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# ANTHELMINTICS RESIDUES IN MILK AND DAIRY PRODUCTS FROM COWS TREATED WITH LEVAMISOLE, OXYCLOZANIDE AND NITROXYNIL AT DIFFERENT ADMINISTRATION DOSES


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# *Chapter 1. Anthelmintic drugs*

## 1.1 INTRODUTION

According to the Regulation (EC) No **470/2009** (**1**), 'residues of pharmacologically active substances' means all pharmacologically active substances, expressed in mg/kg or μg/kg on a fresh weight basis, whether active substances, excipients or degradation products, and their metabolites which remain in food obtained from animals to which the veterinary medicinal product in question has been administered.

The analysis of residues of chemicals in foods of animal origin is a relatively young discipline. A residue may be defined as a trace of a substance, present in a matrix after some kind of administration (e.g. veterinary practice or illegal use) to an animal. In all cases, concentrations levels in the ppb concentration range (ppb or μg kg−1) or even lower (ppt or ng kg−1) have to be detected (**2**).

The substances involved may be divided into two major classes according to council directive **96/23/EC** (**3**): group A and B substances.

ANNEX I

**GROUP A** - *Substances having anabolic effect and unauthorized substances*

(1) Stilbenes, stilbene derivatives, and their salts and esters

(2) Antithyroid agents

(3) Steroids

(4) Resorcylic acid lactones including zeranol

(5) Beta-agonists

(6) Compounds included in Annex IV to Council Regulation (EEC) No 2377/90 of 26 June 1990

There are no maximum residue limits for these substances because they are prohibited in food producing animals in the EU.

**GROUP B** - *Veterinary drugs and contaminants*

(1) Antibacterial substances, including sulphonomides, quinolones

(2) Other veterinary drugs

(a) ***Anthelmintics***

(b) Anticoccidials, including nitroimidazoles

(c) Carbamates and pyrethroids

(d) Sedatives

(e) Non-steroidal anti-inflammatory drugs (NSAIDs)

(f) Other pharmacologically active substances

(3) Other substances and environmental contaminants

(a) Organochlorine compounds including PcBs

(b) Organophosphorus compounds

(d) Chemical elements

(d) Mycotoxins

(e) Dyes

(f) Others

Group B includes the veterinary drug or veterinary medicinal products (VMPs) for which maximum residue limit (MRLs) have been fixed. The safety and residue evaluation is carried out by the Committee for Veterinary Medicinal Products (CVMP) of the European Agency for the Evaluation of Medicinal Products (EMEA) in London, and is

supported by safety and residue experts, upon receipt of a valid application for the establishment of MRLs.

MRLs, listed under European Commission Regulation **37/2010** (**4**), have been set for a number of anthelmintics in milk and edible tissues (muscle, liver, kidney and fat) with the aim of minimizing the risk to human health associated with their consumption.

In general, anthelmintic residues cause no human health risk if veterinary drugs are properly administered and the recommended doses are correctly adhered to.

However, there may be a concern that if withdrawal periods are not adhered to or if products are administered to animals in unapproved applications (e.g. administration to lactating species) those levels may exceed MRLs in foods.

In addiction, whenever chemicals are used, especially with long term low dose exposure, toxicological, teratological and carcinogenetic effects must be considered.

Only a limited number of products are licensed for treatment of animals during the lactating period and have a MRL listed under European Commission Regulation **37/2010** (**Table 1**).

The widespread availability of cheaper generic veterinary medicinal products and the development of drug resistance to the limited number of licensed products have increased the potential for off-label applications.

**Table 1. MRLs for anthelmintic drug residues in milk as listed under European Regulation 37/2010**

| Veterinary drug | Marker Residue(s) | MRLs (µg kg−1) |
|---|---|---|
| Albedazole, albendazole sulphoxide, netobimin | Sum of albendazole-sulphoxide, albendazole-sulphone and albendazole-sulphone amine expressed as albendazole | 100 |
| Fenbendazole, oxfendazole, febantel | Sum of fenbendazole, fenbendazole-sulphoxide and fenbendazole-sulphone expressed as fenbendazole-sulphone | 10 |
| Thiabendazole | Sum of thiabendazole and 5-hydroxythiabendazole | 100 |
| Morantel | Morantel | 50 |
| Oxyclozanide | Oxyclozanide | 10 |
| Eprinomectin | Eprinomectin B1a | 20 |
| Moxidectin | Moxidectin | 40 |
| Closantel* | Closantel | 45 |

* provisional MRL of 45 ug/kg-1 in cattle and sheep milk (EMA/CVMP/584576/2010)

## 1.2 CLASSIFICATION OF ANTHELMINTIC DRUGS

The first treatments used on animals for internal parasites date back to two centuries ago. These kind of 'drugs' were quite rudimentary, since they derived from metals or plant extracts, and they worked by mechanical irritating the parasites from their predilection sites.

In the mid-twentieth century, the introduction of phenothiazine and piperazine as veterinary anthelmintics did provide compounds with real activity against target parasite species and with acceptable tolerance in the host animal species.

However, major progress was made between 1960 and 1980. In these 20 years, drugs with diverse structure, novel activity and enviable safety were produced for a global livestock industry. The discoveries of thiabendazole and levamisole provided compounds with in vivo potency at milligram (per kg of body weight) rather than gram dosages and, in the case of thiabendazole, led to the discovery of a group of structural

analogues, the benzimidazoles, with excellent broad-spectrum activity and safety.

In 1981, a further major step forward was made in chemotherapy with the launch in the animal health market of ivermectin, which had activity in animals when administered at microgram per kg dosages, had excellent broad spectrum activity against nematodes and was also effective against several parasitic arthropods.

The last 20 years have been spent refining existing molecules with niche activity (parasite and host specificity), improving delivery systems and worrying about the inexorable spread of drug resistance (**5**).

Anthelmintics are antiparasite drugs used to treat infections with nematodes, cestodes and trematodes. They are of huge importance both for human tropical medicine and for veterinary medicine.

There is an extensive number of antiparasitic agents licensed as veterinary drugs, such as the benzimidazoles (**6**) macrocyclic lactones, (**7**) and levamisole (**8**). Many of these drugs are licensed for the treatment of meat producing animals but not for dairy animals.

Antiparasite drugs have a great variety, with different chemical constitutions, functions and mechanisms of action.

Anthelmintics may be separated into classes on the basis of similar chemical structure and mode of action (**9**).

## 1.3 PIPERAZINE

This class of drugs was first used as an anthelmintic in the 1950's for the treatment of threadworms in children (White, 1953) (**10**).

The importance of piperazines in designing effective drugs for intestinal and tissue-dwelling helminths was not recognized till 1947 when scientists working at the Lederle Laboratories of the American Cyanamid Company discovered the antifilarial activity of 1,4-disubstituted ***piperazines*** (**11**).

Piperazines have heterocyclic ring structure containing two opposite nitrogen atoms (**Fig.1**).

## 1.4 MODE OF ACTION

Their mode of action is generally by paralyzing parasites, which allows the host body to easily remove or expel the invading organism. This process is mediated by its agonist effects upon the inhibitory GABA (γ-amino butyric acid) receptor. Its selectivity for helminths is because

vertebrates only use GABA in the CNS and the helminths' GABA receptor is a different isoform to the vertebrate's one. Its mode of action has primarily been studied in *Ascaris suum*.

In *A. suum* it acts as a weak GABA-mimetic and causes a flaccid, reversible paralysis of body wall muscle (Martin, 1985) (**12**).

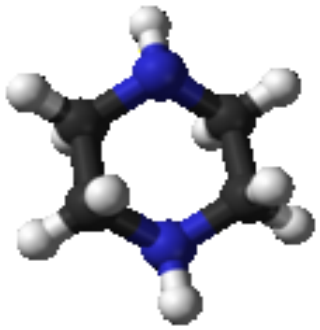


**Figure 1. Piperazine chemical structure**

## 1.5 BENZIMIDAZOLES

Benzimidazoles are anthelmintic drugs widely used for prevention and treatment of parasitic infections in agriculture and aquaculture. Some benzimidazoles have also found applications as pre- or post-harvest fungicides for control of a wide range of fungi affecting field crops, stored fruit and vegetables.

They contain a common 1,2-diaminobenzene nucleus and most of them may also have a carbamate functional group. In general, Benzimidazoles are almost insoluble in water and slightly soluble in alcohols or chlorinated hydrocarbons.

The first of this class, ***thiabendazole*** (TBZ), was discovered in 1961 (Brown et al.) (**13**) and subsequently a number of further benzimidazoles were introduced as broad spectrum anthelmintics. TBZ has been widely used for the control of gastrointestinal nematodes, lungworms and as a fungicidal agent.

After its introduction, a number of alternative drugs offering similar spectrums of activity came on the market, such as ***parabendazole***

(PAR) (**14**), ***cambendazole*** (CAM) (**15**) ***mebendazole*** (MBZ) (**16**), and ***oxibendazole*** (OXI) (**17**).

BZs with sulphide and sulphoxide, such as ***albendazole*** (ABZ) (**18**), ***fenbendazole*** (FBZ) (**19**) and ***oxfendazole*** (OFX) (**20**) functional groups were following introduced, offering a broader spectrum of activity and improved efficacy. These compounds were the first to be successfully used in the treatment of roundworms, lungworms, tapeworms and adult stages of liver fluke.

Later on, in the 1983, was discovered ***triclabendazole*** (TCB) (**21**). This drug, unlike the other benzimidazoles, in the chemical structure has a chlorinated benzene ring but no carbamate group. This drug displays high efficacy against both immature and adult liver fluke (trematodes), but it is not effective against nematodes and cestodes. Since its introduction in the early 1980s, triclabendazole has become the main drug used to treat livestock infections with Fasciola hepatica. A recent study (**22**) has also showed the relative activity of its metabolites ***TCB-SO2*** (sulphone) and ***TCB-SO*** (sulphoxide) against flukicides.

## 1.6 MODE OF ACTION

Different modes of action have been proposed for BZ's. Their anthelmintic efficacy, except for mebendazole, is due to their ability to interfere with the parasite's energy and inhibit the fumarate reductase.

Consequently, this disturbs the adenosine triphosphate formation, because replaces the Krebs cycle in anaerobic way (Lacey et al.)(**23**).

MBZ, instead, compromises the cytoskeleton through a selective interaction with β-tubulin (Borgers and De Nollin, 1975) (**24**).

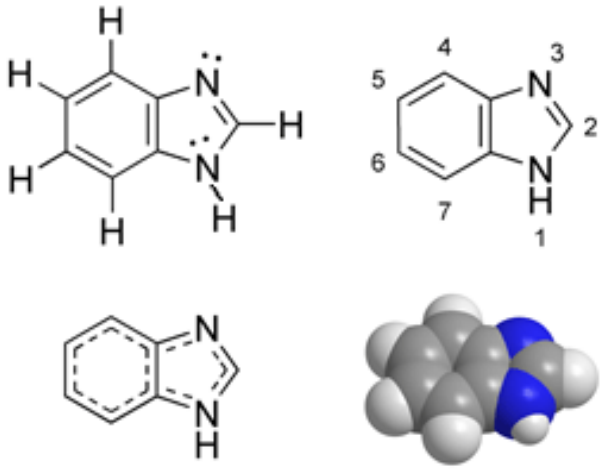



**Figure 2. Benzimidazoles nucleus**

## 1.7 TETRA-HYDRO-IMIDAZOLES

***Tetramisole*** (**25**) and ***levamisole*** (**26**) (**Fig.3**) (imidazothiazoles) with ***pyrantel*** (**27**) and ***morantel*** (**28**) (tetrahydropyrimidines) are nicotinic receptor agonists. Their precise mode of action has been carefully studied at the single-channel level on the body wall muscle preparation of *A. suum*. These compounds act selectively as agonists at synaptic and extrasynaptic nicotinic acetylcholine receptors (nAChR) on nematode muscle cells and produce contraction and spastic paralysis (**29**).

The anthelmintic activity of tetramisole, a racemic mixture, resides in the l-isomer, levamisole. In the earliest studies Tetramisole was used (**30**) and later replaced by the more active levorotatory isomer LEV (**31**). It is commonly used in cattle, sheep, pigs, goats, and poultry to treat nematode infections; it has no activity against flukes and tapeworms.

Levamisole has also immunostimulant effects (**32**) (**33**) at dosage rates higher than those used for anthelmintic activity, and it has been used both in humans and in animals in several diseases. The immunologic

enhancement is due by stimulating lymphoid tissue T-cell system, improving the production of lymphocyte active substances and increasing the phagocytosis of macrophage and neutrophil. Recently many authors have showed the antitumor activity of Levamisole in the colon cancer therapy (**34**) (**35**).

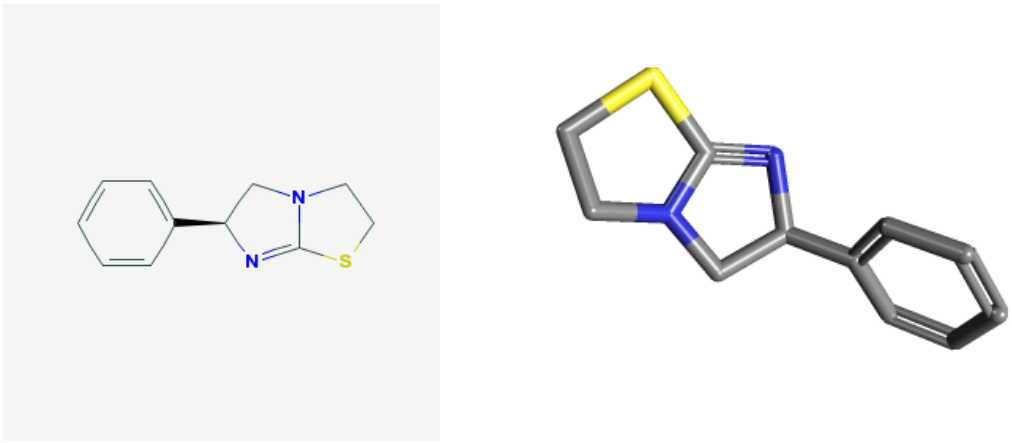



**Figure 3. Levamisole**

The Tetrahydropyrimidines are broad spectrum anthelmintics, expelling eelworm drugs, and they are suitable for various kinds of animal's gastrointestinal parasites. This class includes pyrantel, morantel

and hydroxyl pyrimidine. This last compound is a miracle drug for Trichiuris.

The efficacy of pyrantel (**Fig. 4**) as a veterinary anthelmintic first emerged in 1966 (**36**) just one year after the imidazothiazoles had entered the market. It is available as a citrate, tartrate, embonate, or pamoate salt.

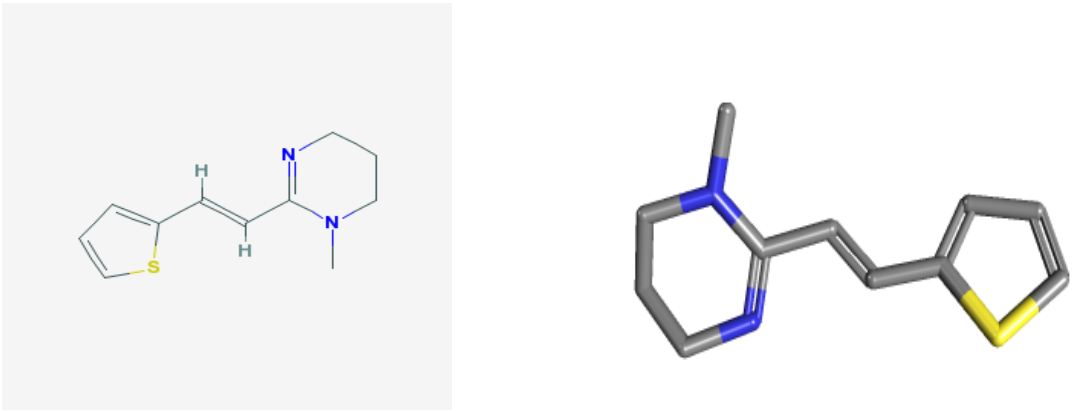



**Fig.4 Pyrantel**

Morantel (**Fig.5**) (**37**) (1,4,5,6-tetrahydro-1-methyl-2-[2-(3-methyl-2-thienyl) ethenyl pyrimidine) is a tetrahydro-pyrimidine anthelmintic, differing from the related analogue pyrantel by the presence of a methyl group on the thiophene ring. It is intended to treat roundworms and tapeworms. It is administered in lactating and non-lactating cattle as

morantel tartrate. The residues in milk must no exceed the limit of 50 µg/kg fixed in the 37/2010 EC.

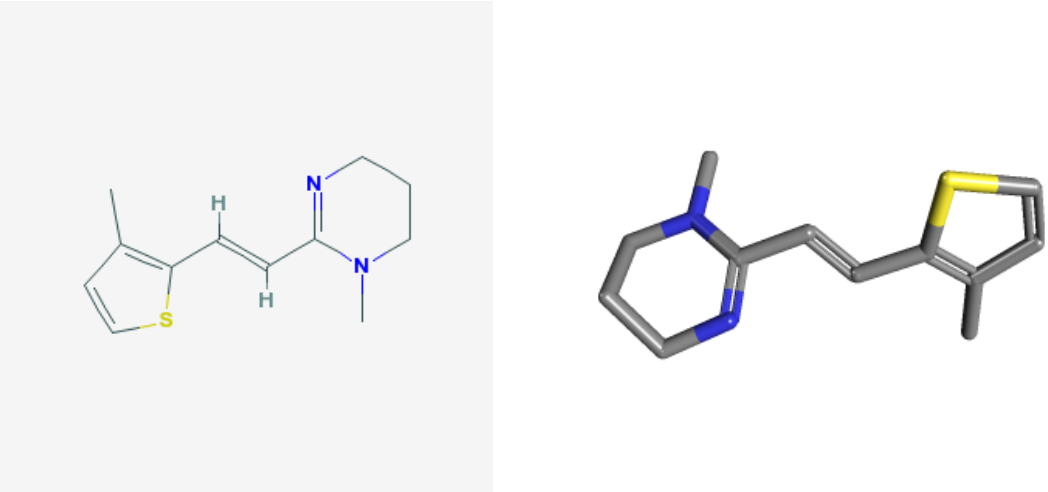



**Figure 5. Morantel**

## 1.8 MACROCYCLIC LACTONES

The macrocyclic lactones (avermectins and milbemycins) are products, or chemical derivatives thereof, of soil microorganisms belonging to the genus *Streptomyces*. The avermectins in commercial use are ivermectin, abamectin, doramectin, eprinomectin, and selamectin. Commercially available milbemycins are milbemycin oxime and moxidectin.

Avermectins are highly lipophilic substances and dissolve in most organic solvents, such as chloroform, acetone, cyclohexane and alcohols. The microorganism that produces the avermectins was isolated from a soil sample collected in Japan (**38**).

The avermectins were initially detected in a program in which thousands of microbial fermentation products were tested in mice for activity against the nematode, *Nematospiroides dubius*. Among the few preparations showing activity in this assay, was the product of a microorganism isolated from soil by researchers at The Kitasato Institute (**39**).

The microorganism was classified as a new species of actinomycete, *Streptomyces avermitilis*. Its anthelmintic activity was shown to reside in 8 closely related macrocyclic lactones which were also found to possess activity against free-living and parasitic arthropods.

One of the natural components, avermectin $B_1$, is now being evaluated as a pesticide for the control of mites of citrus and cotton crops. A chemical derivative, 22,23-dihydroavermectin $B_1$, or ivermectin, has been developed as an antiparasitic agent.

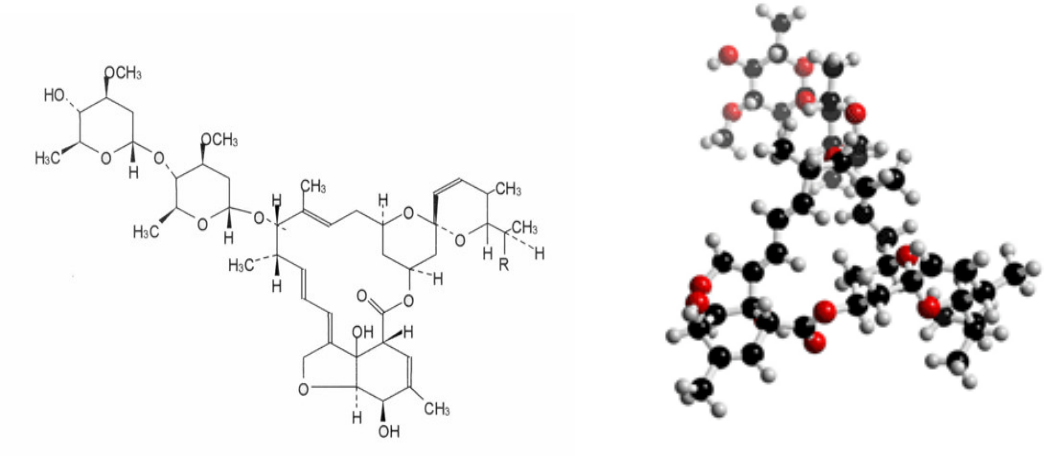



**Fig. 6 Avermectin**

MLs are large complex ringed structures (**Fig.6**); avermectins have a 16-membered macrocyclic ring, containing a spiroketal group, a benzofuran ring and disaccharide functionality or, in the case of

selamectin, have only a monosaccharide group. Milbemycins are structurally similar to avermectins but lack the disaccharide group.

The macrocyclic lactones have a potent, broad antiparasitic spectrum at low dose levels. They are active against many immature nematodes (including hypobiotic larvae) and arthropods, but are no effective against trematodes and cestodes.

Six avermectins and two milbemycins are currently licensed as veterinary drugs and insecticides. ***Abamectin*** (**40**) and ***ivermectin*** (**41**) are the best known, because of their long and extensive employment. MLs were initially investigated for treatment of parasitic infections in animals but their potential for use in human medicine was quickly identified; nowadays is still the first choice in human onchocerciasis (**42**).

Ivermectin (**43**) and ***doramectin*** (**44**) may be used on all mammalian food producing species, with proscription in animals from which milk is produced for human consumption, whereas ***eprinomectin*** (**45**) can be administered on dairy cattle with an MRL set at 20 μg/kg. Approvals have been granted for injectable or oral (bolus) dosage forms

for some of these drugs in cattle, but it is common for these drugs to be applied topically.

Some avermectins, including ***emamectin*** (**46**), have also been shown to be effective for the treatment of fishfin diseases such as sea lice in farm-raised salmon (**47**).

***Moxidectin*** is a synthetic milbemycin drug with similar properties to the avermectins; it has been approved for use in dairy cattle and sheep (**48**) and 40 µg/kg is the MRL fixed for milk.

***Selamectin*** (**49**) is used both as a topical insecticide and anthelmintic on dogs and cats. It controls outbreaks of fleas, heartworm, hookworms, roundworms, and ear mites. Selamectin is not approved for human use.

**Table 2. Macrocyclic lactones**

| COMPOUND | R1 | R2 | R3 |
|---|---|---|---|
| **Abamectin $B_{1a}$** | OH | H | $CHCH_3CH_2CH_3$ |
| **Emamectin $B_{1a}$** | OH | H | $C_6H_{11}$ |
| **Eprinomectin $B_{1a}$** | $C_6H_5COOHCH_3NH$ | H | $CHCH_3CH_2CH_3$ |
| **Doramectin** | $NHCOCH_3$ | H | $CHCH_3CH_2CH_3$ |
| **Ivermectin ($H_2B_{1a}$)** | OH | H | $CHCH_3CH_2CH_3$ |

## 1.9 MODE OF ACTION

The mode of action of macrocyclic lactones (**50**) is to selectively paralyze the parasite by increasing muscle Cl- permeability, specifically avermectins act by binding to glutamate-gated chloride channel receptors in nematode and arthropod nerve cells. This causes the channel to open, allowing an influx of chloride ions. Different chloride channel subunits may show variable sensitivity to macrocyclic lactones and different sites of expression, which could account for the paralytic effects of macrocyclic lactones on different neuromuscular systems at different concentrations. The macrocyclic lactones paralyze the pharynx, the body wall, and the uterine muscles of nematodes.

The effect is similar to that of GABA, but is essentially irreversible. In tissues containing GABA receptors, the avermectin-dependent conductance increase is often accompanied by a loss of sensitivity to exogenously applied GABA and this GABA blocking action may be responsible for the transient tremor observed in mammals. Especially important are the glutamate-gated chloride channels of insect

and nematode skeletal muscle, which may mediate avermectin-induced muscle paralysis in these organisms.

Mammals do not have glutamate-gated chloride channels; the macrocyclic lactones have a low affinity for other mammalian ligand-gated channels and do not readily cross the blood-brain barrier.

Many studies on biological activity and toxicity of avermectins have been done. MLs are relatively safe for approved species such as cattle and sheep. Non teratogenic affects have been observed in these animals (**51**). However, teratogenic and maternotoxic effects have been reported in mice, rats and rabbits. Toxic effects have been noticed in dogs, especially collies, because they are claimed to have more sensitivity to ivermectin then other breeds (**52**). These dogs lack a protein (P-Glycoprotein), which is responsible for pumping out ivermectin and other drugs from the brain. This is caused to a mutation in the multi-drug resistance gene (MDR1), which allows the entrance of toxic substances in the blood-brain barrier.

## 1.10 SALICYLANILIDES AND PHENOL DERIVATES

The discovery of salicylanilides as anthelmintic agents owes its origin to research for new antiseptics. These anthelmintics all have a salicylanilide structure and share common features relating to their mode of action, pharmacokinetics and toxicity.

*Salicylanilide* is a chemical compound which is the amide of salicylic acid and aniline (**Fig. 7**). It is classified as both a salicylamide and ananilide.

Derivatives of salicylanilide have a variety of pharmacological uses. Chlorinated derivatives including niclosamide, oxyclozanide, and rafoxanide are used as antitrematodal and anticestodal agents.

Brominated derivatives including dibromsalan, metabromsalan, and tribromsalan are used as disinfectants with antibacterial and antifungal activities.

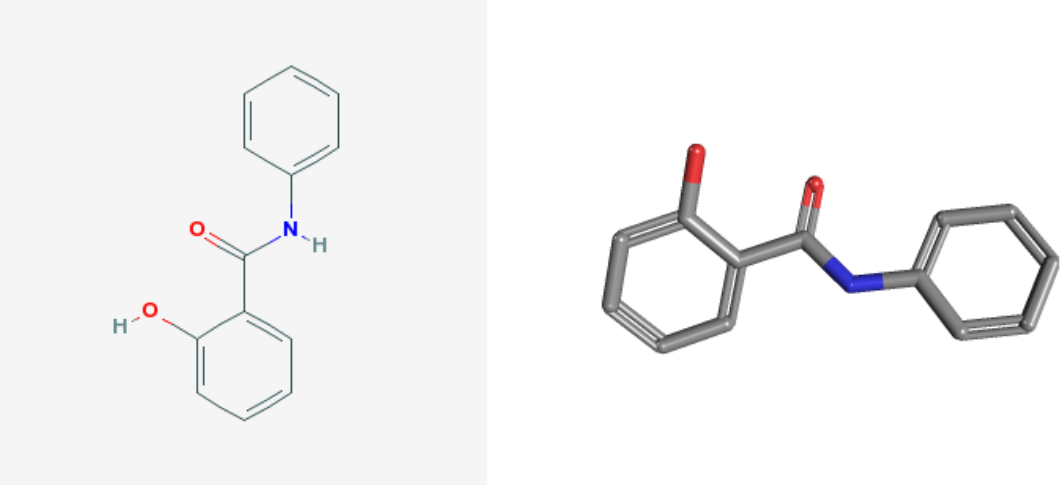



**Fig.7 Salicylanilide structure**

Since the late nineteenth century antibacterial properties of phenol derivatives were commonly known. Further studies led to the introduction of several phenol derivatives among which bithionol and hexachlorophtene. These compounds along with niclofolan and niclosamide were later used to eradicate tapeworms and liver flukes from humans and domestic animals (**53**).

***Niclosamide*** is a highly effective molluscicide and taenicide, which was marketed in 1960. It soon became the drug of choice for treating all gastrointestinal tapeworm infections in humans and domestic animals. This molecule (**Fig. 8**), both in adults and children, is lethal upon contact to taenia solium and saginata but has no effective on mature

proglottides. Niclosamide has also some activity against paramphistomes in ruminants (**54**) and, in the last year, many studies in human medicine have showed its antineoplastic mechanism in leukaemia (**55**) and colon cancer (**56**).

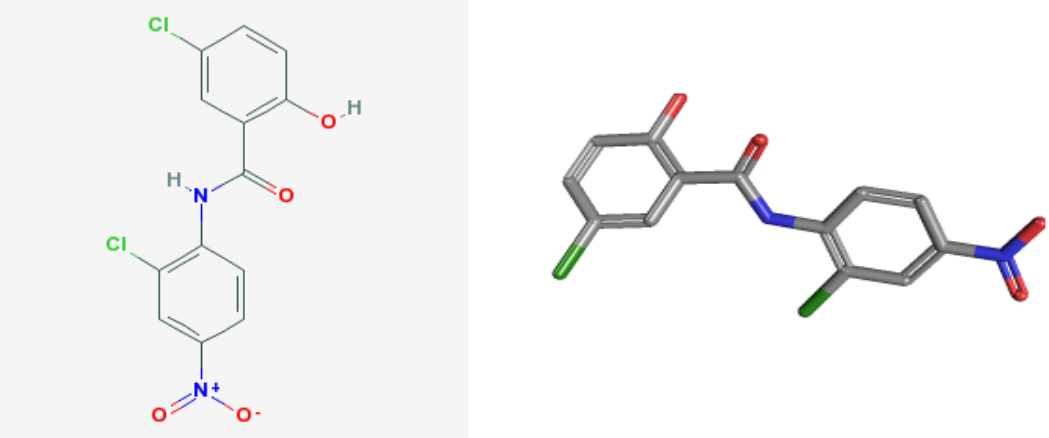



**Fig. 8 Niclosamide**

The pharmacology of drugs used for the treatment of trematodal disease of domestic animals has been afforded much less attention than the antinematodal drug.

A major group of flukicidal drugs became available when the activity of oxyclozanide (**Fig. 9**) against *Fasciola hepatica* was described in 1966 (**57**). Although niclosamide itself possesses two chloro groups, one each in both the benzene rings, scientists considered prominent to study the characteristics of other chloro derivatives.

With its five chloro groups, ***Oxyclozanide*** (**58**) is definitely the most used in the treatment and control of fascioliasis in ruminants and, until now, it was the exclusive flukicide licensed in lactating ruminants. The MRL in milk is 10 µg/kg.

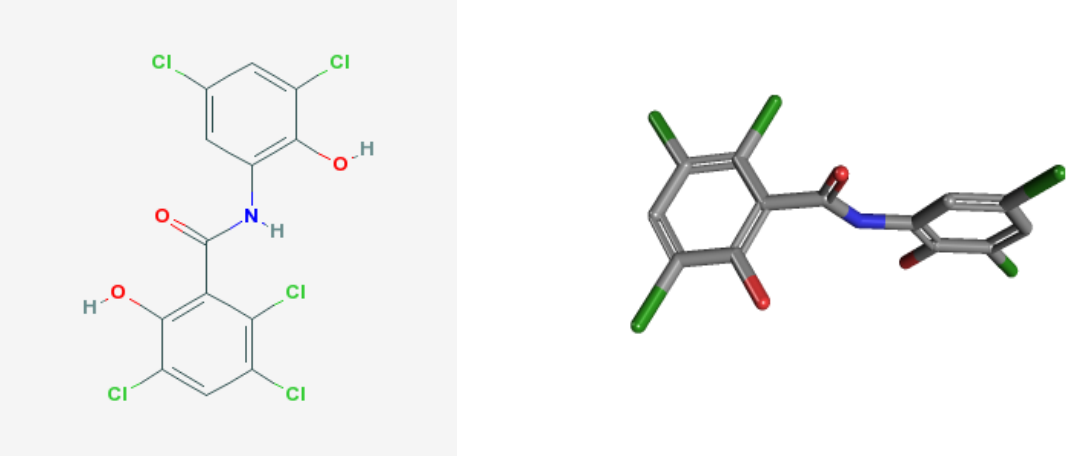



**Fig. 9 Oxyclozanide**

Recently the Committee for Medicinal Products for Veterinary Use of the European Medicines Agency recently adopted a positive opinion recommending the establishment of provisional (expire on the beginning of 2013) maximum residue limits of 45 µg/kg for another flukicide drug, Closantel, in cow and sheep milk.

***Closantel*** (**59**), among with ***Rafoxanide*** (**60**), has a highly antitrematodal activity. This is due to the presence of iodine groups in the benzene ring of the salicylanilides (**Figures 10-11**).

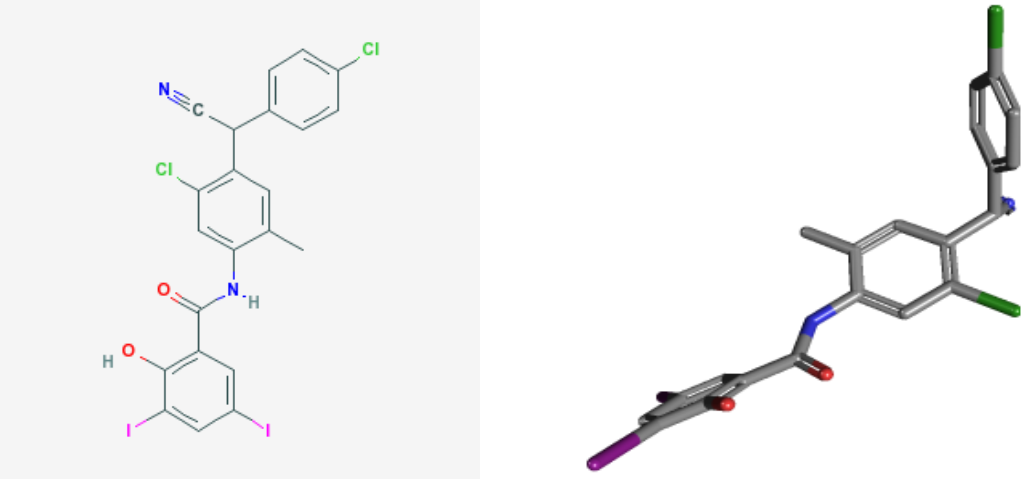



**Fig. 10 Closantel**

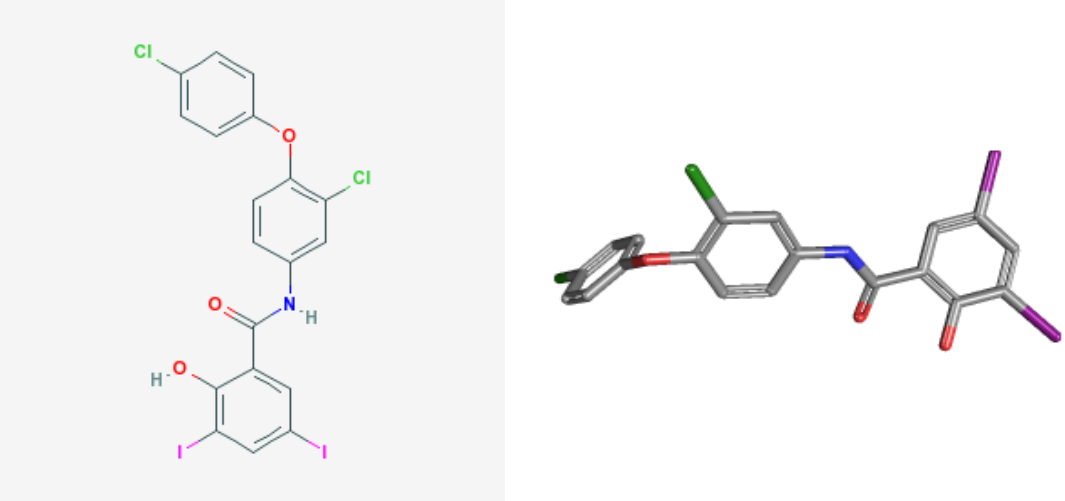



**Fig. 11 Rafoxanide**

These compounds are used extensively for the control of Haemonchus spp. and Fasciola spp. infestations in sheep and cattle and Oestrus ovis in sheep in many parts of the World.

Rafoxanide has MRLs set only for muscle, fat, liver and kidney; this drug cannot be used in animals from which milk is produced for human consumption.

***Nitroxynil*** (3-iodo-4-hydroxy-5-nitrobenzonitrile) is a phenol derivative (**61**) used in cattle and sheep for the control of fascioliasis. When compared with other fasciolicides, this compound shows high activity against both adult and immature liver flukes. The European Union has established Maximum Residue Limits (MRLs) for the marker residue nitroxynil in the tissues liver, kidney, muscle and fat, but no MRL presently is available in milk.

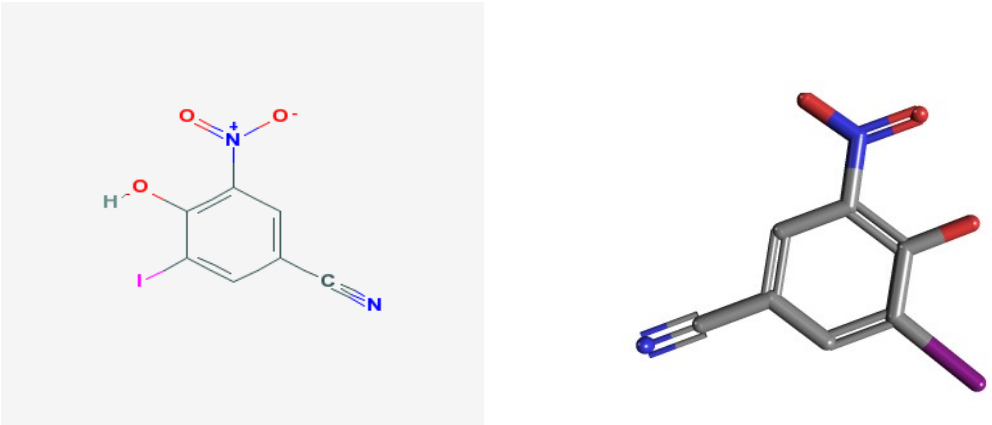



**Fig. 12 Nitroxynil**

## 1.11 MODE OF ACTION

The chemical structure salicylanilides and nitroxynil illustrates that each anthelmintic molecule possesses a detachable proton (H). These molecules are high lipophilic and may shuttle protons across membranes, particularly the inner mitochondrial membrane. The mechanism of action of the proton ionophores has been assumed to be to selectively uncouple oxidative phosphorilation in parasite mitochondria (**62**).

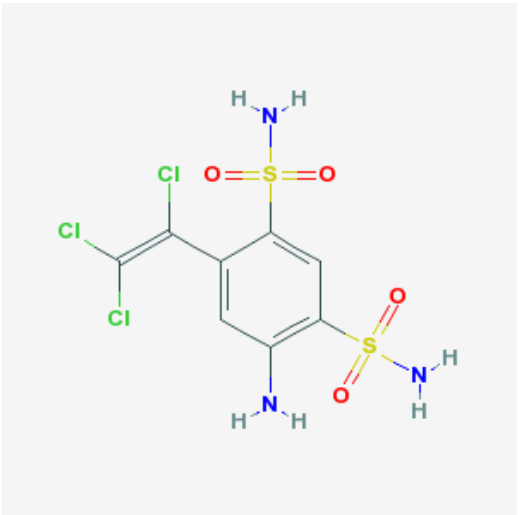
The anthelmintics that have the longest half-life in the body are the salicylanides and nitroxynil. (**63**)

This is explained by their strong plasma protein binding which is more than 99% for the salicylanilides (**64**) and 98% for nitroxynil (**65**). The selective mode of action of these highly protein-bound anthelmintics may be explained, in part, by their effect against blood-sucking parasites, concentrating the anthelmintic in the parasite without the high tissue levels being produced in the host. The high level of protein binding may explain the selective effect of these agents, and the fact that well bled out carcasses have low tissue residue levels. Thus the mode of action of this

group of anthelmintic involves the selective delivery of the proton ionophores to the parasite because of the high level of plasma-protein binding.

## 1.12 **BENZENESULPHONAMIDES**

***Clorsulon*** **(66)** is a compound belonging to the benzenesulphonamide family which is recommended for the control of adult liver flukes in cattle. Frequently is used in association with ivermectin to extend the antiparasite efficacy against roundworms and mites. Throughout the European Union the administration of the 4-Amino-6-(trichlorovinyl)-1,3-benzenedisulfonamide (**Fig. 13**) to dairy cows is not allowed. The Commission Regulation 37/2010 fixed the maximum residue limits only for bovine muscle (35 µg/kg), liver (100 µg/kg) and kidney (200 µg/kg).



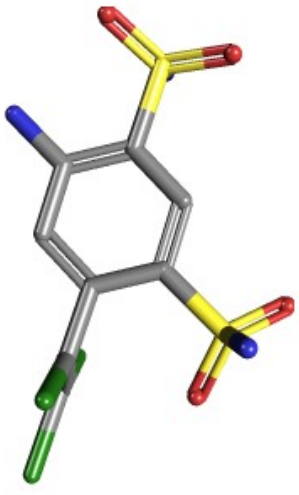



**Figure 13. Clorsulon**

Clorsulon inhibits the enzymes implicated in the glycolytic pathway, the primary source of energy in flukes. Further investigations indicated that clorsulon is a competitive inhibitor of 3-phosphoglycerate kinase and phosphor-glyceromutase and blocks the oxidation of glucose to acetate and propionate. The sulphonamide also depresses the ATP levels in the trematodes (**67**).

A neuromuscular action for clorsulon is unlikely because the flaccid paralysis it induces is essentially long-term in nature. The gradual suppression of motility observed would be compatible with a depletion of energy reserves following glycolysis inhibition.

# *Chapter 2. Methods for the determination of residues in food stuffs*

Residue methods must be able to isolate and detect very small amounts (part per-billion or lower) of an analyte in a variety of complex matrices. The physical and chemical properties of the drugs of interest as well as the accompanying matrix must be evaluated to determine the optimal procedures for extraction, isolation and detection at residue levels.

Because of the potential health risk from exposure to some animal drugs and the low levels at which they are found in edible products, monitoring the food supply for these residues is an important analytical challenge. Tolerances or maximum residue levels are established for drugs that are allowed for use in food animals. For these drugs, analytical methods need to be able to accurately determine if a residue is present above these set limits.

For some compounds (e.g., chloramphenicol, nitrofurans and malachite green) the health concerns are such that no amount of residue is

allowed. In these cases, analytical methods need to be as sensitive and selective as possible to monitor for any residue that might be present.

Several broad types of analytical methods can be described; these include screening, determinative and confirmatory procedures.

## 2.1 SCREENING METHODS

Screening methods are designed to be rapid, easy to use tests that will give a positive or negative response for a drug at a given concentration level in a matrix.

Only those analytical techniques, for which it can be demonstrated in a documented traceable manner that they are validated and have a false compliant rate of < 5 % (β-error) at the level of interest, shall be used for screening purposes in conformity with Directive 96/23/EC (**3**). In the case of a suspected non-compliant result, this result shall be confirmed by a confirmatory method.

## 2.2 CONFIRMATORY METHODS

Determinative methods are designed to separate, quantify and perhaps provide some qualitative information on the analyte of interest. For many of the drugs used in animal husbandry, the determinative method of choice is LC with UV detection using a variable wavelength or diode array detection system.

Confirmatory methods for organic residues or contaminants shall provide information on the chemical structure of the analyte. Consequently methods based only on chromatographic analysis without the use of spectrometric detection are not suitable on their own for use as confirmatory methods. However, if a single technique lacks sufficient specificity, the desired specificity shall be achieved by analytical procedures consisting of suitable combinations of clean-up, chromatographic separations and spectrometric detection.

The **COMMISSION DECISION 2002/657 EC (68),** implementing Council Directive 96/23/EC concerning the performance of analytical

methods and the interpretation of results, is probably the key document of legislation to be consulted by analytical laboratories in veterinary residue control.

It lists performance criteria and other requirements for separation and detection techniques for both screening and confirmatory methods.

It provides for the progressive establishment of minimum required performance limits (**MRPL**) of analytical method for substances for which no permitted limit has been established and in particular for those substances whose use is not authorised, or is specifically prohibited in the Community, in order to ensure harmonised implementation of Directive 96/23/EC.

In this Commission Decision all the performance characteristics that can be attributed to an analytical method have been specified. This includes definitions and descriptions how to assess recovery, repeatability, ruggedness, and detailed requirements for MS detection and identification of targeted substances. Decision limit (CC-alfa), and detection capability (CC-beta), aimed at replacing limit of detection

(LOD) and limit of quantitation (LOQ), are addressed in this document and have received broad attention**(69)**.

The limit of detection (LOD) for any analytical procedure, the point at which analysis is just feasible, may be determined by a statistical approach based on measuring replicate blank (negative) samples or by an empirical approach, consisting of measuring progressively more dilute concentrations of analyte. The limit of quantitation (LOQ), or concentration at which quantitative results can be reported with a high degree of confidence, may likewise be determined by either approach (**70**).

Decision limit (**CCα**) represents the limit at and above which it can be concluded with an error probability of that a sample is non-compliant.

Detection capability (**CCβ**) means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of. In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a

statistical certainty of 1−**β**. In the case of substances with an established permitted limit, this means that the detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1− **β (68)**. The following methods or method combinations are considered suitable for the identification of organic residues or contaminants for the substance groups indicated:

- LC or GC with mass-spectrometric detection (group **a** and **b**)
- LC or GC with IR spectrometric detection (group **a** and **b**)
- LC-full-scan DAD (group b)
- LC -fluorescence(group b)
- 2-D TLC - full-scan UV/VIS(group b)
- GC-Elektron capture detection(group b)
- LC-immunogram(group b)
- LC-UV/VIS (single wavelength) (group b)

In addition to determining how much of a drug residue is present, confirmation of identity is also required for a complete analytical regulatory package. *Mass spectrometry* (**MS**) is ideally suited for this qualitative analysis due to its inherent selectivity and sensitivity. Certain criteria, in terms of the number and quality of ions monitored for any given compound, need to be met to definitively determine if a residue has been positively identified.

## 2.3 MULTICLASS METHODS

An emerging trend in veterinary drug residue analysis is the development of methods that are capable of monitoring a wide variety of residues, regardless of drug class, in a single sample. Screening and confirmatory data are collected by MS; some methods also provide quantification of residues. Both ion trap and triple quadrupole MS-MS instruments have been successfully utilized. Several *multi-class veterinary drug residues methods* have been reported in recent years. In the matter of anthelmintics residues, Kinsella et al. (71) have developed a simple but effective multi-class method for the detection and quantification of 38 anthelmintics residues in bovine milk and liver. This is a QuEChERS ("quick, easy, cheap, effective, rugged and safe") method. Recently another method was optimized by Whelan et al. (72) using UHPLC–MS/MS with fast polarity switching. The method allows the detection of anthelmintic drug residues in milk to $<1\,\mu g\ kg^{-1}$.

This method has been used for these studies on residues of antiparasite drugs allowed and not in milk and dairy products.

# *Chapter 3. Investigation of the persistence of levamisole and oxyclozanide in milk and fate in cheese*

Anthelmintic drugs are widely used in veterinary medicine for protecting or treating animals mainly against gastrointestinal nematodes and trematodes. Levamisole is effective against lungworms and roundworms at both larval and adult stages while oxyclozanide is mainly used in the treatment of adult stages of liver fluke.

The European Regulation 37/2010 (4) has set Maximum Residue Limit in milk for oxyclozanide (10 μg $kg^{-1}$ ) but no MRL is fixed for levamisole. The widespread availability of cheaper generic veterinary medicinal products and the development of drug resistance to the limited number of licensed products have increased the potential for off-label applications (73).

However, levels detected in food are generally well below toxicity thresholds and pose no risk to the consumers, but there are concerns about the presence of residues in milk.

No data has been published in peer reviewed literature concerning the fate of these residues during cheese production. The knowledge generated from this study is particularly useful should these drugs be accidentally administered to dairy cows.

## 3.1 SUMMARY

The aim of this study was to investigate the persistence of ***oxyclozanide*** and ***levamisole*** residues in bovine milk after treatment with a combination product and their subsequent fate during cheese production. Animals were milked twice daily up to 16 days post treatment. Soft (3 d ripening), hard (35 d ripening) and whey cheeses were produced from the milk samples collected from first two milkings. Milk and cheese samples were subsequently analysed by UPLC-MS/MS. Highest levels of levamisole (<600 $\mu g/kg^{-1}$) and oxyclozanide (<25 $\mu g/kg^{-1}$) were determinate at first and third milking respectively.

Levamisole residues were found to concentrate in all cheese types. There was a three fold concentration effect for levamisole in mature

cheese. Oxyclozanide residues were found to occur at lower levels in soft and hard cheese than milk with a 10-fold concentration in whey cheese compared to milk.

Residues were found at higher levels in cheeses compared to the milk. The results of this study demonstrate that levamisole and oxyclozanide residues are rapidly excreted in dairy cows and milk is compliant after few days, but survive the fermentation process to persist in cheese.

## 3.2 MATERIALS AND METHODS

### 3.2.a Animal Studies

Six Friesian cows weighing between 400 and 500 kg were selected for the study. The six cows were treated with the maximum dose (150 mL for animals 300 Kg and over) of TOLOXAN, containing levamisole (5mg $kg^{-1}$ bodyweight) and oxyclozanide (10mg $kg^{-1}$ bw). The first milk sample was taking at 6 p.m., 9.5 h after administration. Milk samples were subsequently taken from the animals twice daily, morning (6 a.m.)

and evening (6 p.m.) over a 16 days period and the samples were frozen (-20 °C) until analysis.

Mature, soft and whey cheese were produced from the first two milkings collected 9.5 and 21.5 h after administration by the following procedure. Pooled raw milk from the six animals was collected (60 L) and heated to 38 °C, a curdling agent (cagliodoro) was added and kept for 30 min. The curdle was then broken and kept for 15 min after which the curdle and liquid whey were separated. The curdle was then placed in a mould and the surface was salted to produce soft cheese after 3 d of maturation. Half of the soft cheese was refrigerated for 35 d to produce mature cheese. The liquid whey was boiled and placed immediately in a mould to obtain the whey cheese. All samples were frozen (-20 °C) until analysis.

**3.2.b Materials and Reagents**

Pre-weighed 50 mL polystyrene centrifuge tubes containing 4 g anhydrous (anh.) magnesium sulphate ($MgSO_4$) and 1 g sodium chloride

(NaCl) (Tube 1), and 1.5 g anh. $MgSO_4$ and 0.5 g $C_{18}$ (Tube 2) were obtained from UCT, Inc. (Bristol, PA; USA).

### 3.2.c Extraction and clean-up

As described in Whelan et al. (**72**), milk samples (10 g ± 0.1 g) were weighed into centrifuge tubes (50 mL) and fortified with internal standard and left to sit for 15 min. Cheese samples (4 g ± 0.04 g) and Millipore water (6 g ± 0.06 g) were weighed into centrifuge tubes (50 mL) and placed in a water bath at 50 °C until the cheese and water become homogeneous, they are then fortified with internal standard and left to sit for 15 min. MeCN (12 mL) was added to tube one containing $MgSO_4$ (4 g) and NaCl (1 g). The contents of tube one was added to the sample and shaken immediately to extract the residues into the MeCN layer. The sample was centrifuged for 12 min at 3,500 rpm (959 *g*). A dispersive-SPE cleanup step was performed by pouring the supernatant from tube one into tube two (50 mL) containing $MgSO_4$ (1.5 g) and $C_{18}$

(0.5 g). The samples were vortexed for 30 s and centrifuged for 10 min at 2,500 rpm (489 *g*). The supernatant (6 mL) and DMSO (0.25 mL) were added to a starstedt tube (15 mL) and vortexed for one min. The MeCN layer was evaporated under nitrogen at 50 °C to 0.25 mL. Extracts were filtered through 0.2 μm PTFE syringe filters (Whatman Rezist) and injected onto the UPLC-MS/MS system. Any samples that fell outside the calibration range were diluted in negative milk and reanalysed.

### 3.2.d UPLC-MS/MS analysis

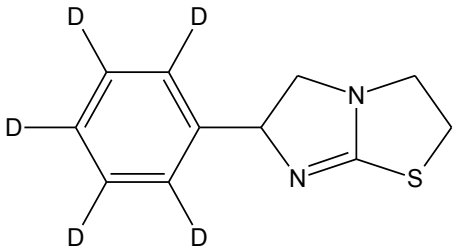
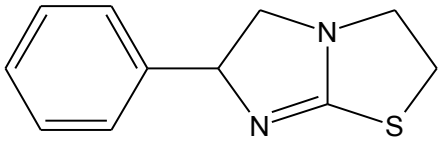
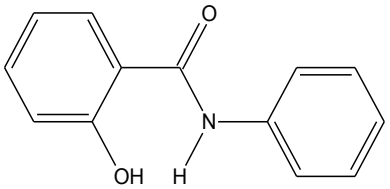
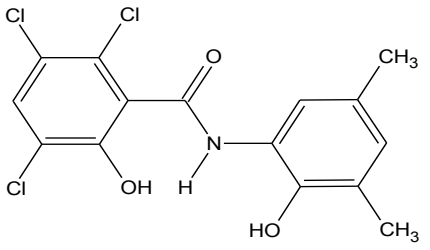
Chromatographic separations were performed using a Waters (Milford MA; USA) Acquity UPLC system, the column used was a 100 mm × 2.1 mm i.d., 1.8 μm, Acquity HSS T3, with an in-line filter unit with 0.2 μm stainless steel replacement filters (Waters). The column oven was maintained at a temperature of 60 ºC and the Acquity pump was maintained at a flow rate of 0.6 mL/min. Analytes were separated using the following gradient elution comprising of mobile phase A, 0.01%

HOAc in water:MeCN (90:10 v/v) and mobile phase B, 5mM ammonium formate in MeOH:MeCN (75:25, v/v). The gradient profile was as follows: 0 – 0.5 min, 100% A; 5 min, 50% A; 7 min, 10% A; 8.5 min, 10% A; 8.51 min, 0% A; 9.5 min, 0% A; 9.51 min, 100% A; 13 min 100% A (**72**).

A Waters Quattro Premier XE mass spectrometer was used to quantify the veterinary drug residues found in the milk samples The electrospray ionisation (ESI) UPLC-MS/MS system was controlled by MassLynx software and chromatographic data was processed using TargetLynx Software (both from Waters). Injection volume was 2 µL. The analytes and internal standards (**Table 3**) were tuned on the UPLC-MS/MS and the optimum conditions were obtained during tuning and they were input into the MS settings. The following transitions were input into multiple reaction monitoring (MRM) windows, 210.10→183.08 (*m/z*), $d_5$-levamisole; 204.93→122.89 (*m/z*) and 204.93→177.94 (*m/z*), levamisole; 212.05→92.00 (*m/z*), salicylanilide; 397.80→175.75 (*m/z*) and 397.80→201.80 (*m/z*), oxyclozanide. $d_5$-

Levamisole retention time 2.82 min and levamisole retention time 2.83 min were input to MRM channel 1 and detected in positive ion mode, salicylanilide, retention time 5.95 min and oxyclozanide, retention time 6.83 min were input to MRM channel 2. The MRM's were time-sectored; dwell time, inter-scan delay and inter-channel delays were set to get maximum response from the instrument.

**Table 3. UHPLC condition. Analytes and internal standards.**

| ANALYTE | $t_R$ (min) | Transition *(m/z)* | Cone (V) | CE (V) | ESI Polarity | IS |
|---|---|---|---|---|---|---|
| **Levamisole D5**  | 1.58 | 210.10→183.08 | 40 | 20 | + | IS |
| **Levamisole**  | 1.59 | 204.93→122.89<br>204.93→177.94 | 35<br>35 | 27<br>14 | +<br>+ | LEVA-D5 |
| **Salicylanilide**  | 5.65 | 212.05→92.00 | 35 | 28 | - | IS |
| **Oxyclozanide**  | 6.63 | 397.80→175.75<br>397.80→201.80 | 32<br>32 | 26<br>20 | - | SALI |

### 3.2.e Calibration

Levamisole, oxyclozanide and salicylanilide were purchased from Sigma-Aldrich. $d_5$-Levamisole was purchased from Witega Laboratories. Primary stock standard solutions were prepared at concentrations of 4 and 2 mg/mL for oxyclozanide and levamisole respectively. Internal standards (salicylanilide and $d_5$-levamisole) were prepared at concentration of 1 mg/mL. $d_5$-Levamisole was prepared in deuterated methanol (in case of deuterium exchange in solution) and the remaining powders were prepared in methanol.

Extracted matrix calibrants were prepared by fortifying negative milk samples prior to extraction with working standard mixes, prepared at the following concentrations (in μg/mL): 10, 5 (Std 7) 5, 4 (Std 6), for levamisole and oxyclozanide respectively and 2 (Std 5), 1 (Std 4), 0.5 (Std 3), 0.2 (Std 2), and 0.1 (Std 1) for both analytes. Matrix-matched calibration curves were prepared by fortifying matrix blanks before extraction with 100 μL of the standards to give working standard curves in the sample equivalent range of 1 to 100 and 1 to 50 μg/kg for

levamisole and oxyclozanide respectively. An additional four blank matrix samples (recovery controls) were fortified after extraction, two with Std 2 (50 µL) and two with Std 5 (50 µL) to monitor for loss of analytes during extraction.

### 3.2.f Development of the method

The extraction, clean up procedure and UPLC-MS/MS method used in this investigation were developed previously (**72**). The extraction and clean-up method did not require modifications as the method performance was satisfactory. This was demonstrated for levamisole by participating in a proficiency study. As this was not a multi method and the sensitivity of levamisole and oxyclozanide was not critical, it was possible to reduce the injection volume from 5 to 2 µL and still detect sub µg/kg levels. This resulted in an extended linear range from 1 – 50 µg/kg to 1 – 100 µg/kg for levamisole, and oxyclozanide remained 1 – 50 µg/kg with an improvement in the $r^2$ value due to the reduced amount of matrix injected onto the system. A preliminary run was carried out to determine

the concentration of the analytes in the samples. The samples were then diluted in negative milk samples to fit the calibration range and the samples were re-extracted.

The method was extended to cheese samples. The only modification was to the initial sample, 4 g of cheese and 6 g of water was added to the centrifuge and the sample was heated to 50 $^{o}$C. This resulted in a liquid suspension of cheese and water. The cheese suspension was then treated the same as the milk samples.

## 3.3 RESULTS AND DISCUSSION

### 3.3.a Levamisole and oxyclozanide residues in bovine milk

There are surprising reports in literature of the persistence of levamisole residues in the milk of dairy cows after oral administration. Archambault et al. (**74**) investigated the persistence of levamisole in dairy cows after a subcutaneous injection (10 mg/kg bw). The analytical method employed was only to measure levamisole residues to the limit of detection (LOD) of the assay, 100 μg/kg. Levamisole residues persisted

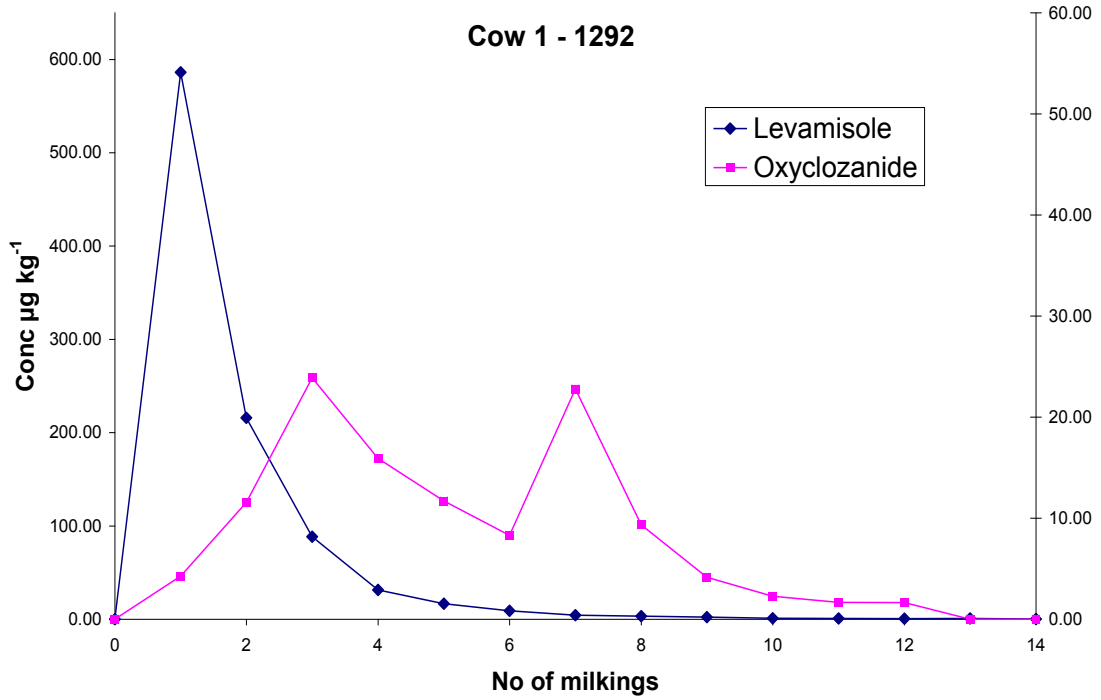
for approximately 12 d above the LOD post treatment. Osterdahl et al. (**75**) treated cows (n = 42) infected with lungworm by an intramuscular injection (7 mg/kg bw). Levamisole residues were less than the LOD, 40 µg/L in all animals by 29 h post treatment. De Ruyck et al. (**76**) treated cows (n = 4 each dose) topically with 10 and 20 mg/kg bw levamisole and levels declined to 21 and 83 µg/L respectively by 79 h post treatment. At 7 h post-treatment, mean concentrations of levamisole residues were 1896 (low dose) and 6027 µg/kg (high dose). Paulson and Feil (**77**) treated dairy cows with oral (n = 1) and s.c. injectable doses (n = 1) with radiolabelled levamisole (8 mg/kg bw). Highest concentrations after oral and subcutaneous doses were 191 and 208 µg/kg, respectively. Levamisole residues were measurable at approximately 1 µg/kg (levamisole parent drug) after both treatments. Simkins et al. (**78**) carried out an extensive study in four groups of animals (n = 5 animals per group), which were treated with different formulations, oral drench, pellets, bolus and subcutaneous injection containing 8 mg/kg bw levamisole. Levamisole residues were less than 10 µg/kg at 48 h for all

formulations. Highest levamisole residues were detectable at 12 h post-treatment: oral dose (240 to 750 µg/kg), pellet (160 to 900 µg/kg), bolus (220 to 840 µg/kg) and s.c injection (140 to 830 µg/kg).

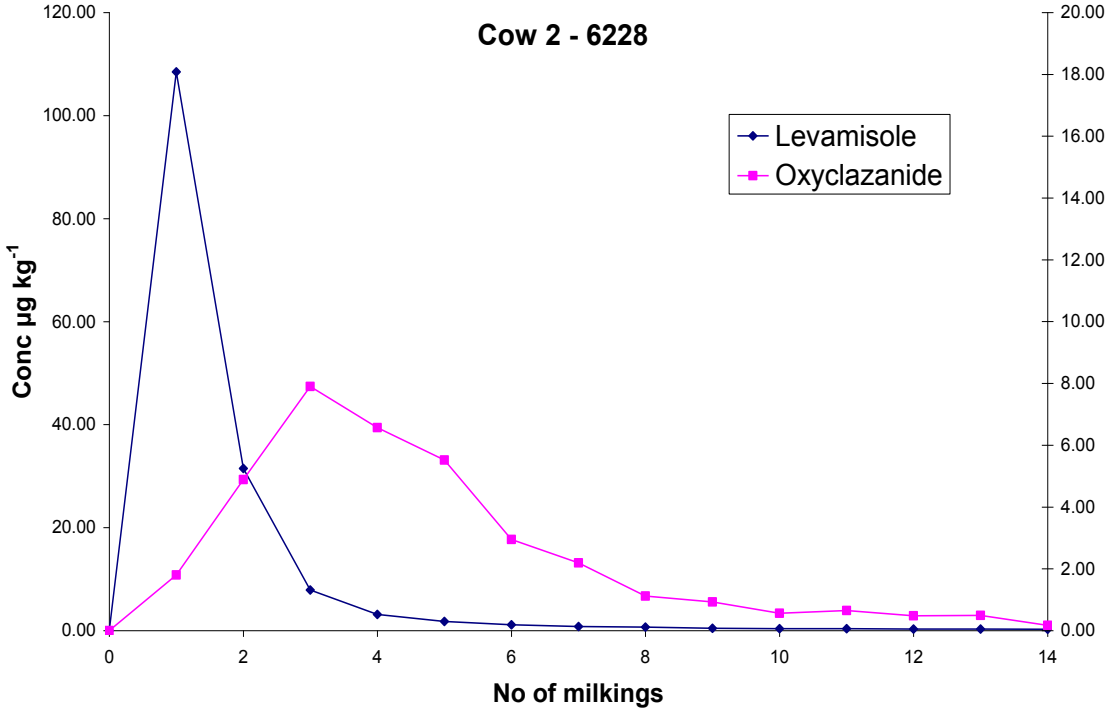
In this study, dairy cows (n = six) were treated with a combination product containing levamisole and oxyclozanide. Milk samples were collected at morning (6 a.m.) and evening (6 p.m.) milking for 16 d post-treatment and analysis was carried out by UPLC-MS/MS. In agreement with previous studies, highest levels of levamisole residues were detected at the first milking and ranged from 108 to 586 µg/kg (**Figure 14**). Levamisole residues were found to be detectable at above the limit of reporting for levamisole ($CC\alpha$ = 0.83 µg/kg) (72). Residues of levamisole were <LOR on the $11^{th}$, $7^{th}$, $8^{th}$, $7^{th}$, $9^{th}$ and $8^{th}$ milking for cow one to six respectively. The results of the study show that using LC-MS/MS technology that levamisole residues were compliant 130 h post-treatment.

**Figure 14. Depletion profile of residues in bovine milk (A-F). Animal 1 to 6 respectively. Scale for Levamisole (left) and oxyclozanide (right)**

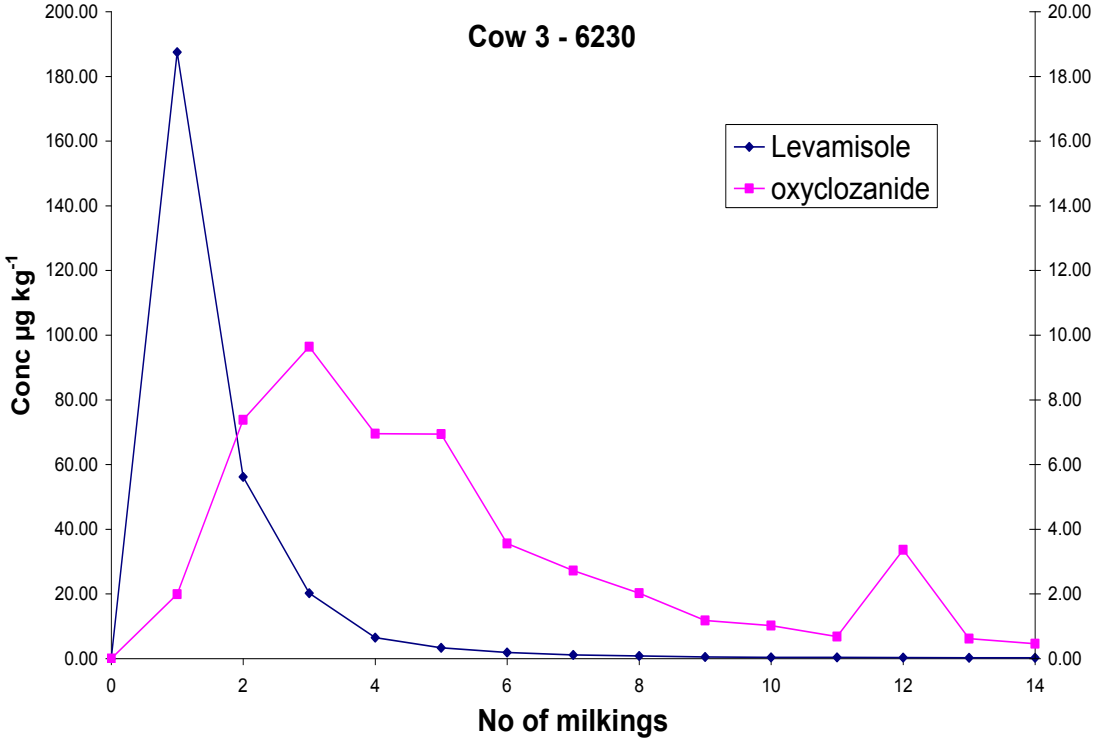
**(A)**



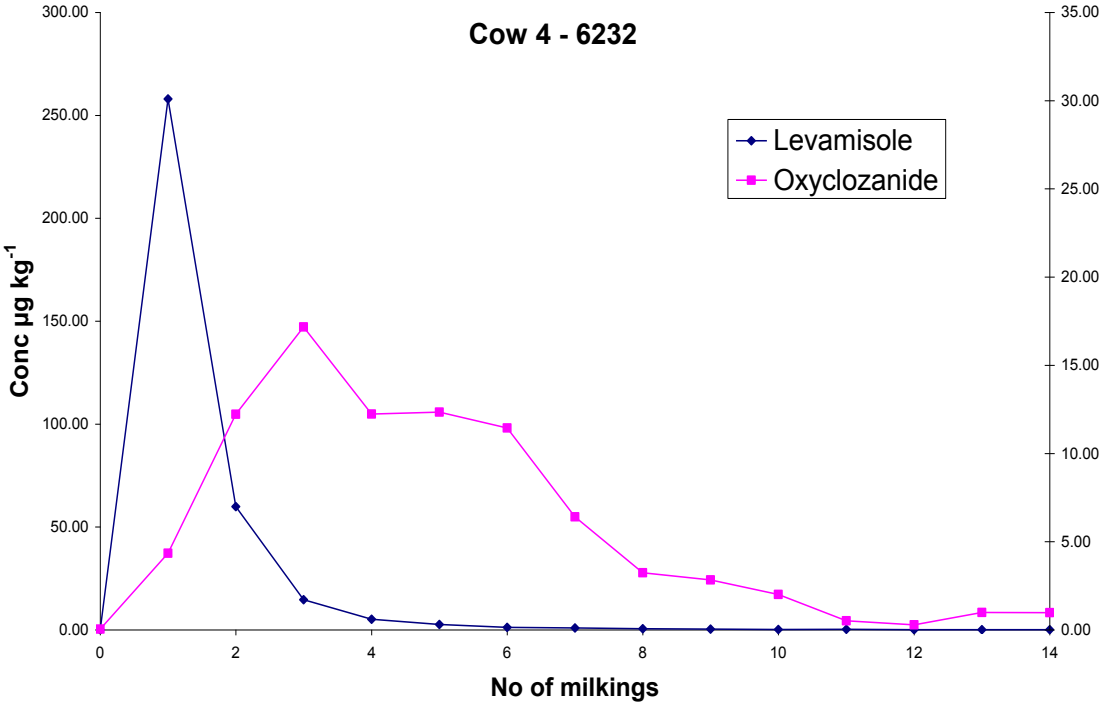

**(B)**



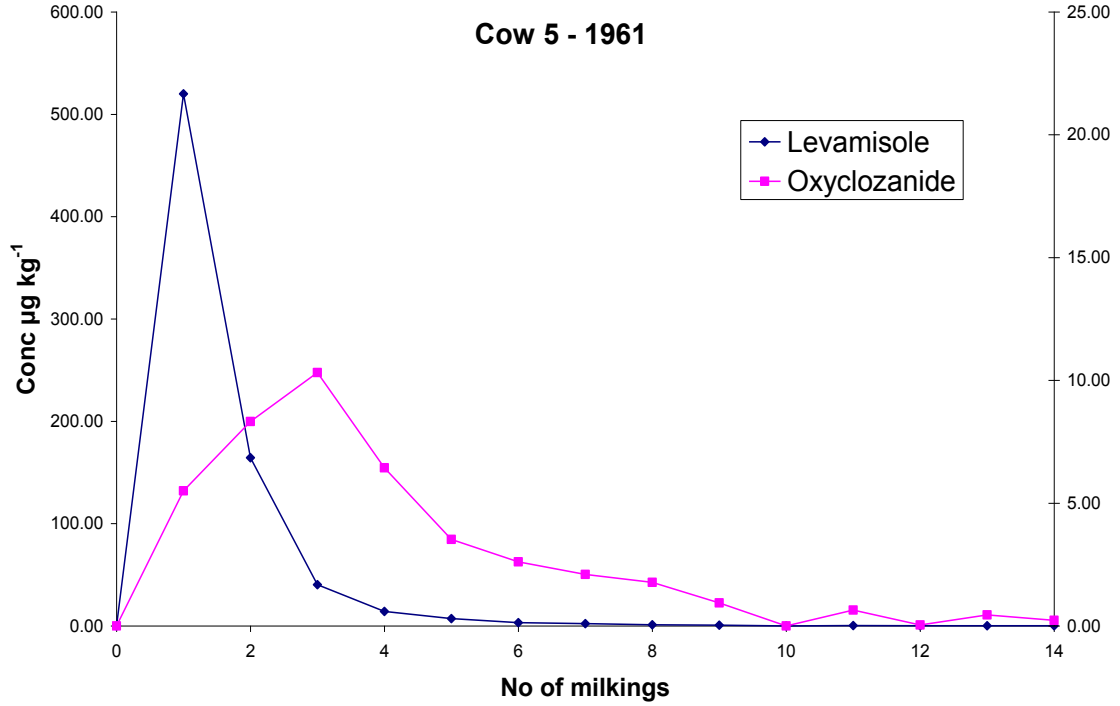
**(C)**


Cow 3 - 6230
Levamisole
oxyclozanide
Conc µg kg-1
No of milkings
200.00
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
2
4
6
8
10
12
14

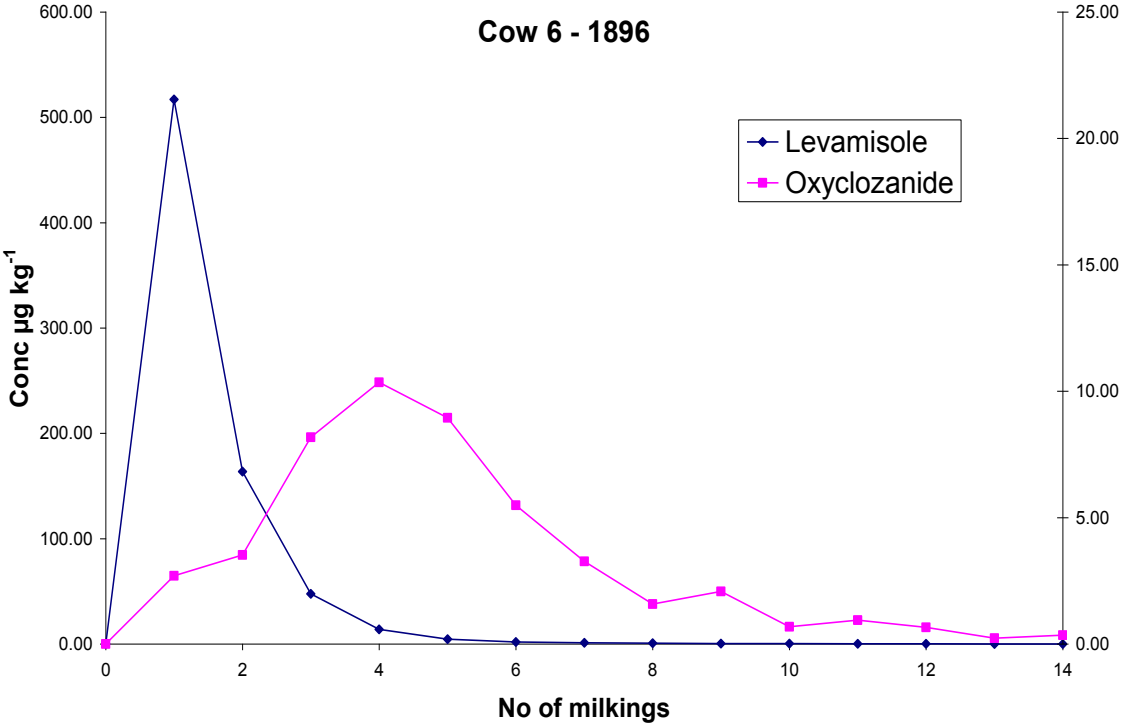
**(D)**


Cow 4 - 6232
Levamisole
Oxyclozanide
Conc µg kg-1
No of milkings
300.00
250.00
200.00
150.00
100.00
50.00
0.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0
2
4
6
8
10
12
14

**(E)**


Cow 5 - 1961
Levamisole
Oxyclozanide
Conc µg kg-1
No of milkings
600.00
500.00
400.00
300.00
200.00
100.00
0.00
25.00
20.00
15.00
10.00
5.00
0.00
0
2
4
6
8
10
12
14

**(F)**


Cow 6 - 1896
Levamisole
Oxyclozanide
Conc µg kg-1
No of milkings
600.00
500.00
400.00
300.00
200.00
100.00
0.00
25.00
20.00
15.00
10.00
5.00
0.00
0
2
4
6
8
10
12
14

Only two papers have been reported on the persistence of oxyclozanide in milk. Fujinuma et al. treated cows orally with 10 mg/kg bw oxyclozanide and residues were detectable until 30-47 h above 1 µg/kg (79). Residues were typically at <10 µg/kg in all animals at all time-points. Bluthgen et al. treated cows orally with 10 mg/kg bw oxyclozanide and residues were detected at 130 µg/kg at 48 h post treatment and 10 µg/kg at the prescribed withholding period of 120 h (80).

In this study the highest levels of oxyclozanide ranged between 8 and 24 µg/kg and were determined at the third milking. Oxyclozanide residues were <1 µg/kg at 13$^{th}$, 8$^{th}$, 11$^{th}$, 11$^{th}$, 9$^{th}$, and 10$^{th}$ milking for animal one to six respectively. Residues were below the MRL (10 µg/kg) at 8$^{th}$, 7$^{th}$, 4$^{th}$ and 5$^{th}$ milking for cows one, four, five and six, and below the MRL for all time points for cows two and three.

The seventh and 12$^{th}$ milking for animal one and three respectively were at higher concentrations than the previous samples in the depletion profile. These samples were reanalysed to ensure results were correct and repeat analyses confirmed previous results. The results were further

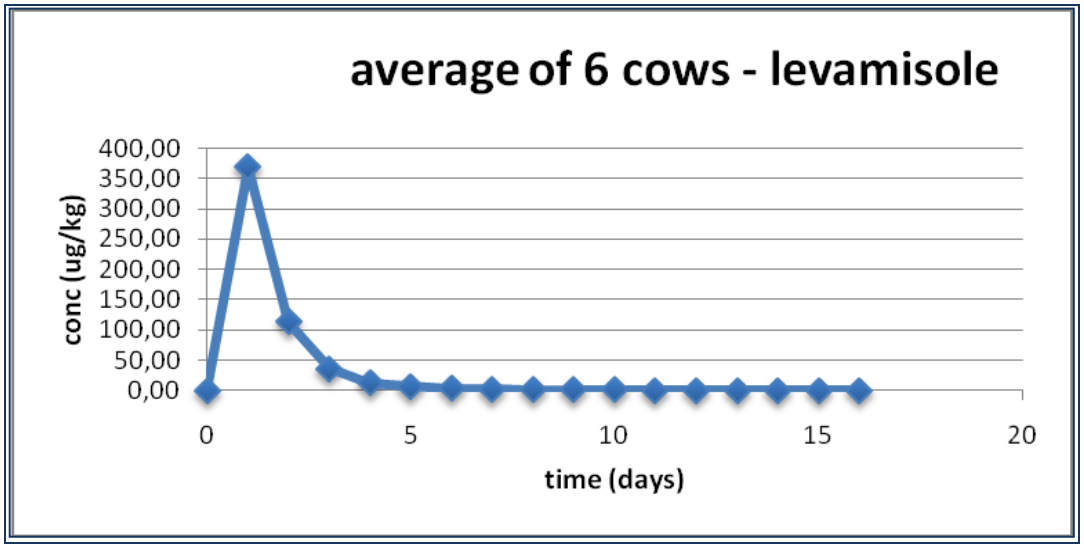
verified as the concentration of levamisole in the samples was as expected in the depletion profile.

**Table 4. Depletion of Levamisole and Oxyclozanide in Milk – average of 6 cows**

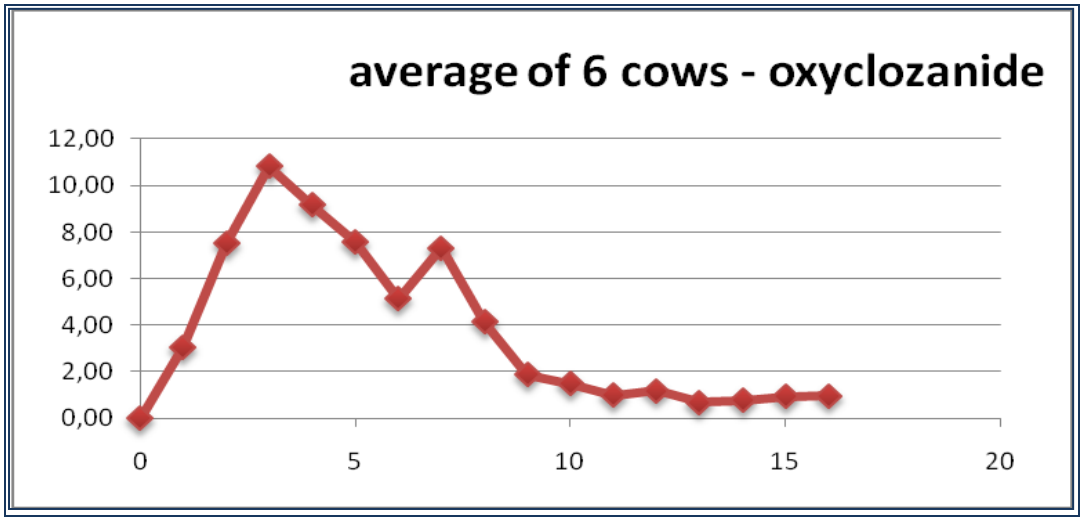
| Time point | Levamisole | Oxyclozanide | CCα Levamisole | MRL Oxyclozanide |
|---|---|---|---|---|
| | ppb | ppb | µg $kg^{-1}$ | µg $kg^{-1}$ |
| **0** | 0,00 | 0,00 | 0,83 | 10 |
| **0,5** | 339,87 | 3,23 | 0,83 | 10 |
| **1** | 113,08 | 7,50 | 0,83 | 10 |
| **1,5** | 36,14 | 10,83 | 0,83 | 10 |
| **2** | 12,24 | 9,18 | 0,83 | 10 |
| **2,5** | 6,07 | 7,57 | 0,83 | 10 |
| **3** | 3,13 | 5,14 | 0,83 | 10 |
| **3,5** | 1,68 | 7,31 | 0,83 | 10 |
| **4** | 1,44 | 4,14 | 0,83 | 10 |
| **4,5** | 0,78 | 1,87 | 0,83 | 10 |
| **5** | 0,59 | 1,48 | 0,83 | 10 |
| **5,5** | 0,42 | 0,99 | 0,83 | 10 |
| **6** | 0,35 | 1,20 | 0,83 | 10 |
| **6,5** | 0,29 | 0,69 | 0,83 | 10 |
| **7** | 0,18 | 0,78 | 0,83 | 10 |

**Figura 15. depletion of levamisole (A) and oxyclozanide (B) - average in the 6 cows**

(A)




(B)



## 3.3.b FATE OF RESIDUES IN CHEESES

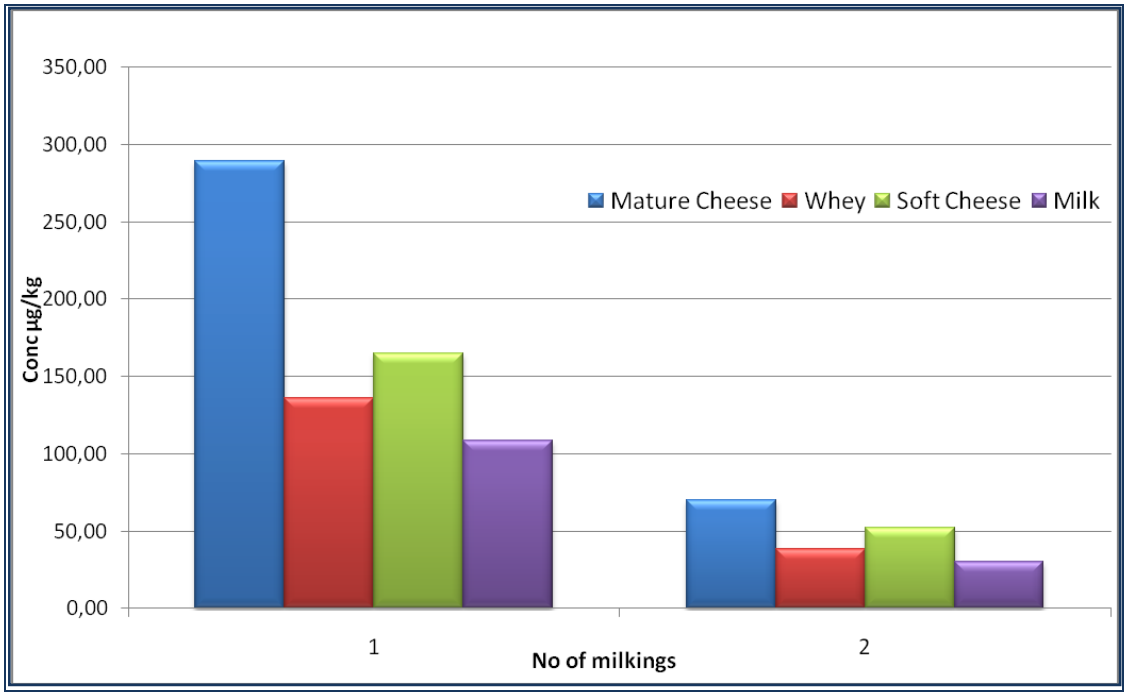
Three types of cheese were produced from pooled milk of the six animals from the first two milkings 9.5 and 21.5 h after administration; soft, mature and whey cheese. The cheese samples were produced from milk incurred with levamisole and oxyclozanide.

### Levamisole residues in cheese

In general, the amount of levamisole residues found in the soft, mature and whey cheeses produced from the first two milking had similar patterns (Figure 14). The concentration of levamisole in the pooled milk was 108 and 30 µg/kg for the first and second milking respectively. The cheeses produced from the first milking had the following concentrations: 290, 136 and 165 µg/kg for mature, whey and soft cheese respectively. In the cheese produced from the second milking 70, 38 and 52 µg/kg levamisole residues were detected in the mature, whey and soft cheese

respectively. The values observed in cheese deserve careful attention as the concentration was higher than that found in milk. The results indicate that the levamisole residue binds more strongly to the fat in the curdle than the proteins in the whey. Levamisole residues concentrated in mature cheese samples as water content decreased. The results found indicate that levamisole residues survive the fermentation process and the whey heat treatment and are stable during storage.

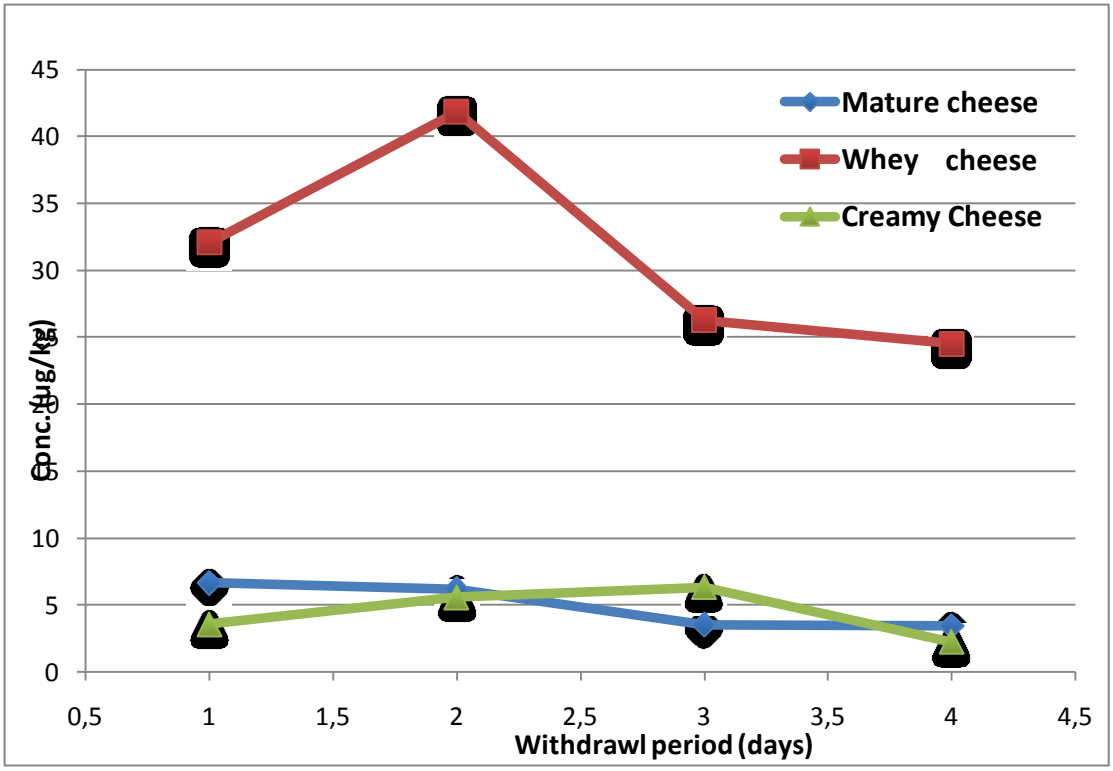
**Figure 14. Depletion of levamisole in cheeses**



**Oxyclozanide residues in cheese**

The levels of oxyclozanide found in mature, whey and soft cheese produced from the first two milkings indicates oxyclozanide is stable during storage and survives the fermentation process (Figure 15). The concentration of oxyclozanide in the pooled milk was 7.0 and 13.4 µg/kg for the first and second milking respectively. In the cheese produced from the first milking 6.7, 32.1 and 3.6 µg/kg oxyclozanide residues were detected in the mature, whey and soft cheese respectively. In the cheese produced from the second milking 6.2, 41.9 and 5.6 µg/kg oxyclozanide was detected in mature, whey and soft cheese respectively. Oxyclozanide residues were found to 10-fold concentrate in whey cheese, which is likely due to strong binding of acidic drugs residue to the proteins in the whey.

**Figure 15. Depletion of oxyclozanide in cheeses**



## 3.4 CONCLUSIONS

This study showed that levamisole and oxyclozanide are rapidly excreted in milk and residues are compliant after the 11th and 13th milkings, respectively. Combination products containing levamisole and oxyclozanide are currently not allowed for the treatment of dairy animals and the results of this study show the consequences of hypothetical illegal use. Levamisole is well absorbed after oral administration as seen in Table 5. However, the results indicate that a short withdrawal period would be needed to ensure milk is compliant in the event that a MRL is set for levamisole. Presently, no MRL is set for levamisole and detection of its residues in milk or dairy products will deem a sample non-compliant. This study also shows levamisole and oxyclozanide are stable during storage and survive the fermentation process with an increase in concentration compared to the concentration found in milk.

# *Chapter 4. Persistence of nitroxynil residues in bovine milk by ultra performance liquid chromatography tandem mass spectrometry (UPLC/MS-MS) following a single administration in the dry period.*

## 4.1 SUMMARY

Nitroxynil (61) is a halogenated phenol used to control fascioliasis in cattle and sheep. 35 pregnant dairy cows were treated in this study with a nitroxynil 340 mg/ml solution for injection at the recommended dose of 10 mg/nitroxynil per kg body weight at the start of the dry period, 53 to 74 days before the expected calving. Calving occurred between 43 days and 79 days after treatment. Subsequently, the concentrations of nitroxynil in the milk were monitored for up to 120 days after calving. Nitroxynil was detected by UHPLC-MS/MS (ultra high performance liquid chromatography coupled to tandem mass spectrometry).

The present study quantified the residue concentrations of nitroxynil in the milk of cows which were treated by subcutaneous administration at the recommended dose of 10 mg nitroxynil per kg body

weight at the beginning of the dry period. The occurrence and the depletion of the residues in milk after calving was investigated.

## 4.2 INTRODUCTION

Nitroxynil is a flukicide belonging to the family of phenol derivatives and according to the Commission Regulation No 37/2010 (**4**) can not be used in animals from which milk is produced for human consumption. Until December 2009 in a few countries of the European Union a limited number of compounds, one of which is Nitroxynil, were licensed for use in animals treated during dry cow period. The probably of finding non compliant samples after calving is more then virtual (**65**) and for this reason products containing nitroxynil are no longer allowed in dairy cows.

Nowadays there is no Maximum Residue Limit fixed for this drug in milk, the European Union has established MRLs for the marker residue Nitroxynil (**4**) only in bovine and ovine muscle (400 μg/kg $^{-1}$), liver (20 μg/kg $^{-1}$), fat (200 μg/kg $^{-1}$) and kidney (400 μg/kg $^{-1}$). Aim of the study

was to determinate the persistence and the depletion of Nitroxynil in dried off cows.

## 4.3 EXPERIMENTAL

35 healthy pregnant Friesian-Holstein dairy cows from "Animal & Grassland Research and Innovation Centre", Teagasc Moorepark, Fermoy, Co. Cork were selected for the experiment. The cows used in this study, with an average body weight of 600kg, had not been treated with products containing nitroxynil within 12 months before the start of this study as documented by the farm records. The cows were treated at the start of the dry period, from **53** to **74** days before the expected calving with TRODAX 340 mg/ml (Merial ®). This corresponds to 1.5ml Solution for Injection per 50 kg body weight. The dose to administer was calculated on the basis of the estimated average body weight of the cows. Milk samples were taken daily for the first four days and then every three days for up to 120$^{th}$ day and stored at -20°C until analysis.

## 4.4 REAGENTS AND SAMPLES

Ultra-pure water (18.2 MOhm) was generated in house using a Millipore (Cork, Ireland) water purification system. HPLC-grade methanol (MeOH) and acetonitrile (MeCN), 99.5% deuterated methanol (MeOH-d), and ammonium formate (puriss p.a) were sourced from Sigma-Aldrich (Dublin, Ireland). Analar grades of DMSO, isopropyl-alcohol (IPA), and glacial acetic acid (HOAc) were obtained from BDH Chemicals Ltd., (Poole, UK). Pre-weighed mixtures of 4 g anhydrous magnesium sulphate ($MgSO_4$) and 1 g sodium chloride (NaCl) in 50 mL centrifuge tubes, and 1.5 g $MgSO_4$ and 0.5 g $C_{18}$ in 50 mL centrifuge tubes were obtained from UCT, Inc. (Bristol, PA; USA). Organic milk was purchased in supermarkets and tested for residues prior to analysis.

## 4.5 STANDARDS, INTERNAL STANDARDS AND STOCK SOLUTIONS

Nitroxynil and $^{13}C$ Nitroxynil were purchased from Sigma-Aldrich and Witega laboratories respectively. Primary stock standard solution was prepared at concentrations of 4,000 and 1,000 μg $mL^{-1}$ for nitroxynil and $^{13}C$ Nitroxynil respectively in MeOH.

Intermediate working standard solution was prepared at a concentration of 50 μg $mL^{-1}$ for nitroxynil in MeOH. A working internal standard solution was prepared at 4μg $mL^{-1}$ for $^{13}C$ Nitroxynil in MeOH-d.

Extracted matrix calibrants were prepared by fortifying negative milk samples prior to extraction with a working standard mix, prepared at the following concentrations (in μg $mL^{-1}$): 0.1, 0.25, 0.5, 1, 2.5, and 5; Standard 1 to 6 respectively. Matrix-matched calibration curves were prepared by fortifying matrix blanks before extraction with 100 μL of the standards to give working standard curves in the sample equivalent range of 1 to 50 μg $kg^{-1}$.

Samples with values above the concentration of 50 µg kg $^{-1}$ were diluted with control milk. An additional four blank matrix samples (recovery controls) were fortified after extraction, two with Std 2 (50 µL) and two with Std 5 (50 µL) to monitor for loss of analytes during extraction.

## 4.6 APPARATUS

A glass dispenser (Dispensette® lll, Brand) was used for aliquoting MeCN extraction solvent, a Mistral 3000i centrifuge, micro centrifuge (Eppendorf), a multi-vortexer, a Caliper Life Sciences (Runcorn, UK) Turbovap LV evaporator, and a Transsonic 780LH ultrasonic bath were used for the extraction.

Separations were performed using a Waters (Milford MA; USA) Acquity UPLC system comprising of a stainless steel HSS T3 analytical column (100 × 2.1 mm, particle size 1.8 µm) equipped with an in-line filter unit containing a 0.2 µm stainless steel replacement filter maintained at a temperature of 60ºC and the pump was operated at a flow

rate of 0.6 mL $min^{-1}$. Analytes were separated using a binary gradient elution containing a mobile phase A water:MeCN (90:10, v/v) with 0.01% HOAc and mobile phase B MeOH:MeCN (75:25, v/v) with 5mM ammonium formate. The gradient profile was as follows: (1) 0 – 0.5 min, 100% A, (2) 5 min, 50% A, (3) 7 min, 10% A, (4) 8.5 min, 10% A, (5) 8.51 min, 0% A, (6) 9.5 min, 0% A, (7) 9.51 min, 100% A, (8) 13 min 100% A. Injection volume was 5 µL.

The veterinary drug residues and their metabolites were quantified using a Waters Quattro Premier XE triple quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) interface. The UPLC-MS/MS system was controlled by MassLynx™ software and data was processed using TargetLynx™ Software (both from Waters).

## 4.7 SAMPLE PREPARATION

Milk samples (**6**) (10 g ± 0.1 g) were weighed into centrifuge tubes (50 mL) and fortified with internal standard and left to sit for 15 min. Acetonitrile (12 mL) was added to tube one containing $MgSO_4$ (4 g) and

NaCl (1 g). The contents of tube one was added to the sample and shaken immediately to extract the residues into the MeCN layer. The sample was centrifuged for 12 min at 3,500 RPM (959 *g*). A dispersive-SPE cleanup step was performed by pouring the supernatant from tube one into tube two (50 mL) containing $MgSO_4$ (1.5 g) and $C_{18}$ (0.5 g). The samples were vortexed for 30 s and centrifuged for 10 min at 2,500 RPM (489 *g*). The supernatant (6 mL) and DMSO (0.25 mL) were added to a starstedt tube (15 mL) and vortexed for one min. The MeCN layer was evaporated under nitrogen at 50°C to 0.25 mL.

Extracts were filtered through 0.2 µm PTFE syringe filters (Whatman Rezist®) and injected onto the UPLC-MS/MS system. Any samples that fell outside the calibration range were diluted in negative milk and reanalysed.

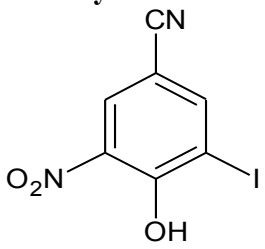
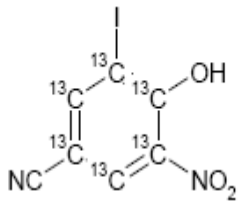
## 4.8 RESULTS AND DISCUSSION

### 4.8.a Method

The method used for the detection of Nitroxynil was developed previously (72) for 38 anthelmintic residues in milk. In this study the samples were only monitored for nitroxynil. The MRM window (Table 6) only contains two transitions, one for nitroxynil and one for $^{13}C6$ Nitroxynil. The following transitions were input into multiple reaction monitoring (MRM) windows, 288.90→126.86 (*m/z*) and 288.90→161.95 for nitroxynil; 295→126.69 for the $^{13}C6$ nitroxynil internal standard. The retention time, 3.46 min, was the same for both nitroxynil and $^{13}C6$ nitroxynil that have been detected in negative ion mode. In the method developed by Whelan et al., the incurred samples were mostly highly positive and the sensitivity for negative ionization mode was not so acceptable. Nitroxynil was linear in the range 1 - 50 µg kg. Maximum concentration of the drug was 657 µg kg, which is outside the linear range of the calibration curve. Samples were typically 10 time more

concentrated then the range of the curve so, after the first quantification, samples were re-extracted with a dilution in organic milk (analyzed previously) by a factor of 1 in 10 or 1 in 5 depending on the initial concentration found in the first extraction. Using rapid polarity switching in electrospray ionization, a single injection was capable of detecting charged ions in a 13 minutes run time.

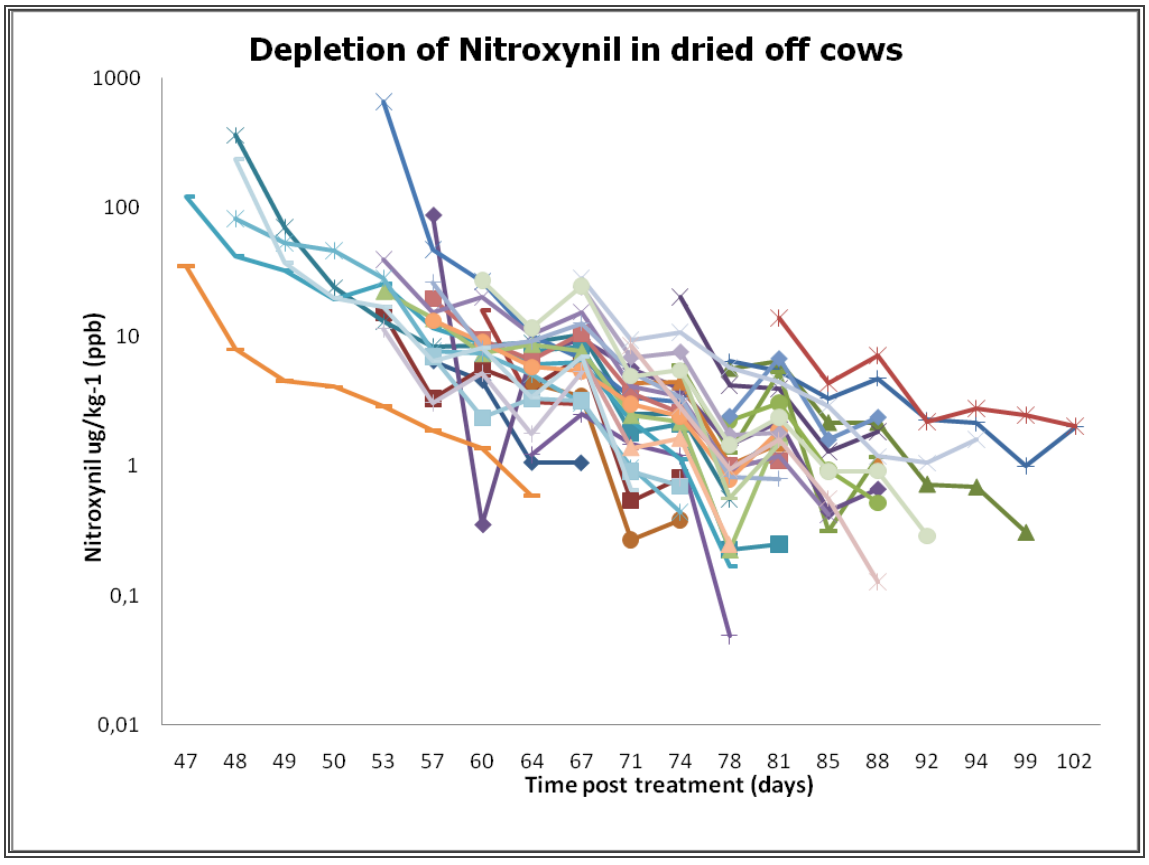
**Table 6. UPLC-MS/MS Conditions**

| Analyte | $t_R$ (min) | Transition (m/z) | Cone (V) | CE (V) | MRM window | ESI Polarity | IS |
|---|---|---|---|---|---|---|---|
| **Nitroxynil**  | 3.46 | 288.90→126.86<br>288.90→161.95 | 36<br>36 | 24<br>20 | 3<br>3 | -<br>- | $^{13}$C |
| **$^{13}$C 6 Nitroxynil**  | 3.46 | 295→126.69 | 40 | 25 | 3 | - | IS |

### 4.8.b Persistence of nitroxynil residues

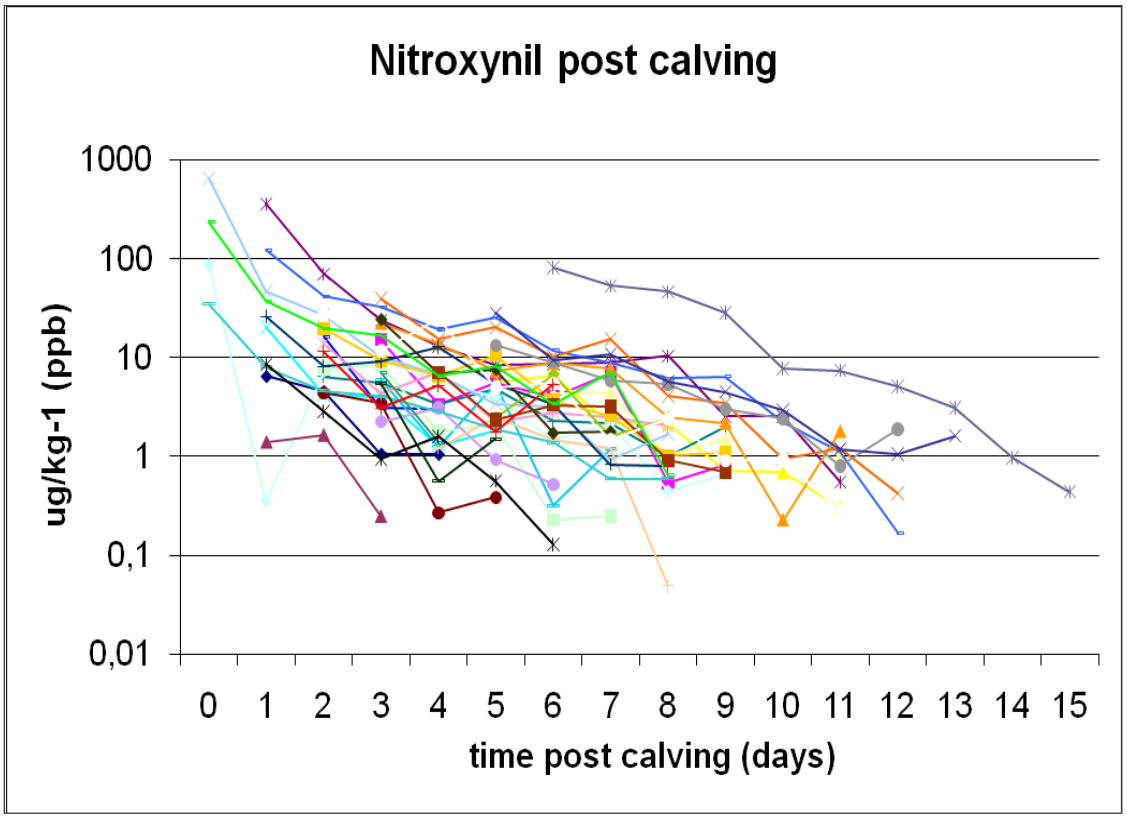
The results of this study show that nitroxynil residues are very persistent but low in bovine milk of treated cows during the drying period. In two of 35 animals, were found to be detectable at 102 days after treatment with concentration of 2 µg $kg^{-1}$. The cc alpha for nitroxynil is 0.24 µg $kg^{-1}$. The highest levels of the drug were detected in two animals (Figure 16) at the 48$^{th}$ and 53$^{rd}$ day post treatment with concentrations respectively of 362 and 657 µg $kg^{-1}$.

**Figure 16. Depletion of Nitroxynil – time post treatment**



The depletion profile is represented in the graph below that shows the reduction of the drug in the post calving period of each cow. This study was conducted in pregnant dairy cows at the beginning of the dry period because these are the target animal population for the investigated treatment.

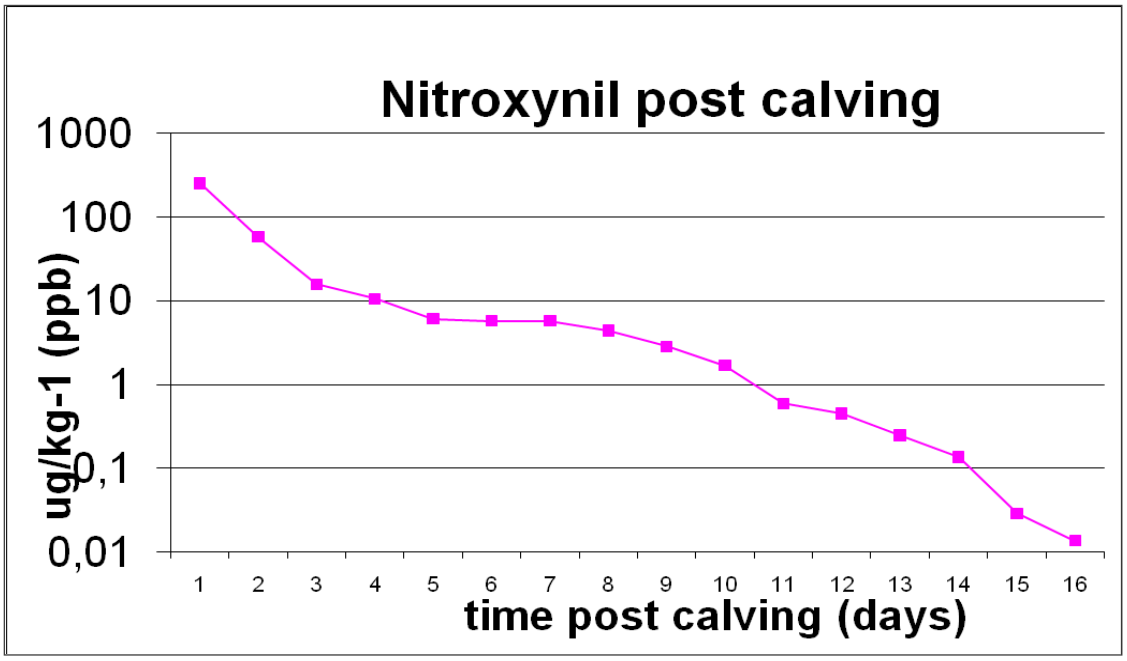
**Figure 17. Depletion of Nitroxynil - post calving**




The figure 18 shows the concentration of the antitrematodal drug in the mean of the animals, that decreases to ~ 10 µg/kg at 4 days post calving.

**Figure 18. Concentration of the drug in post the calving period**

**a) average of 35 cows**




**b) standard deviation**

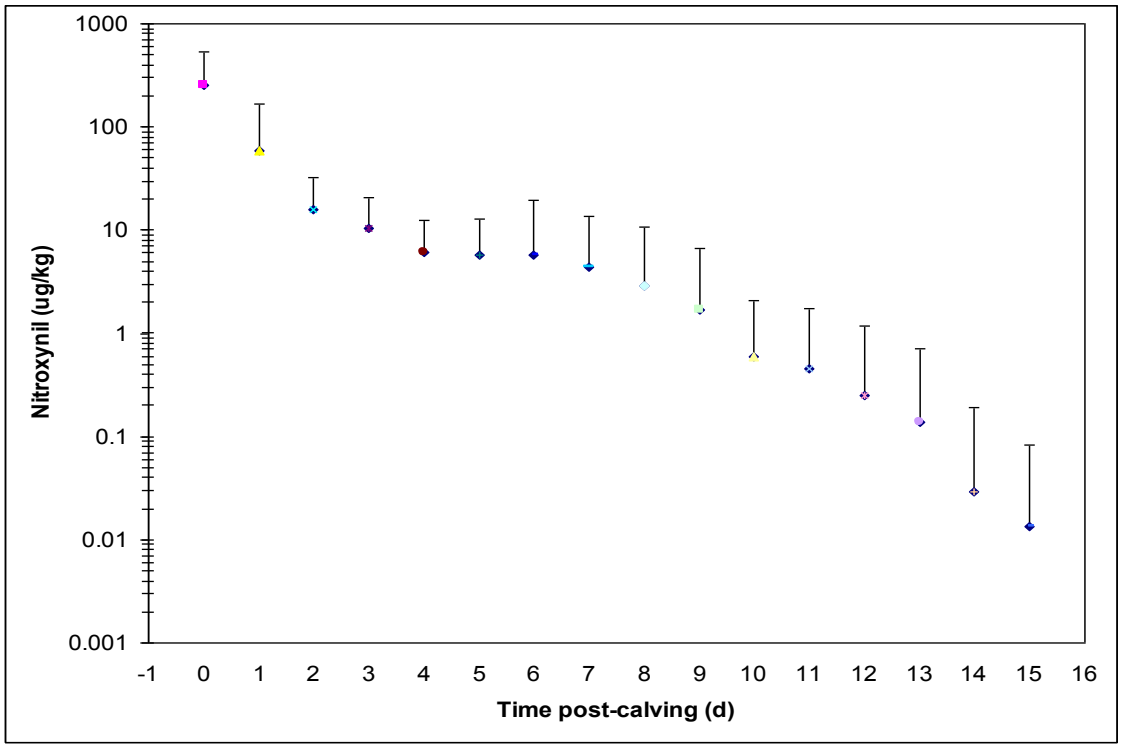


**Table 7. Descriptive statistical parameters for the concentrations (µg/kg) of nitroxynil in the milk of dairy cows post administration of 10 mg nitroxynil per kg body weight at the beginning of the dry period - Data on animals in post-calving period.**

| Cow No. | Wt (kg) | Date calved | Day calved | Cmax (µg/kg) | Cmax (d) | Tmax (d) | <LOD (PT d) | <LOD (PC d) |
|---|---|---|---|---|---|---|---|---|
| 2545 | 706 | 01/02/10 | 56 | 6.5 | 57 | 67 | 71 | 15 |
| 2645 | 674 | 26/01/10 | 50 | 15.3 | 53 | 74 | 78 | 28 |
| 2719 | 664 | 18/02/10 | 73 | 6.5 | 81 | 99 | 102 | 29 |
| 2809 | 674 | 18/02/10 | 73 | 20.4 | 74 | 88 | 92 | 19 |
| 2850 | 620 | 23/01/10 | 47 | 362.4 | 48 | 78 | 81 | 34 |
| 2972 | 612 | 07/02/10 | 62 | 4.4 | 64 | 74 | 78 | 16 |
| 3089 | 688 | 21/02/10 | 77 | 6.4 | 78 | 102 | 106 | 29 |
| 3175 | 638 | 03/02/10 | 58 | 16.2 | 60 | 67 | 71 | 13 |
| 3261 | 646 | 16/02/10 | 71 | 6 | 74 | 88 | 92 | 21 |
| 3282 | 580 | 02/02/10 | 57 | 86.9 | 57 | 88 | 92 | 25 |
| 3304 | 604 | 07/02/10 | 62 | 7.8 | 64 | 81 | 85 | 23 |
| 3354 | 493 | 13/02/10 | 68 | 4.5 | 74 | 88 | 92 | 24 |
| 3362 | 542 | 29/01/10 | 53 | 656 | 53 | 81 | 85 | 32 |
| 3381 | 528 | 24/02/10 | 80 | 14 | 81 | 102 | 106 | 26 |
| 3385 | 522 | 20/02/10 | 76 | 3.1 | 81 | 88 | 92 | 16 |
| 3389 | 524 | 05/02/10 | 60 | 2.5 | 67 | 78 | 81 | 21 |
| 3408 | 550 | 22/01/10 | 46 | 122.4 | 47 | 78 | 81 | 35 |
| 3429 | 662 | 23/01/10 | 47 | 35 | 47 | 64 | 67 | 20 |
| 3494 | 632 | 18/02/10 | 73 | 6.9 | 81 | 88 | 92 | 19 |
| 3589 | 500 | 31/01/10 | 55 | 19.7 | 57 | 81 | 85 | 30 |
| 3592 | 584 | 26/01/10 | 50 | 22.6 | 53 | 81 | 85 | 35 |
| 3610 | 588 | 26/01/10 | 50 | 39.7 | 53 | 85 | 88 | 38 |
| 3611 | 526 | 19/01/10 | 43 | 81.7 | 49 | 78 | 81 | 38 |
| 3625 | 544 | 28/01/10 | 52 | 13.3 | 57 | 81 | 85 | 33 |
| 3689 | 598 | 01/02/10 | 56 | 26.3 | 57 | 81 | 85 | 29 |
| 3773 | 544 | 09/02/10 | 64 | 7.1 | 67 | 71 | 74 | 10 |
| 4247 | 732 | 16/02/10 | 71 | 5.6 | 74 | 81 | 85 | 14 |
| 8116 | 720 | 09/02/10 | 64 | 24.1 | 67 | 81 | 85 | 21 |
| 8201 | 702 | 29/01/10 | 53 | 7.1 | 57 | 74 | 78 | 25 |
| 8229 | 672 | 15/02/10 | 70 | 1.6 | 74 | 78 | 81 | 11 |
| 8240 | 634 | 07/02/10 | 62 | 28.4 | 67 | 94 | 99 | 37 |
| 8259 | 650 | 15/02/10 | 70 | 8.6 | 71 | 88 | 92 | 22 |
| 8269 | 742 | 03/02/10 | 58 | 27.4 | 60 | 92 | 94 | 36 |
| 8285 | 574 | 27/01/10 | 51 | 11.6 | 53 | 74 | 78 | 27 |
| 8295 | 640 | 24/01/10 | 48 | 237.4 | 48 | 71 | 74 | 26 |

## 4.9 CONCLUSIONS

Nitroxynil residues were found to very persistent in milk compared to other anthelmintic drug residues (65-81). When dairy cows are treated with Trodax 340 mg/ml Solution for injection at the highest recommended dose of 10 mg nitroxynil per kg body weight at the beginning of a dry period (about 53 to 80 days before calving – expected calving dates), residues of this flukicide are quantifiable in the milk of the 35 cows until at least 13 days after calving (decision limit - cc $\alpha$ = 0.24 $\mu g\ kg^{-1}$).

Highest concentrations of the drug were detected at the first milkings and ranged from 656 µg kg in the cow 3362 to 362 µg kg in the cow 2850 at $53^{rd}$ and $67^{th}$ day after treatment respectively. Nitroxynil residues were below the limit of detection of the method between $67^{th}$ and $106^{th}$ day post treatment.

This could due to the size difference in body compartments and the amount of fat present in each animal. However, there was considerable variation in levels between animals, this could be due to size difference in

body compartments and the amount of fat of each animal. The age and health of the animal also can have an effect on the results, the drugs behave differently in animals with/without parasitic infections.

In conclusion highest concentrations of nitroxynil occur in the milk produced shortly after calving. This colostrum milk is not suitable for human consumption and is collected separately from the milk that enters the bulk tank on the dairy farm. After the colostrum period, this is from about 3 days after calving, the concentrations in milk decrease to 10 μg $kg^{-1}$or below and residues are non-detectable 10 to 38 days post-calving. The results of this study could be interesting for the establishment of a Maximum Residue limit for nitroxynil in bovine milk.

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