Chapter 1

Introduction

1.1 Introduction

Animal nutrition has been developed rapidly in recent years since it is an integral part of animal production. From the end of the 19th century, many reports had shown proteins, fats and carbohydrates to be the required compositions of animal feed. After 1920, animal feed development included the adding of vitamins, amino acids, fatty acid, macro and micro minerals and antibiotics. Thus, changing its composition from the past (Verstegen and Tamminga, 2001 and Elliott; Situ 2005).

Antibiotics are an important composition in animal feed and have been used in animal feed for over 60 years since their discovery. Many reports showed that antibiotics could increase feed efficiency and growth rate (Wegener, 2003), treating clinically sick animals and preventing or reducing the incidence of infectious disease in the animals (Gibbons, 1979). The benefits of antibiotic lead to their widespread use in animal feed in many countries. The amounts of antibiotics used in animal agriculture were estimated and calculated by the Institute of Medicine, the Animal Health Institute, a trade organization, and the Union of concerned Scientists to be 1.36 – 14.64 million kilogram/year (Doyle, 2001). The most often used antibiotics in animal agriculture are tetracycline, penicillin, sulfas, diethylstilbestrol (DES) and nitrofurans (Gibbons, 1979).

Nitrofurans are a group of antibacterial compounds. The main members of this group are furazolidone, nitrofurantoin and nitrofurazone (Moittier, *et al.*, 2005). They are relatively broad – spectrum bacterial drugs, and widely used as feed additives to act against *Salmonella* spp., *Escherichia coli*, *Mycoplasma* spp., *Coccidia* spp., and some protozoa which were bacterial enteritis in cattle, fish, swine and poultry (Samuelen *et al.*, 1991). The widespread use of drugs in livestock production has led to increasing concern over potential adverse effects on human

health. The reason are the residues of drugs found in animal products such as milk (Díaz *et al.*, 1997), eggs (Draisci *et al.*, 1997) and edible tissue (McCracken *et al.*, 1995) that is potentially carcinogenic and may be passed to consumers. Therefore, these chemicals have been placed in Annex IV of European Union (EU) regulation in 1995, a regulation for prohibition use in food producing animals (Tomlinson *et al.*, 2004). Furthermore, Food and Drug Administration (FDA) of the United States of America prohibited nitrofuran drugs use in food producing animals since 2002. Other countries such as Australia, Philippine and Brazil also ban all use of nitrofurans in food producing animals (Khong *et al.*, 2004). Therefore, Thailand which exported agricultural commodities *i.e.* animal and aquaculture products to Europe, Asia and Africa has been affected by these prohibitions.

Thailand has been the world largest exporter of farmed shrimp and the fourth largest exporter of poultry (FAO, 2004). In March 2002, the EU reported the finding of trace amount of nitrofurans in imported animal products from Thailand. The European Commission then required that all consignments must be 100 percent tested for the presence of nitrofurans and any consignment found with their residues would be destroyed. This regulation is not only reduced the volume of imports entering the EU, but also lowered the confidence of consumers in the safety of imported food. This caused the drop in the value of Thailand's exports during 2002 (Nidhiprabha, 2002). So, the Ministry of Agriculture issued a proclamation prohibited the use of nitrofurans in animal feed in 2003 due to their genotoxic and carcinogenic properties (Ministry of Agriculture, 2003). Thus, to prevent any illegal use it is necessary to analyze nitrofurans in animal feed for the presence of these drugs which will later constitute a hazard to the health of the consumer.

This thesis emphasized on the methodology for a simple, rapid and high recovery yields analysis of nitrofurans *i.e.* nitrofurazone, nitrofurantoin and furazolidone in animal feeds. The method consists of the optimizations of a High Performance Liquid Chromatography with UV detector (HPLC – UV) and UV – Vis spectrophotometer, and the investigation of a sample preparation technique.

1.2 Background

1.2.1 Name, synonym and chemical structure

The name, synonym and chemical structure of the three nitrofurans investigated in this work are as follows.

Furazolidone

Synonyms : 3-[[(5-Nitro-2-furanyl)methylene]amino]-2-oxazolidinone

Nifurazolidonum

Trade name : Furoxone

Molecular formula : $C_8H_7N_3O_5$

Chemical structure

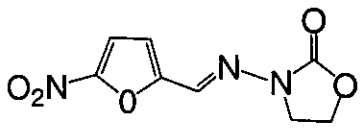

Figure 1 Structure of furazolidone

(Mill and Roberson, 1987)

Nitrofurantoin

Synonyms : 1-[[(5-Nitro-2-furanyl)methylene]amino]-2,4-

Imidazolidinedione

Trade name : Furadantin

Ivadantin

Macrodantin

Nitrex

Molecular formula : $C_8H_6N_4O_5$

Chemical structure

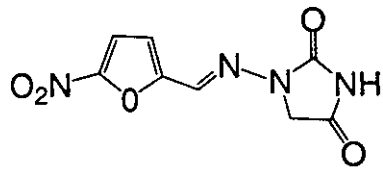

Figure 2 Structure of nitrofurantoin

(Mill and Roberson, 1987)

Nitrofurazone

Synonym : 2-[(5-Nitro-2-furanyl)methylene]hydrazine carboxamide

Trade name : Furacin

Molecular formula : $C_6H_6N_4O_4$

Chemical structure

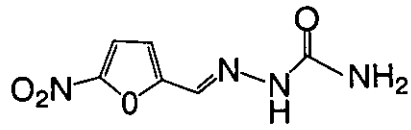

Figure 3 Structure of Nitrofurazone

(Mill and Roberson, 1987)

1.2.2 Physical properties

The physical properties of the studied nitrofurans are summarized in Table 1.

Table 1 Physical properties of the studied nitrofurans

Nitrofuran	Color	Physical state	Odor	Molecular weight ($gmol^{-1}$)	Melting point (°C)	Solubility	Storage
Furazolidone	Yellow	Crystalline powder	Odorless or almost odorless	225.2	259	Dimethylformamide	Avoid explore to direct sunlight
Nitrofurantoin	Yellow	Crystalline powder	Odorless or almost odorless	238.2	271	Dimethylformamide	Avoid explore to direct sunlight
Nitrofurazone	Yellow or brownish-yellow	Crystalline powder	Odorless or almost odorless	198.1	236	Dimethylformamide	Avoid explore to direct sunlight

Source: IARC 1983, 1990 and Moffat *et al.*, 1986

1.2.3 Uses

Nitrofurans; nitrofurantoin, furazolidone and nitrofurazone, are veterinary drugs that have been used in animals and humans (Díaz *et al.*, 1997). They are commonly used in dairy, livestock, poultry and aquaculture production industries. These drugs are given to the animals by orally administered, mixed with animal feed or drinking water to stimulate growth and prevent and control a number of diseases (McCracken and Kennedy, 1997). Nitrofurazone is an antibacterial agent used mainly for local treatment and prophylaxis of skin infections in animals. It has also been used in the prevention of some animal ailments, such as coccidiosis in poultry, due to the salmonella bacteria like, pullorum disease or typhoid in chickens, blackhead and hexamitiasis in turkeys and enteritis disease in swine (Dykstra, 1961, Humphreys, 1988, Wanishphongphun, 2002).

Nitrofurantoin, has activity against almost all bacterial species that cause urinary infections, *i.e.*, several gram negative and gram positive organisms (Kagan, 1980), including *E. coli*, *Streptococcus faecalis*, staphylococci and micrococci (Garrod *et al.*, 1985). In addition, it is effective in treating renal cortex or perinephic abscess (Hammam, 2002).

Furazolidone is used as antibacterial veterinary drug, effective against *Salmonella* spp. and *Escherichia coli* and also as an antiprotozoan (McCracken and Kennedy, 1997). It is commonly used in cattle, pig and poultry to treat infectious diarrhea, enteritis, cholera and bacteremic salmonellosis caused by bacteria or protozoan (IARC, 1983, Garrod *et al.*, 1981).

1.2.4 Toxicological data and the need of analysis

Nitrofuran drugs are a group of antibiotics which are widely used for the treatment and prevention of disease in animals. They are inexpensive and effective veterinary medicines, so they are used most extensively in the rearing of animals, especially pigs, poultry and shrimp (Dykstra, 1961). A high level of nitrofurans can lead to side effect where the nervous system of animals is seriously damaged (Humphreys, 1988). Several other studies have also shown the toxicological data of these drugs. Mutagenic activity has been observed in yeast, fungi, bacterial

and sub-mammalian systems (Dracisci *et al.*, 1997). Moreover nitrofurans have been shown to be tumorigenic in rats and mice and cytotoxic to mammalian cells in culture (FSANZ, 2004). Due to their toxicity, in 1993, EU banned nitrofurantoin and nitrofurazone in food-producing animals. Furazolidone was banned by the EU in 1995, Australia in 1993, the Philippines in 2001 and Brazil in 2001 (Khong, *et al.*, 2004). In 2002, the Ministry of Public Health of Thailand also prohibited the use of nitrofuran antibiotic in animal feeds (Ministry of Public Health, 2002).

The prohibition of nitrofurans in food producing animal by the EU and several other countries including Thailand brought out the need of monitoring these animal products. However, it is difficult to measure the residues of the parent drugs due to their instability and rapid metabolism. They have a short half-life which causes them to disappear rapidly from blood and tissue, but there are the occurrences of the metabolites of nitrofuran covalently bind to cellular protein *in vivo* (Pereira *et al.*, 2004). The metabolites of nitrofurazone, nitrofurantoin and furazolidone are semicarbazide (SEM), 1-aminohydantoin (AHD) and 3-amino-2-oxazolidone (AOZ), respectively. The chemical structures of these nitrofuran drugs and their metabolites are shown in Figure 4.

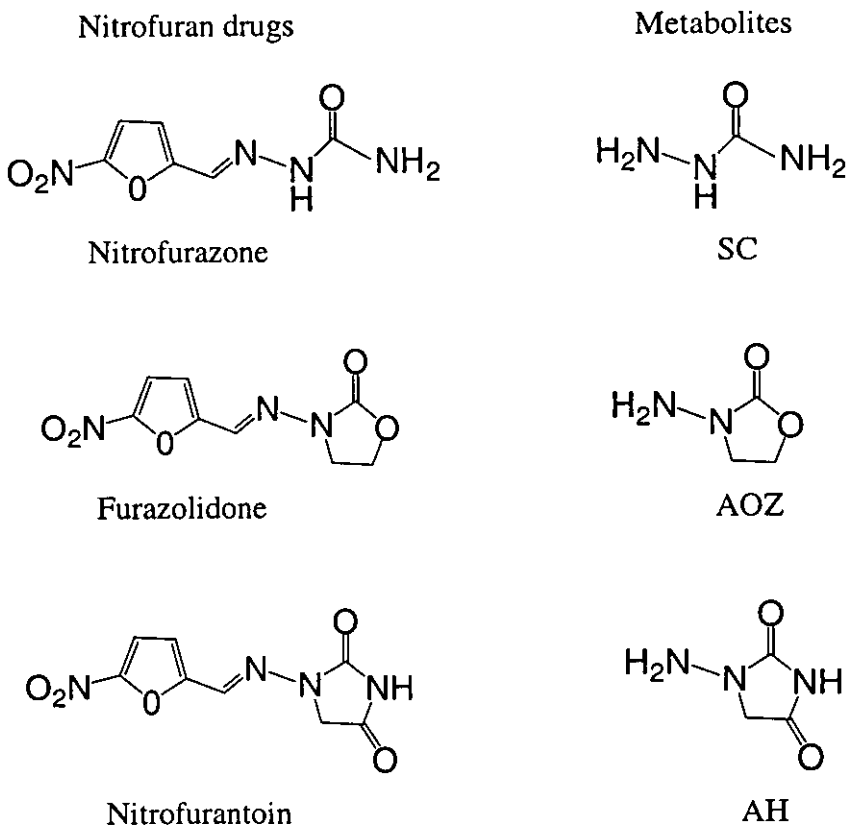

Figure 4 Chemical structures of 3 nitrofurans and their correspond metabolites (Leitner *et al.*, 2001)

The protein bound metabolites of these drugs are stable even after long storage and can be detected in the tissue of animals up to 7 weeks after withdrawal of nitrofuran parent drugs (Conneely, *et al.*, 2002). McCracken *et al.* (1997) reported that these metabolite residues do not significantly degrade during food preparation techniques like cooking, baking, grilled and microwaving of the tissue bound residues (McCracken *et al.*, 1997 and Mottier *et al.*, 2005). Furthermore, the metabolites of these drugs can be released from the bound residues under mild acidic conditions like in human stomach, therefore, the ingestion of protein bound residues in meat or shrimp products may result in the releasing of metabolites in the human body during digestion. Thus, causing toxicological risk to the consumers since these drugs have been shown to cause mutagenic and carcinogenic effects (Vass *et al.*,

2005). The minimum required performance limits (MRPLs), as restricted in Annex II of Regulation 2003/181/EC on 13 March 2003 by the EU, for nitrofuran metabolite residues in poultry meat and aquaculture products, is 1 μgkg^{-1} (Commission Decision (EC), 2003).

Thus, to prevent the use of these drugs in animal feeds a technique is needed to evaluate residual nitrofuran drugs in animal feeds because of concerns about their possible effects on consumer health.

1.3 Review of literature

In any instrumental analysis technique, the system performances rely mainly on sample preparation technique as well as analysis method and these are the subject of the review in this section.

1.3.1 Sample preparation

Sample preparation techniques are necessary before the identification and quantification of nitrofurans residue in the interested samples. Several methods have been reported for determination of nitrofurans in various samples.

Solvent extraction method is one of the methods used for the analysis of nitrofurans in animal feed samples. This is a simple and convenient technique used to separate interested compounds from solution, aqueous suspension or solid where they are present by extraction solvent (Brondi and Lançcas, 2005). Smallidge (1985) used dimethylformamide to extract furazolidone in premixes feed sample. The recovery of 96.0% was obtained. In 2001, Lin Jeng also used this method to determine nitrofurazone and furazolidone in pig feed, 95.0% dimethylformamide in water was used as extraction solvent, and the clean up was by a column of alumina oxide. This work gave the percent recovery as 97.3 and 109.9% for nitrofurazone and furazolidone, respectively. The main disadvantages of this technique were the use of large volumes of organic solvent and time consuming.

To solve these problems, solid-phase extraction (SPE) method

was developed. This method was an extraction technique based on the isolation of one or more components between two phases, one of which was a solid sorbent. The second phase typically was a liquid phase. It was usually used to clean up a sample before using a chromatographic or other analytical method to quantitate the amount of analyte(s) in the sample (McDonald, 2001). Díaz *et al.* (1997) reported the determination of nitrofurantoin, furazolidone and furaltadone in milk sample using C_{18} contained in a cartridge as solid sorbent to isolate nitrofurans and obtained recoveries of 83-98%. In addition, Leitner *et al.* (2001) used SPE method to analyse metabolites of nitrofuran in pig muscle. In the sample preparation process metabolites of nitrofurans were derivatised with 2-nitrobenzaldehyde (NBA), to produce a chromophore which can be detected. These were passed through the SPE with a polystyrene sorbent for clean up. Percent recovery of metabolite was 66%. Later in 2002, Conneely *et al.* modified SPE method for the isolation and clean up of a derivatised furazolidone metabolite (AOZ) from pig liver sample. They used dual SPE cartridges, SPE Oasis™ MAX cartridges. Furazolidone was derivatised with 2-nitrobenzaldehyde (NPAOZ) and SPE cartridge was used to remove excess NBA which caused interference in the detection of metabolite in the sample. The developed dual SPE system served to remove NBA > 99.0% out of the metabolite. It helped to improve the recovery of AOZ to up to 72% (Conneely *et al.*, 2002). In general solvent use and lab time were reduced by SPE method (Choi *et al.* 2004).

Supercritical fluid extraction (SFE) was a more recent technique, it uses a supercritical fluid as an extraction solvent to facilitate the extraction of interested compounds from solid samples (Langezaal *et al.*, 1990). When a fluid was taken above a particular temperature and pressure (critical point of the respective fluid), it existed in a condition called the supercritical fluid state. The physio-chemical properties of a fluid in the supercritical state were in between those of a typical gas and liquid. Carbon dioxide is certainly the most popular fluid because of its physiological compatibility, non-toxicity, inflammability, easy, availability, convenient critical parameters, inexpensiveness and environmental friendliness (Kumar *et al.*, 2005). Arancibia *et al.* (2003) used this method to study the extraction of nitrofurantoin and its metabolite from urine samples with acetonitrile-modified

supercritical CO_2 (2500 psi). The efficiency achieved was 99.8%. SFE technique is fast and uses minimum amount of solvents per sample. But the limitation of SFE were a limited sample amount (<10 g) and high capital investment for equipment (Brondi and Lanças, 2005).

1.3.2 Analysis method

Several methods have been reported for the identification and quantification of nitrofurans in various samples. In 1973, Barefield used the screening method, *i.e.*, distinct colors of nitrofurans when brought into contact with methanolic potassium hydroxide for a preliminary guide. Barefield used this method for identification of furazolidone in feeds. The drug is identified by the color produced in dimethylformamide and potassium hydroxide solution, *i.e.* furazolidone gives intense blue color (Barefield, 1973). The screening method was later investigated for the identification of furazolidone, nitrofurantoin and nitrofurazone (Moffat *et al.*, 1986). The derived colors appear deep blue, yellow-orange and red, respectively. The advantage of screening method is easy and rapid for qualitative analysis. Color forming agent was also used to quantify nitrofuran. Díaz *et al.* (1994) reported the determination of furazolidone in animal feed samples by reacted it with sodium hydroxide solution to form the orange color and measured at the maximum absorption wavelength of 430 nm. The detection limit was 0.17 $\mu g\ mL^{-1}$. This method is simple and rapid for the quantification of nitrofurans in samples (Biswas and Ghosh, 1980).

The high performance liquid chromatography (HPLC) coupled with different detector has been widely employed to determine several nitrofurans simultaneously. Cieri (1979) demonstrated that furazolidone and nitrofurazone in animal feeds could be assayed by HPLC coupled with an ultraviolet detector at 365 nm, with 30 cm × 3.9 mm I.D., μBondapak C_{18} column and 30% acetonitrile in water as the mobile phase, and showed the lowest amount of 0.5 ppm. Furazolidone was also determined in feed by HPLC-UV, LiChrosorb RP-18 (25 cm × 4.6 mm I.D.) column with a mobile phase, acetonitrile-2% acetic acid (20:80). The detection limit was 0.005% furazolidone (Smallidge *et al.*, 1985). Later McCracken and Kennedy (1997) reported the analysis of furazolidone in animal feeds, with the limit of

detection of 1 mg kg^{-1}, by HPLC with UV detector at 365 nm. The HPLC conditions were 300 × 3.9 mm I.D., μBondapak, C_{18} reversed-phase column and 0.01 M sodium acetate pH 6.0 and acetonitrile (8:2) as a mobile phase.

HPLC with gradient program has also been used for quantitative and simultaneous determination of the five drugs *i.e.* Carbadox, Olaquindox, Furazolidone, Nitrofurazone and Nitrovin in feeds (Lin and Jeng, 2001). The chromatographic conditions were; Kratos sphere-5, RP-8, 5 μm, 250 × 4.6 mm with water Guard-Pak C-18 guard column and the gradient concentration of acetonitrile with flow rate of 1 mL min^{-1}. Five drugs could be completely eluted in 10 minutes. Although HPLC with UV-Vis detector has been used it has some limitation, *i.e.*, it can not detect the non-chromophore molecules or monitor many wavelengths simultaneously.

To overcome this limitation diode array detector (DAD) that can simultaneously monitor several compound with fast scanning rate and wide range of wavelengths (Parriott, 1993) has been studied. In 1997, Draisci *et al.* used HPLC-DAD for the determination of nitrofuran residues in avian egg, using a Spherisorb octadecylsilyl (ODS) column with the mixture of 20 mM sodium acetate (pH 4.6): acetonitrile (79 : 21) as mobile phase at a detector wavelength of 362 nm. The detection limits were reported at 2.5 μg kg^{-1} for nitrofurazone and 5.0 μg kg^{-1} for furaltadone. In a more recent work HPLC equipped with a photodiode array detector has been used to evaluate 13 veterinary drugs residues (Kao *et al.*, 2001). These included sulfadimethoxine, clopidol, sulfadiazine, sulfamerazine, ormethoprim, sulfamethazine, ethopabate, sulfathiazole, carbadox sulfamonomethoxine, sulfamethoxazole, sulfaquinoxaline, and furazolidone in chicken and swine muscles. The analytical column was a Luna C_{18} (25 cm × 4.6 mm) using a gradient elution of acetonitrile and 0.05 M sodium dihydrogen phosphate. The detection limits were 0.04 μg mL^{-1} for sulfathiazole and 0.02 μg mL^{-1} for the other 12 drugs.

Another technique which has been reported for the identification or quantitative measurement of nitrofurans in samples was HPLC coupled with mass spectrometry (HPLC-MS) (Parriott, 1993). In 1995, McCracken *et al.* used HPLC-MS to confirm the identity of nitrofurans in porcine tissue by comparing it with reference

(standard) spectra. The HPLC-MS system separation was done on the Lichrospher RP18 (125 × 4 mm I.D., 5 μm) column, mobile phase consisted of 0.1 mol L^{-1} ammonium acetate-acetonitrile (65:35) and selected-ion monitoring (SIM) of the ion m/z 243. The detection limit of 1 μg kg^{-1} was reported. Later Fuh *et al.* (2000) has also used HPLC-MS to determine 13 antibacterial drugs, including furazolidone, by using the mobile phase of 0.05% acetic acid with 1 μM sodium acetate and acetonitrile. The column is Cosmosil C_{18}, (125 × 2.0 mm) 4 μm with gradient elution was used for separation of the analytes. The detection limit of each compounds were in the range of 0.05-0.20 ppm. Table 2-4 summarized the methods used for nitrofuran analysis.

Each of these methods, could be used for quantitative analysis of residual nitrofurans in various samples, *i.e.*, animal tissue, biological sample, animal product or animal feed. This thesis emphasized on the simultaneous analysis of three nitrofurans (nitrofurazone, nitrofurantoin and furazolidone) in animal feed sample by HPLC and spectrophotometric techniques.

In these previous works, there were no any report on the evaluation of the analysis of mixture of two or three nitrofurans. Therefore, this is one of the focus of this thesis.

Table 2 Identification of nitrofurans by screening method.

Chemical reagent	Observed color	Sample matrix	Author
Potassium hydroxide solution	Intense blue (for furazolidone)	Feed	Barefield (1973)
Methanolic potassium hydroxide solution	Deep blue (for furazolidone) Yellow-orange (for nitrofurantoin) Red (for nitrofurazone)	Drug	Moffat *et al.* (1986)

Table 3 Determination of nitrofurans by UV-Visible spectrophotometry.

Chemical reagent	Observed color	Analytical wavelength (nm)	Sample matrix	Author
Sodium hydroxide solution	Orange (for furazolidone)	430 nm	Animal feed	Díaz, *et al.* (1994)

Table 4 Nitrofurans analysis by HPLC

Mobile phase	Column	Detection	Sample matrix	Author
30% Acetonitrile in water	μBondapak C_{18}, (30 cm × 3.9 mm)	UV (365 nm)	Animal feeds	Cieri (1979)
Acetonitrile - 2% acetic acid (20:80)	LiChrosorb RP-18 (25 cm × 4.6 mm)	UV (365 nm)	Animal feeds premixes	Smallidge *et al.* (1985)
0.01 M sodium acetate pH 6.0 containing acetonitrile (8:2)	μBondapak, C_{18} (30 cm × 3.9 mm)	UV (365 nm)	Animal feeds	McCracken and Kennedy (1997)
Acetonitrile in water	Kratos sphere-5, RP-8 (25 cm × 4.6 mm)	UV (365 nm)	Feed	Lin and Jeng (2001)
20 mM Sodium acetate (pH 4.6) containing acetonitrile (79 : 21)	Spherisorb octadecylsilyl (ODS) (25 cm × 4.6 mm)	DAD (362nm)	Avian egg	Draisci, *et al.* (1997)

Table 4 (Continued)

Mobile phase	Column	Detection	Sample matrix	Author
Acetonitrile containing 0.05 M sodium dihydrogen phosphate	Luna C_{18} (25 cm × 4.6 mm)	DAD (368 nm)	Chicken Swine muscles	Kao *et al.* (2001)
0.1 mol L^{-1} ammonium acetate containing acetonitrile (65 : 35)	Lichrospher RP18 (12.5 cm × 4 mm)	MS (m/z 243) for furazolidone	Porcine tissue	McCracken *et al.* (1995)
0.05% acetic acid with 1 μM sodium acetate and acetonitrile	Cosmosil C_{18} (12.5 cm × 2.0 mm)	MS (m/z 243) for furazolidone	Antibacterial drugs	Fuh *et al.* (2000)

1.4 Objectives

1. To study the appropriate sample preparation and analysis of nitrofurans in animal feeds.

2. To optimize spectrophotometric and high performance liquid chromatographic conditions for qualitative and quantitative analysis of nitrofurans.