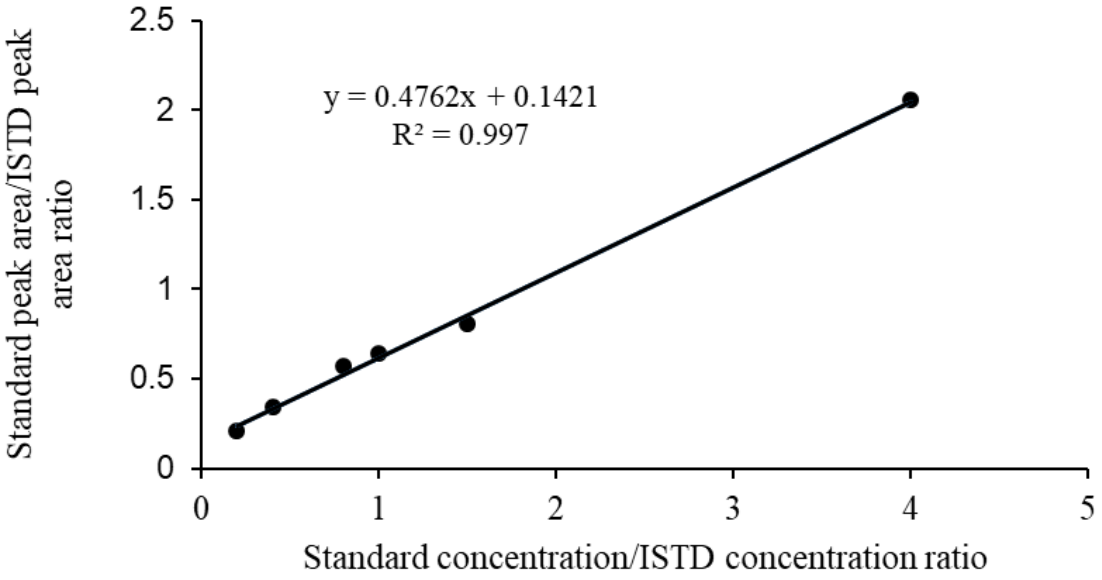

Fig. A12: Matrix matched calibration curve for sulfamethoxazole

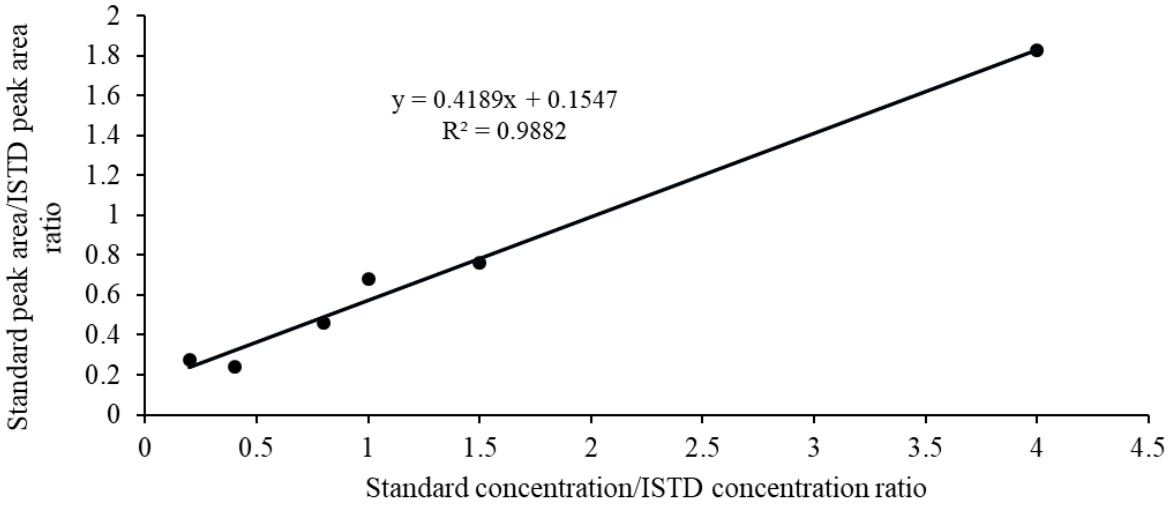

Fig. A13: Matrix matched calibration curve for sulfaquinoxaline

Appendix B: Paper VI

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Research paper

Determination of Cd, Mn and Ni accumulated in fruits, vegetables and soil in the Thohoyandou town area, South Africa

Babra Moyo[1], Vhahangwele Matodzi[1], Malebogo A Legodi[1], Vusumzi E Pakade[2] and Nikita T Tavengwa[1]

[1]*Department of Chemistry, School of Mathematical and Natural Sciences, University of Venda, Private Bag X5050, Thohoyandou, 0950, South Africa*
[2]*Department of Chemistry, Vaal University of Technology, Private Bag X021, Vanderbijlpark, 1900, South Africa*

The accumulation of heavy metals such as Cd, Mn and Ni was investigated in seven different vegetables, fruits and soil samples from Thohoyandou, Limpopo Province, South Africa. Heavy metals were quantified using graphite furnace atomic absorption spectrometry. Concentrations of heavy metals in fruits and vegetables were in the range of 0.23–2.94 mg·kg^{-1} for Cd, 11.72–50.16 mg·kg^{-1} for Mn and 5.73 – 44.11 mg·kg^{-1} for Ni on a dry weight basis. Analysis of soils from where fruits and vegetables were sampled showed that Cd in the soil was in the range of 0.08–1.07 mg·kg^{-1}, Mn levels were 204.99–249.13 mg·kg^{-1} and Ni levels were 48.47–88.23 mg·kg^{-1}. Cd was below the instrument detection limit for soils on which onions and bananas were grown. Vegetables showed different accumulation abilities, with leafy vegetables being the highest accumulators of heavy metals. The obtained results showed that concentrations of Cd in fruits, vegetables and soils exceeded the recommended maximum acceptable levels proposed by FAO/WHO and, hence, may pose a health risk to consumers. Ni concentrations in bananas, onion, beetroot, spinach and Chinese cabbage exceeded recommended standards by FAO/WHO.

CORRESPONDENCE
Nikita Tavengwa

EMAIL
nikita.tavengwa@univen.ac.za

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INTRODUCTION

Food contamination by heavy metals is commonly due to environmental and industrial contamination from sources such as industrial emissions and irrigation water (Huang et al., 2014). Dust emission from cement production is among the anthropogenic activities that contribute to environmental pollution (Bermudez et al., 2010). Heavy metals, particulates and dioxins are some pollutants that are contained in cement dust, which may pose a health risk to humans (Tajudeen et al., 2011). Soils and plants serve as sinks for atmospheric deposition of heavy metals from industrial emissions (Bermudez et al., 2010; Hao et al., 2009; Hernández-Martínez and Navarro-Blasco, 2012). The accumulation of cement dust in soils and on plants may be a result of wind and seepage waters (Taghipour et al., 2013; Li et al., 2015; Xu et al., 2014). Heavy metals that may be contained in cement dust include As, Cd, Pb, Hg, Tl, Al, Be, Cr, Cu, Mn, Ni, and Zn (Schuhmacher et al., 2002; Engelbrecht et al., 2013; Ogunbileje et al., 2013).

Excessive accumulation of these heavy metals in agricultural soils is a result of phytotoxicity and elevated heavy metal uptake by food crops, hence causing food insecurity (Kabata-Pendias and Mukherjee, 2007; Nagajyoti et al., 2010). Furthermore, the potential of heavy metals to bioaccumulate in the food chain has led to health concerns. Excessive bioaccumulation of toxic heavy metals in vegetables may result in the unavailability of dietary nutrients to humans or cause health problems for both humans and the ecosystem (Ogunkunle et al., 2013; Wuana and Okieimen, 2011; Hu et al., 2013; Yang et al., 2009). Moreover, cement dust deposition on plants can cause stomatal clogging and thereby affect plant growth (Abdel-Rahman and Ibrahim, 2012; Prajapati and Tripathi, 2008).

Non-biodegradability, long biological half-lives and their persistent nature make heavy metals harmful (Arora et al., 2008; Shalini et al., 2017). Consumption of unsafe concentrations of heavy metals continuously through food may lead to chronic accumulation of heavy metals in the human kidney and liver, consequently disrupting numerous biochemical processes and leading to cardiovascular, nerve, kidney and bone diseases (Zhou et al., 2016; Sharma et al., 2009).

Exposure to chronic Cd may trigger acute liver and lung toxicity, induce nephrotoxicity and osteotoxicity, and impair the functions of the immune system (Klaassen et al., 2009; Patrick, 2003). Clinical symptoms such as nausea, vomiting, abdominal discomfort, diarrhoea, visual disturbance, headache, giddiness and cough indicate acute health impacts of high concentrations of Ni (Duda-Chodak and Blaszczyk, 2008).

Vegetables are a rich source of vitamins, minerals and fibre, whereas fruits are a rich source of carbohydrates, proteins, vitamins, minerals and fibre, which are essential for good human health (Cherfi et al., 2014). Zn, Cu, Mn, Ni and Co are essential heavy metals that might be contained in fruits and vegetables. However, they can be toxic when their concentrations exceed the tolerable limit in living organisms. Non-essential heavy metals such as Hg, Pb, As, Cr and Cd are toxic to humans even at low concentrations (Izah et al., 2016).

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In this study, the presence of Mn, Cd and Ni was investigated in soil, fruits and vegetables from a small-scale farm in the vicinity of a cement brick manufacturing company in Thohoyandou, South Africa. The close proximity of this farm to the cement brick company might result in the contamination of soil, vegetables and fruits with heavy metals from cement dust. Moreover, this small-scale farm is close to one of the busiest roads in Thohoyandou. There have been few studies that have focused on heavy metal contamination of fruits and vegetables in South Africa (Bvenura and Afolayan, 2012; Kisten et al., 2017). Furthermore, none of the previous studies examined heavy metals in soil, vegetables and fruits simultaneously in Thohoyandou. Monitoring levels of heavy metals can provide useful information for promoting food safety in South African food industries and setting national standard limits since there are presently none.

EXPERIMENTAL

Chemicals

Nitric acid, hydrochloric acid, manganese sulphate, cadmium sulphate and nickel sulphate analytical reagents were purchased from Merck (Johannesburg, South Africa). Polyethylene bags and all the glassware were purchased from Lasec (Johannesburg, South Africa).

Instruments and materials

Heavy metal concentrations were analysed using a graphite furnace atomic absorption spectrometer (GFAAS) technique (Perkin Elmer Model Pinnacle 900T, Perkin Elmer, Germany), fully automated and PC-controlled using Syngistix AA. A Mars 5 microwave assisted digestion system (CEM Corporation, USA) was used for the digestion of fruits and vegetables. A Restch grinder and mesh sieves of 2 mm, 1 mm, 500 µm, 250 µm and 75 µm sizes were purchased from Retsch GmbH (Haan, Germany). The pH and electrical conductivity (EC) of soil samples were measured using a portable multi-probe Boeco pH meter that was purchased from Rochelle (Johannesburg, South Africa).

Study area

Thohoyandou is in the province of Limpopo in South Africa. It is an administrative centre of the Vhembe District Municipality and Thulamela Local Municipality. Daily temperatures in the town vary between 20°C and 40°C in wet seasons and 12°C and 22°C in dry seasons. The average annual rainfall in the town is approximately 800 mm, but ranges between 340 mm and 2 000 mm. In summer and the winter months, the prevailing wind direction is east to southeast. The average wind speed is 11 $km \cdot h^{-1}$ in summer and 15 $km \cdot h^{-1}$ in winter (Mzezewa et al., 2010). The town is in an urbanization and development stage, with a modern shopping mall being the most recent large development. There are also houses being built since the town is expanding. There are two brick-making companies which supply building materials and which are situated on the western part of town. The company which is the possible source of heavy metals to the site of interest is about 1 km away from the sampling site in this study. Figure 1 shows the sampling area and the sampling points (indicated by red circles).

Sample collection

A sampling method described by Zhou et al. (2016) and Sharma et al. (2009) was used. Bananas/*Musa acuminate* and 7 vegetable samples of different vegetable species (spinach/*Spinacia oleracea*, Chinese cabbage/*Brassica rapa*, onion/*Allium cepa*, beetroot/*Beta vulgaris*, sweet potatoes/*Ipomoea batatas*, tomatoes/*Lycopersicon esculentum* and cabbage/*Brassica pekinensis*) were collected from a small-scale farm close to a brick-making company in Thohoyandou, using the random sampling method. Tomatoes are a solanaceous vegetable species, onion is an allimus vegetable, and sweet potatoes and beetroot are root vegetables, whereas Chinese cabbage, spinach and cabbage are leafy vegetables. All samples were stored at a constant temperature of 4°C in polyethylene bags for transport. Soil samples were collected from the upper soil layer (0–20 cm) in the same location where vegetables were sampled, using a stainless-steel spade. Polyethylene bags were used to store samples for transport.

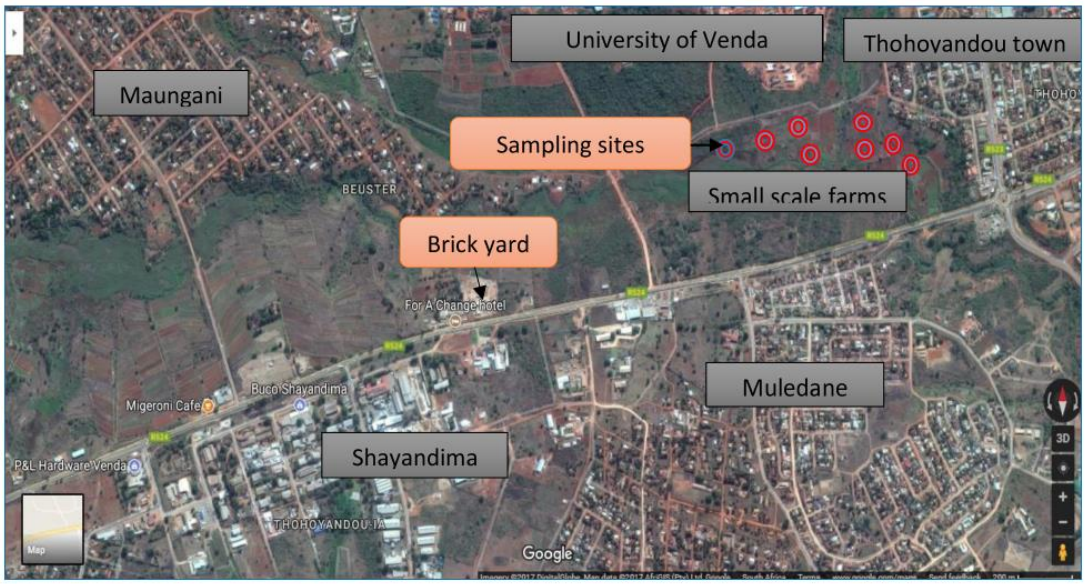

Figure 1. Sampling area showing a brick-making company and the nearby small-scale farms; sampling points shown as red circles

Sample preparation

Fruit and vegetable samples were cleaned with deionised water to remove dust and soil. The edible parts of the vegetables were separated from the plants, chopped into small pieces and air-dried to constant mass. A Restch grinder was used to grind all samples to fine powder; the samples were passed through a series of sieves and the 75 µm fraction was used for analysis. Approximately, 0.2 g of each sample was transferred into a Teflon vessel and digested in 12 mL of HNO_3 using a Mars-5 microwave-assisted digestion system according to the programme shown in Table 1. The resulting solutions were filtered using Whatman No. 42 filter papers into 50 mL volumetric flasks and filled to the mark with deionised water and then analysed for concentrations of Mn, Cd and Ni using a graphite furnace atomic absorption spectrometer. Standard solutions of the three elements under study were prepared. White clover certified reference material (CRM) (BCR-402) was used for quality assurance. Hollow cathode lamps of Mn, Cd and Ni at wavelengths of 193.7, 228.8 and 279.49 nm, respectively, were used to do measurements.

Soil samples were air-dried at room temperature to constant mass and were passed through a 75 µm sieve to eliminate plant roots and other waste materials and stored in sealable plastic bags until analysis. The pH and electrical conductivity (EC) of the soil slurry were measured with a pH multi-meter at 1:5 (w/v) ratio soil to water. For the analysis of the total concentrations of soil metals, approximately, 0.2 g of each sample was weighed into a 250 mL beaker and digested using aqua regia (5 mL HNO_3 and 15 mL HCl). The mixture was heated for 3 h on a hot plate to near-complete evaporation; then 20 mL of 2% (v/v) HNO_3 was added into the beaker. The solution was filtered through Whatman No. 42 filter paper into a 100 mL volumetric flask and filled to the mark using deionised water. The samples were analysed using the GFAAS.

RESULTS AND DISCUSSION

Chemical properties of soil

Table 2 shows some chemical properties of the composite soil sample. The pH of the composite soil sample was 6.73 indicating that the soil was near neutral pH. Soil acidification has the effect of reducing the supply of nutrients and increasing the dissolution of heavy metals such as Mn and Cd and hence increasing their absorption by plants (Dorraji et al., 2010). The pH of the soil is a critical factor in controlling the bioavailability of trace elements, especially for Cd (Adriano, 2001; Kabata-Pendias and Pendias, 2001). The soil electrical conductivity was 95.3 $mS{\cdot}m^{-1}$. The soil in this study was clayey, hence it has the ability to store and bind cations. X-ray fluorescence (XRF) results showed that the composite sample contained 87.1 $mg{\cdot}kg^{-1}$ of Ni which was above the standard value as stipulated by FAO/WHO. Cd was not detected.

Quality assurance

White clover CRM (BCR - 402) from the Community Bureau of Reference of the Commission of the European Communities was analysed for quality assurance purposes. The obtained results were compared with the certified value (Table 3). Measured values of Ni compared well with a certified value of 8.25 mg kg^{-1}.

Table 1. Digestion programme for the vegetables and fruits with a microwave-assisted acid digestion system

Stage	Power (%)	Ramp time (min)	Pressure (psi)	Temperature (°C)	Hold time (min)
1	100	4	800	180	8
2	100	5	800	180	5

Table 2. Chemical properties of soil

Sample name	pH	EC ($mS{\cdot}m^{-1}$)	Mn ($mg{\cdot}kg^{-1}$)	Ni ($mg{\cdot}kg^{-1}$)	Cd ($mg{\cdot}kg^{-1}$)
Composite	6.73	95.3	–	87.1	0
Standard value in soil*	–	–	–	50	0.3

*– not available, *FAO/WHO, 2011*

Table 3. Concentrations of heavy metals in edible parts of fruits and vegetables ($mg{\cdot}kg^{-1}$ dry weight) from a small-scale farm in Thohoyandou

Sample name	Cd	Mn	Ni
Maximum permissible limit† ($mg{\cdot}kg^{-1}$)	0.05 (fruits) † 0.05 (vegetables) *	NA (fruits) NA (vegetables)	0.80 (fruits) † 10.00 (vegetables)*
Cabbage green outer leaves	0.29	13.68	5.73
Cabbage inner layer leaves	0.54	22.58	6.55
Onion leaves	0.74	30.65	12.51
Onion bulb	0.91	22.65	10.84
Spinach	2.94	50.16	44.12
Chinese cabbage	0.77	31.78	11.38
Beetroot	1.08	29.95	19.07
Sweet potatoes	0.57	23.92	9.34
Tomatoes	0.60	15.75	7.97
Bananas	0.23	11.72	8.54
CRM (White clover)	–	–	8.16

*ND – not detected; NA – not available; †FAO/WHO, 2002; *FAO/WHO, 2011*

Table 4. Concentrations of heavy metals in soil collected under the mentioned fruit/vegetable (mg·kg^{-1} dry weight)

Sample name	Cd	Mn	Ni
Maximum permissible limit† (mg·kg^{-1})	0.30*	N/A	50.00*
Cabbage	0.20	211.81	48.47
Onion	ND	223.26	74.71
Spinach	1.07	229.13	88.23
Chinese cabbage	0.03	204.99	68.83
Beetroot	0.08	210.14	83.50
Sweet potatoes	0.12	230.27	80.20
Tomatoes	0.32	245.13	74.32
Bananas	ND	249.13	66.52

*ND – not detected, *FAO/WHO, 2011*

Heavy metal concentrations in edible parts of fruits and vegetables

The concentrations of Cd, Mn and Ni found in the fruits and vegetables are shown in Table 3. The range of concentrations of heavy metals in fruits and vegetables was in the order Mn > Ni > Cd. The same trend was obtained by Kisten et al. (2017) in vegetables. The obtained concentration ranges were 0.23–2.94 mg·kg^{-1}, 11.72–50.16 mg·kg^{-1} and 5.73–44.11 mg·kg^{-1} for Cd, Mn and Ni, respectively. The concentration of Cd in fruits and vegetables exceeded the recommended maximum acceptable levels proposed by FAO/WHO (2002 and 2011). Ni concentrations in bananas, onion, beetroot, spinach and Chinese cabbage exceeded recommended standards by FAO/WHO (2002 and 2011). The lowest concentrations of Cd, Mn and Ni were obtained in bananas and cabbage green outer leaves, whereas the highest concentrations were found in the spinach. Generally, vegetable species differ in their ability to take up and accumulate heavy metals (Saumel et al., 2012). The accumulation of Cd in vegetable species decreased in the order of leafy vegetables > root vegetables > allimus vegetables > solanaceous vegetables. Onion leaves showed a higher accumulation of Cd compared to the onion bulb. The concentrations of Mn were in descending order, leafy vegetables (spinach, Chinese cabbage, cabbage inner layer leaves) > allimus vegetables (onion leaves) > root vegetables (beetroot and sweet potatoes) > solanaceous (tomatoes) > fruit (bananas). This trend was similar to that found by Zhou et al. (2016). However, the concentration of cabbage outer green leaves was lower compared to the rest of the leafy vegetables. The highest concentration of Ni was observed in spinach (leafy vegetable). However, there is no clear trend for the rest of the vegetables according to their species. The interception of heavy metals emitted in the atmosphere by leaves might result in high levels of heavy metals in leafy vegetables. These heavy metals may remain on the leaf surface or enter leaf tissues, even though metal contents in leaf tissues can also be a result of selective metal uptake by roots (Maisto et al., 2013). The exposed surface area of the leaves may influence aerial dust deposition (Prajapati, 2002).

Results of this study are in agreement with the results obtained by Ali and Al-Qahtaini (2012), who reported that Mn and Cd concentrations ranged from 4.16–94.16 mg·kg^{-1} and 0.92–4.13 mg·kg^{-1}, respectively, in different vegetables. The concentrations of Cd and Mn in cabbage, onion, spinach and tomatoes correspond to the results reported by Bvenura and Afolayan (2012), except that the Cd concentration in spinach samples in this study was higher. In a more recent study by Shaheen et al. (2016), a concentration of 0.05 mg·kg^{-1} of Cd in tomatoes was reported which was above the recommended standard limit by FAO/WHO. The high concentration levels of heavy metals might be due to atmospheric deposition of contaminated dust on the leaves. Cement dust from the brick-making company might be responsible for the presence of heavy metals in vegetables and fruits. Cement dust may be carried by wind and deposited on the vegetables and the soil. Automobiles might also be a source of heavy metal contamination. The sampling site is less than 1 km away from one of the busiest roads that connect Thohoyandou and the town of Louis Trichardt.

Heavy metal concentrations in soil

The concentrations of Cd, Mn and Ni in soil samples collected where fruits and vegetables were grown are shown in Table 4. The concentrations of Cd, Mn and Ni ranged from 0.03–1.07 mg·kg^{-1}, 204.99–249.13 mg·kg^{-1} and 48.47–88.23 mg·kg^{-1}, respectively. The concentrations of heavy metals in soils were in the order Mn > Ni > Cd; the same order applied to the samples of fruits and vegetables. Cd was below instrumental detection limits for soils where onions and bananas were grown. The Cd concentrations of soils where tomatoes and spinach were grown were above FAO/WHO standards. The concentration of Ni was below the FAO/WHO standard only for the soil where cabbage was grown. The concentrations of Cd obtained in this study were similar to the results obtained by Liu et al. (2015) in which Cd concentrations ranged between 0.0541 and 0.8487 mg·kg^{-1} in vegetable soils. The concentrations of Cd and Mn were comparable to the results obtained by Bvenura and Afolayan (2012), although the concentration of Cd in the soil where spinach was grown was higher. However, Mn concentrations obtained by Bvenura and Afolayan (2012) are slightly higher and ranged between 377.61 mg·kg^{-1} and 499.68 mg·kg^{-1}.

Phosphate fertilizers are a major source of heavy metals that enter agricultural soils, particularly Cd (Nicholson et al., 2003). Pollution related to traffic is another source of Cd, primarily caused by the aging and wear of automobile tyres, gasoline and car body and brake lining wear (Weckwerth, 2001).

Bioaccumulation factor in fruits and vegetables

The bioaccumulation factor (BF) can be used to estimate the ability of plants to accumulate heavy metals in their edible tissues. The bioaccumulation factor was calculated using the following equation.

$$\text{Bioaccumulation factor (BF)} = \frac{C_{plant}}{C_{soil}} \quad (1)$$

where C_{plant} is the heavy metal concentration in edible tissues of a plant and C_{soil} is the heavy metal concentration in the soil.

The bioaccumulation factors for Cd, Mn and Ni were 1.45–25.67, 0.05–0.22 and 0.11–0.50, respectively (Table 5). Cd has the

Table 5. Bioaccumulation factors of fruits and vegetables from a small-scale farm in Thohoyandou

Sample name	Cd	Mn	Ni
Cabbage green outer leaves	1.45	0.06	0.12
Cabbage inner layer leaves	2.70	0.11	0.14
Onion leaves	–	0.14	0.17
Onion bulb	–	0.10	0.15
Spinach	2.75	0.22	0.50
Chinese cabbage	25.67	0.15	0.16
Beetroot	13.33	0.14	0.23
Sweet potatoes	7.58	0.10	0.12
Tomatoes	1.88	0.06	0.11
Bananas	–	0.05	0.13

– *not available*

highest BF, hence the uptake of Cd by vegetables is higher than for Mn and Ni. The trend of the bioaccumulation factor for Mn and Ni is similar to the trend of the concentrations of these heavy metals in fruits and vegetables, with spinach having the highest BF for both heavy metals, showing that leafy vegetables have a greater ability to bioaccumulate heavy metals. Chinese cabbage (leafy vegetable) had the highest bioaccumulation factor for Cd and this corresponds to the findings by Zhou et al. (2016). The strong ability to accumulate Cd by all the vegetables might be due to acidity of the soil and water used for irrigation. Acidity increases the solubility of heavy metals, hence making them readily available for absorption by plants.

CONCLUSION

The concentration of heavy metals in fruits and vegetables was in the order Mn > Ni > Cd. The obtained results showed that concentrations of Cd in fruits, vegetables and soils exceeded the recommended maximum acceptable levels proposed by FAO/WHO and hence may pose a risk to public health. The concentrations of Ni in bananas, onion, beetroot, spinach and Chinese cabbage exceeded recommended standards by FAO/WHO. Vegetables showed different heavy metal accumulation abilities, with leafy vegetables being the highest accumulators of heavy metals. Heavy metal uptake and accumulation was high for leafy vegetables and low for tomatoes (solanaceous vegetables) and bananas.

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