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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 cephalopyrin, 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

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	75	—
	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 pK_a 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 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 $\mu\text{g kg}^{-1}$ for liver, muscle and kidney tissues, respectively for enrofloxacin and ciprofloxacin. The MRL set by the EU Committee for veterinary medicinal products is 200 $\mu\text{g kg}^{-1}$ in muscles, liver and kidneys of animal origin, 40 $\mu\text{g kg}^{-1}$ in milk, and 150 $\mu\text{g kg}^{-1}$ in eggs for the macro-lide drugs. **Table 2** shows some MRL values for different foods of animal origin.

Class of veterinary drugs	Target veterinary drug	Matrix	MRL ($\mu\text{g kg}^{-1}$)
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

Class of veterinary drugs	Target veterinary drug	Matrix	MRL ($\mu\text{g kg}^{-1}$)
	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 $\mu\text{g kg}^{-1}$	2.79 $\mu\text{g kg}^{-1}$	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 $\mu\text{g kg}^{-1}$	0.3–3.0 $\mu\text{g kg}^{-1}$	73.8–127.4	[43]
Three SAs	Chicken breast	HPLC-DAD	QuEChERS based on acetonitrile and water with 1% CH ₃ CO ₂ H followed by a cleanup using d-SPE Oasis HLB as a sorbent.	—	10 and 13 $\mu\text{g kg}^{-1}$	25–30 $\mu\text{g kg}^{-1}$	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 $\mu\text{g kg}^{-1}$	2.2–3.6 $\mu\text{g kg}^{-1}$	7.4–11.5 $\mu\text{g kg}^{-1}$	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 $\mu\text{g L}^{-1}$	—	92.9–104.7	[36]
Six FQs	Milk	HPLC-UV	DLLME was coupled to QuEChERS	—	—	2.5–15 $\mu\text{g kg}^{-1}$	74.1–101.4	[35]
Four TCs	Milk and eggs	HPLC-UV	IL-DLLME ([C ₆ MIM][PF ₆] as an extraction solvent, FIL-NOSM a disperser solvent)	—	0.08–1.12 $\mu\text{g kg}^{-1}$	—	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 $\mu\text{g kg}^{-1}$ florfenicol	12.2 and 12.5 $\mu\text{g kg}^{-1}$	36.6 and 37.5 $\mu\text{g kg}^{-1}$	—	[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 $\mu\text{g L}^{-1}$	5–15 $\mu\text{g L}^{-1}$	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 $\mu\text{g L}^{-1}$	0.5–1.0 $\mu\text{g L}^{-1}$	0.5–1.0 $\mu\text{g L}^{-1}$	-	[39]
Tylosin	Milk	UV/Vis	HFLPME-TiO ₂ (TiO ₂ was dispersed in 1-octanol)	—	0.21 $\mu\text{g L}^{-1}$	—	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]

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	5.0–75 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.

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 deproteinization. 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 $\mu\text{g kg}^{-1}$ 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 $\mu\text{g kg}^{-1}$) and LOQ (0.3–3.0 $\mu\text{g kg}^{-1}$) 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 $\mu\text{g kg}^{-1}$ and the LOQs ranged between 25 and 30 $\mu\text{g kg}^{-1}$ 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 $\mu\text{g kg}^{-1}$) ranged from 80–105%. LODs and LOQs ranged from 2.2 to 3.6 $\mu\text{g kg}^{-1}$ and from 7.4 to 11.5 $\mu\text{g kg}^{-1}$ respectively. Concentrations of chlortetracycline and oxytetracycline were detected in bovine muscle samples to be between 38.4 and 82.3 $\mu\text{g kg}^{-1}$ which is lower than the stipulated European Union MRL of 100 $\mu\text{g kg}^{-1}$. 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

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 $2.5 \mu\text{g kg}^{-1}$ for danofloxacin and below $15 \mu\text{g kg}^{-1}$ 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 ($0.73\text{--}1.21 \mu\text{g L}^{-1}$) than QuEChERS ($1.15\text{--}2.73 \mu\text{g L}^{-1}$) and higher recoveries (92.9–104.7% compared to 83.6–97.1%, when samples were spiked with sulfonamides at $150 \mu\text{g L}^{-1}$). 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 $600 \mu\text{g}$ 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 $12.2 \mu\text{g kg}^{-1}$ respectively whereas the LOQs were 37.5 and $36.6 \mu\text{g kg}^{-1}$ 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 $62.4 \mu\text{g kg}^{-1}$.

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 ($[\text{C}_4\text{MIM}][\text{NPA}]$) with strong acidity for the determination of TCs in milk and eggs. The use of $[\text{C}_4\text{MIM}][\text{NPA}]$ 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 $1.12 \mu\text{g kg}^{-1}$ 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 HFLPME 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 TiO₂ 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.

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 C₁₈, 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 $\mu\text{g kg}^{-1}$ 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 C₁₈ 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 C₁₈) 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

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.

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