STUDIES ON DISTRIBUTION AND PERSISTENCE OF HALQUINOL AND ITS METABOLITES IN VARIOUS TISSUES OF CHICKEN

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STUDIES ON DISTRIBUTION AND PERSISTENCE OF HALQUINOL AND ITS METABOLITES IN VARIOUS TISSUES OF CHICKEN

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By

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DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

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CERTIFICATE

This is to certify that the thesis entitled "**Studies on distribution and persistence of halquinol and its metabolites in various tissues of chicken**". submitted by **Indu Tripathi I.D. No. DVHK 1018 in partial fulfillment of the requirements for the award of Doctor of Philosophy** in **Veterinary Pharmacology and Toxicology** of the Karnataka Veterinary, Animal and Fisheries Sciences University Bidar, is a bonafide record of research work done by her during the period of her study in this University under my guidance and supervision, and that the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar titles.

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Dedicated to

My Param Pujya Guruji

My beloved Parents

and

Late Dr. K. Jayakumar

Whose incessant blessings has led me so far

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LIST OF ABBREVIATIONS

ADI	Acceptable daily intake
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ATR	Attenuated total reflectance
b. wt.	Body weight
BUN	Blood urea nitrogen
°C	Degree Celsius
CVMP	Committee for veterinary medicinal products
EDTA	Ethylenediamine tetra acetic acid
EU MRL	European union maximum residue limit
FAA	Free amino acid
FCR	Feed conversion ratio
Fig.	Figure
FTIR	Fourier transform infrared spectroscopy
g	gram
H or h	hour
HPTLC	High Performance Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
IR	Infrared
JECFA	Joint expert committee for food additives
KBr	Potassium bromide
kg	kilogram
L	Litre

LD_{50}	Lethal dose in 50 per cent of population
M	Molarity
mM	millimolar
ml	millilitre
mm	millimeter
min	Minute
N	Normality
MRL	Maximum residue limit
NOEL	No observed effect level
NOAEL	No observed adverse effect level
OECD	Organization for Economic Cooperation and Development
PVDF	Polyvinylidene fluoride
rpm	Rotations per minute
SE	Standard Error
TLC	Thin layer chromatography
MRL/MRLs	Maximum residue limit/s
MRPL	Minimum required performance limit
PDA	Photodiode array
PFA	Prevention of food adulteration
WP/WPs	Withdrawal period/s
NMR	Nuclear magnetic resonance
USFDA	United States Food and Drug Administration
USDA	United States Department of Agriculture

UV-Vis	Ultraviolet visible
p	Probability
ppm	Parts per million
mg	Milligram
µl	microlitre
µ	Micron
pH	-log[H^+]/ negative log of hydrogen ion concentration
%	Per cent
r^2	Regression coefficient
R_t	Retention time
R_f	Retention factor
nm	nanometer
ng	Nanogram
g	gram
@	At the rate of
v/v	Volume by volume
w/w	Weight by weight
w/v	Weight by volume
i/v	intravenous

INTRODUCTION

I. INTRODUCTION

In recent years, concerns about the use of antimicrobials in food producing animals have focused on human food safety because foods of animal origin are vehicles of food borne disease in humans. The selection of drug-resistant bacteria populations is a consequence of exposure to antimicrobial drugs and can occur from human and animal uses. Hence the use of non-antibiotic growth promoter came into existence. Halquinol is one such drug which is being extensively used in Asian as well as European countries to promote growth in poultry and swine production.

Halquinol is a non-antibiotic antimicrobial mixture of chlorohydroxyquinolines. Its powerful antimicrobial activity makes it a superior growth promoter in poultry. It is a mixture obtained by chlorinating 8-hydroxy quinoline. It generally contains 5,7-dichloroquinolin-8-ol (57 to 74%), 5-chloroquinolin-8-ol (23 to 40%), and 7-chloroquinolin-8-ol (up to 4%) . Halquinol is used as an antibacterial, antifungal and antiprotozoal feed additive for poultry and as a growth promoter in swine. It is known to potentiate the effect of anticoccidial drugs in poultry. It is also useful to overcome malabsorbtion syndrome as it has wide spectrum of activity and slows down peristalsis in the gut (Nischal *et al* ., 2012).

It has various advantages over other compounds used for similar purpose. It is mainly a gut acting compound and not absorbed from the gastrointestinal tract. It is a triple acting antidiarrheal product effective against bacteria, fungi and protozoa. Because of its specificity in action against bacteria, it induces minimum or no resistance in bacteria even on prolonged use.

It is a broad-spectrum antimicrobial having antibacterial, antifungal and antiprotozoal activities, and is being used in India and other countries to overcome several challenges in modern poultry and swine farming, like microbial infections, and for growth promotional aspects, by incorporating it with feed at different levels. Besides, halquinol bolus has been used in large animal practice to treat enteric infections. Halquinol administration is found to be beneficial in controlling vibriosis in fishes and acts as a growth promoter in fresh water aquaculture (Wojtowicz, 1984).

In the early 1960s, several antibiotics were used as growth promoters at low doses, apart from being used to treat bacterial infections in man and animals. But soon there was a concern that the practice of using as growth promoters, though helpful in achieving higher meat production, may turn out to be a public health problem. This is because continued use of any antibiotics at sub-therapeutic doses over a period of time may lead to local bacterial populations acquiring resistance to the antibiotic (Sadanand *et al*., 2006).

In the early 1970s, the UK banned the use of tetracycline and penicillin for growth promotional purposes, spurring other European countries to take the same precaution shortly after. In the mid 1970s, the Food and Drug Administration (FDA) proposed a similar ban in the USA. Today, the organizations such as, the European Commission, the World Health Organization, the centers for disease control and prevention (CDC) and the American Public Health Association are supporting the immediate prohibition of antibiotic growth promoters that are the same as, or closely related to, antibiotics used in humans. In March 1999, Centre for Science in the public

interest, the Environmental Defense Fund, and others petitioned the FDA to ban, for purposes of growth promotion, six antibiotics used in or related to those used in human medicine, including penicillin, tetracycline, erythromycin, lincomycin, tylosin and virginiamycin (FAO, 2005).

In considering phasing out or banning antibiotic growth promoters, other alternatives are considered along with better security and hygiene on the farm. Thus, this non-antibiotic growth promoter, halquinol is in use from 1960s and is still being used in India and other Asian countries as well as Latin American countries to overcome common challenges of modern poultry and swine farming. Halquinol is a broad spectrum antimicrobial having weak antibacterial, antifungal and antiprotozoal activity (Ellenrieder and Sensch, 1972; Fiedler and Kaben, 1966; Heseltine and Campbell, 1960; Lamy, 1964; Cosgrove and Baines, 1978; Forster and Duggan, 1974).

Studies in rats indicated poor absorption after oral administration. But, further investigations in man and other species showed that a substantial amount is excreted in the urine after oral administration (Heseltine and Freeman, 1959). Bories and Tulliez (1972) reported that the urinary and faecal elimination of halquinol was rapid, with more than 90 per cent being eliminated within 48 h in the rats. They reported that urinary elimination of halquinol was predominant in calves while faecal elimination in rats.

Halquinol is one of the popular molecules marketed in South American countries and Asian countries including India, for use in poultry as growth promoter. However, there is paucity of scientific data in poultry with respect to tissue distribution, its metabolism and residue kinetics of halquinol and its metabolites in edible tissues. It was

realized that there was no simple and convenient analytical method for regular quality control checks in any dosage form reported for this particular compound. In addition, the pre-slaughter withdrawal time in poultry has not been specified. It is, therefore, of great interest to develop analytical procedures capable of determining accurately animal tissue concentrations of halquinol and its metabolites in edible animal products because of demands from the EU to protect human health.

Metabolomics is the downstream complement to genomics, transcriptomics and proteomics, offering a global assessment of the physiological state of a biological system. Metabonomics is a systems approach for studying *in vivo* metabolic profiles, which promises to provide information on drug toxicity, disease processes and gene function at several stages in the discovery and development process. It represents the end point of genetic regulation and its impact on the altered enzymatic activities and endogenous biochemical reactions in a cell (Villas *et al*., 2005). Metabolomic profiling thus aims to provide a comprehensive assessment of the alterations in the metabolite levels in blood, urine, tissue, or cells (Dunn, 2008).

Recent technological advancements in NMR (Nuclear Magnetic Resonance) spectroscopy and mass spectrometry (MS) have led to wide use of these technologies for precise measurements of metabolites with improved sensitivity, resolution and mass accuracy. Although the exact number of metabolites of any drug/compound is unknown, they are estimated to range from thousands to tens of thousands. Several metabolic pathways, including glucose, fatty acid, and lipid metabolism, have been reported to play an important role in drug response (Vinayavekhin *et al*., 2009).

Extraction is the key step in the analysis of food samples, from either plant or animal sources for residues of veterinary drugs and pesticides. Such residues pose an unacceptable risk to the consumer and information is insufficient to allow a full safety assessment.

Metabolomics is the comparative analysis of metabolites found in sets of similar biological samples. Since metabolites play vital roles in biological systems, metabolomics can be useful for finding and identifying biomarkers or for obtaining a better understanding of the effects of drugs or diseases on both known and unexpected biological pathways. Successful metabolomic research requires effective metabolite extraction. For non-targeted metabolomics, extraction methods need to capture a broad range of cellular and biofluid metabolites while excluding components such as proteins that are not intended for analysis. Extraction is made more challenging by the physico-chemical diversity of metabolites and by metabolite abundances that can vary by many orders of magnitude.

Biphasic, liquid-liquid extraction by using advanced techniques like LCMS or GC-MS are often used to extract metabolites. The nature of the organic and aqueous solvents, their volumes, solvent ratios and aqueous solvent pH, however, must be considered carefully. They can significantly affect the total number of metabolites extracted and experimental reproducibility.

The comprehensive characterization of the metabolome, however, is a daunting task, as the endogenous metabolites vary widely in their physical and chemical properties, which in turn, makes their concurrent extraction, separation, and detection of a

major challenge (Aerts *et al*., 1995). Although a number of metabolite extraction procedures have been described for microbial systems and human bio-fluids, currently, there is a paucity of wide ranging metabolite extraction methodologies for growth promoters. So, the present study aimed at studying the overall distribution of halquinol and its metabolites in edible tissues of chicken and calculation of the time period when the residual effect of the parent compound as well as its metabolites vanishes and when the meat products would be safe for human consumption. It is therefore of great interest to develop analytical procedures capable of determining accurately animal tissue concentrations of halquinol and its metabolites in edible animal products because of demands from the regulatory agencies to protect human health.

Keeping the above points in view, the present study was undertaken in broiler chickens with the following objectives:

A. To determine the distribution of halquinol and its metabolites in various tissues following dietary inclusion in chickens.

B. To study the halquinol as well as its metabolites in various tissues of chicken using Nuclear Magnetic Resonance (NMR) and other advanced techniques.

C. To establish meat withdrawal period of halquinol and its metabolites in chicken.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Quinoline derivatives are a group of synthetic compounds derived from quinoline nucleus. They are broadly classified into following groups diiodohydroxyquinoline, iodochlorhydroxyquinoline, broxyquinoline and chlorhydroxyquinoline which are all halogenated oxyquinolines, while chloroquine is a 4-aminoquinoline derivative. All the halogenated oxyquinolines have weak antibacterial and antifungal properties (Satoskar and Bhandarkar, 1988).

2.1 Halquinol

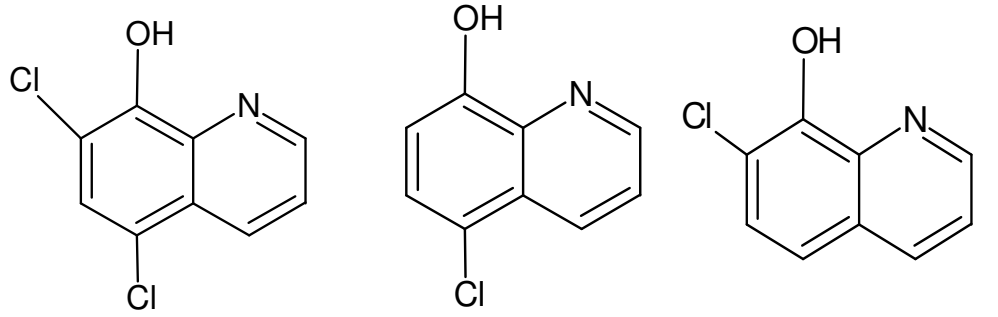
Halquinol, a chlorhydroxyquinoline compound is the mixture obtained by chlorinating quinolin-8-ol. It is a broad spectrum antimicrobial agent used in veterinary medicine to treat gastrointestinal tract infections and to augment growth in poultry and swine. It possesses an antimicrobial activity greater than iodochlorhydroxyquinoline or diiodohydroxyquinoline (Readett, 1965).

2.1.1 Nomenclature and chemistry

Halquinol is the approved name (British Pharmacopoeia Commission) for a chemically controlled group of chlorinated derivatives of quinolin-8-ol.

Chlorinating quinolin-8-ol yields a mixture, generically called as halquinol which contains not less than 57.0 per cent and not more than 74.0 per cent of 5,7-dichloroquinolin-8-ol, not less than 23.0 per cent and not more than 40.0 per cent of 5-chloroquinolin-8-ol and not more than 4.0 per cent of 7-chloroquinolin-8-ol, and the total

content of the three components is not less than 95.0 per cent and not more than 98.0 per cent, all calculated with reference to the dried substance (Anon., 1980).



5,7-dichloroquinolin-8-ol 5-chloroquinolin-8-ol 7-chloroquinolin-8-ol

Stuctures of 5,7-dichloroquinolin-8-ol, 5-chloroquinolin-8-ol and 7-chloroquinolin-8-ol

2.1.2 Physicochemical properties

Halquinol is a yellowish-white to yellowish-grey voluminous powder; having faint and characteristic of cresol odour. It is practically insoluble in water; soluble in 250 parts of ethanol (96 %), in 130 parts of ether and in 50 parts of chloroform, soluble in acids and alkali. Halquinol should be protected from light and kept free from contact with metal as it has got strong metal chelating property (Anon., 1980).

2.1.2.1 Molecular formula

The molecular formula of constituent molecules of halquinol is depicted below:

5,7-dichloroquinolin-8-ol : $C_9H_5Cl_2NO$

5-chloroquinolin-8-ol : C_9H_6ClNO

7-chloroquinolin-8-ol : C_9H_6ClNO

2.1.2.2 Molecular weight

The molecular weight of three constituent molecules of halquinol is:

5,7-dicloroquinolin-8-ol : 212.9

5-chloroquinolin-8-ol : 179

7-chloroquinolin-8-ol : 179

2.1.3 Mechanism of action

Halquinol combines with metallic prosthetic group of respiratory enzymes in cytoplasmic membrane of bacteria, fungi depriving the respiration and thus completely destroying them (Stewart, 1958).

2.1.4. Metabolism

Clioquinol a member of quinoline compounds undergo conjugation reaction in the process of metabolism. Halquinol also undergoes conjugation reaction in liver and their major metabolic products are 5-chloro-quinolin-8-ol sulfate, 5-chloro-quinolin-8-ol glucuronide, 5,7, dichloro-quinolin-8-ol sulfate, 5,7, dichloro-quinolin-8-ol glucuronide (Verdon *et al.,*2005*).*

2.1.5 Spectrum of activity

Halquinol has activity against a wide variety of bacteria, fungi, protozoa and mycoplasmal organisms (Heseltine and Campbell, 1960; Lamy, 1964; Fiedler and Kaben, 1966; Ellenrieder and Sensch, 1972; Forster and Duggan, 1974; Cosgrove and Baines, 1978; Cosgrove, 1977).

Halquinol was found to be active against a variety of pathogenic Gram-positive and Gram-negative bacteria (Heseltine and Campbell, 1960). Among Gram-negative bacteria, it is effective against *Escherichia coli, Salmonella typhimurium, Proteus vulgaris* (Cosgrove and Forster, 1980; Cosgrove *et al.,* 1981; Readett, 1965). *Salmonella typhi, Salmonella paratyphiA, Salmonella enteritidis, Shigella sonnei, Shigella flexneri, Shigella dysenteriae* were also found to be sensitive to chlorhydroxyquinoline (Heseltine and Freeman, 1959). It has little or no effect on *Pseudomonas aeruginosa* (Heseltine and Campbell, 1960). Halquinol has activity against *Vibrio anguillarum,* a Gram-negative bacteria which is the casual agent of vibriosis in fishes and also effective against *Vibrio parahaemolyticus* (Austin *et al.,* 1982). Halquinol was found to be active against *Vibrio cholera* as well, the causal agent of cholera in humans (Neogy and Nandy , 1965; Tranter, 1968).

Among Gram-positive bacteria, it has activity against *Staphylococcus aureus, Streptococcus faecalis, Streptococccus bovis* (Cosgrove and Forster, 1980).

Halquinol has good activity against fungus *Candida albicans* (Swick, 1996; Readett, 1965). *Epidermophyton floccosum*, *Trichophyton mentagrophyte*, *Microsporum gypseum* were also found to be sensitive (Heseltine and Freeman, 1959). It was also found to be effective against *Aspergillus niger, Aspergillus terreus* (Heseltine and Campbell, 1960).

Halquinol has significant antimycoplasmal activity being active against different species of mycoplasma, *viz: Mycoplasma synoviae, Mycoplasma gallisepticum,*

Mycoplasma agalactiae var bovis, Mycoplasma hyopneumoniae and Mycoplasma hyorhinis (Cosgrove and Baines, 1978).

Among protozoa, halquinol has good activity against *Cryptosporidium parvum* (Armson *et al.,* 1999). Chlorhydroxyquinoline is also effective against protozoa, *Entamoeba histolytica* (Heseltine and Campbell, 1960; Lamy, 1964).

2.1.6 Resistance to microbes

Halquinol inducing development of resistance against microbes was a rare phenomenon. It has been shown that neither of the two organisms *Escherichia coli and Salmonella* species developed resistance to halquinol *in vitro* (Forster and Duggan, 1974).

A study by Cosgrove *et al.* (1981) has established that addition of halquinol at 120 ppm in a standard grower diet, when fed to pigs over a period of six weeks, did not induce the development of resistance to *Escherichia coli.* Similarly, halquinol incorporation as feed additive did not alter the resistance pattern of *Escherichia coli* to other commonly used therapeutic anti-bacterials, but on the contrary standard commercial ration lacking halquinol changed the sensitivity patterns of *Escherichia coli.*

2.1.7 Therapeutic uses

As halquinol was found to be absorbable to little extent when given to animals by the oral route (Heseltine and Freeman, 1959) and to exhibit activity against a variety of bacteria and fungi, it has potential in the treatment of infections localized within the intestinal tract.

2.1.7.1 Bovines

Halquinol has been proved to be effective in treating balantidiosis in bovines (Singh, 1979).Verma (1982) has investigated the efficacy of halquinol in improving symptoms of liver disorder and found halquinol to be effective in alleviating symptoms of chronic hepatitis such as loss of appetite, vague digestive disturbances and progressive emaciation.

Therapeutic trial by Kaur *et al.* (2002) has shown that halquinol was effective in treating colibacillosis in neonatal calves.

2.1.7.2 Equines

Halquinol was found to be effective in treating chronic diarrhoea in equines (Obrien, 1981). The beneficial effect was related to their action on colonic microbial fermentative activity and by modifying volatile fatty acid production (Minder *et al.,* 1980).

2.1.7.3 Pigs

Halquinol was recommended for the prevention and treatment of scours in pigs (Burrows, 1975). Halquinol in feed at 60 ppm was successful in curing *Campylobacter* associated intestinal pathology in pigs (Pointon, 1989).

2.1.7.4 Caprines and ovines

Halquinol was found superior in combating acute clinical coccidiosis in goats and effecting cure in two to four days (Baxi *et al.,* 1973) where *Eimeria arloingi* was predominant in the faeces of affected animals.

A clinical trial by Singh (1979) has reported that halquinol was efficacious in treating non-specific diarrhoea in adult sheep, goat and kids.

2.1.7.5 Fishes

Vibriosis is a systemic bacterial infection of primarily marine and estuarine fishes, caused by bacteria of the genus *Vibrio,* a major cause of mortality in Marine culture operations. *Vibrio anguillarum* is regarded as the dominant species causing vibriosis.

Usefulness of substituted quinolines, in particular 5,7-dichloroquinolin-8-ol and halquinol for the control of vibriosis in turbot was evident from the study by Austin *et al*. (1982), where these chemicals inactivated rapidly the bacterial isolates *in vitro* and controlled the manifestation of vibriosis in fishes. From a comparison of 121 antimicrobial agents, the usefulness of substituted quinolines, in particular halquinol, has been indicated for the control of vibriosis in marine fish by Austin *et al.* (1981). *In vitro* and *in vivo* experiments have deduced that halquinol controlled the disease manifestation in turbot.

2.1.7.6 Poultry

The study by Mathew (1990) has proved the usefulness of chlorhydroxyquinoline in diseases of poultry caused by mixed infections due to *Escherichia coli* and *Salmonella;*

Escherichia coli and *Klebsiella; Escherichia coli* and *Proteus sps.* which are complicated with *Aspergillu*s infection or *Aflatoxicosis*. Halquinol potentiates the effect of anticoccidial drugs in poultry. Swick (1996) has observed that halquinol was useful in the control of crop mycosis in poultry caused by *Candida albicans*. Of late, it has been reported that halquinol potentiates the action of maduramycin in the control of *Eimeria acervulina* infection in poultry (Cardoso *et al*., 2002).

Halquinol was found to be useful to combat conditions of malabsorption and necrotic enteritis in poultry as it possesses wide spectrum of activity and slows down peristalsis in the gut (Swick, 1996).

2.1.7.7 Humans

Normally 3 to 4 g of halquinol daily in divided doses for adults up to five days has been recommended in humans in the control of amoebiasis. Further, halquinol has also been administered in doses of 500 mg, 3 to 4 times daily up to a maximum total dose of 7.5 g in the treatment of non-specific diarrhoea in humans (Riviere and Sundlof, 2001). Halquinol had been used for a long time in the treatment of human amoebic and bacillary dysenteries (Heseltine and Campbell, 1960; Heseltine and Freeman, 1959). Damayanthi *et al*. (1973) has undertaken a clinical trial to determine the efficacy of Quixalin® tablets in children suffering from diarrhoea. They have observed that halquinol (Quixalin®) was highly effective in 39 out of 45 cases when it was used @ 30 to 50 mg / kg /day.

Halquinol was used as a topical anti-infective in man (Anon., 1976). Halquinol was used in topical preparation containing 0.75% in conjunction with corticosteroids in

the treatment of skin disorders (Anon., 1994) and incorporated in shampoo (2 % w/v) in the treatment of seborrhoeic dermatitis of the scalp (Anon., 1999). A clinical trial of halquinol in impetigo suggests that halquinol cream (0.75%) has an effective antibacterial activity (Readett, 1965).

2.1.8 Toxicological properties

2.1.8.1 Rat

Swetha *et al.* (2006) has reported that halquinol at high (1000 mg/kg) and intermediate dose (450 mg/kg) was hepatotoxic and nephrotoxic in male rats. There was significant increase ($p \leq 0.01$) in ALP, ALT, BUN and creatinine, and increased ($p \leq 0.01$) organ to body weight ratio of liver and kidney. Histopathology of liver and kidney revealed marked pathological alterations at high and intermediate doses. Swetha *et al.* (2009) indicated that halquinol at high dose (1000 mg/kg, p.o.) and intermediate dose (450 mg/kg, p.o.) exert hepatotoxic and nephrotoxic effect in female rats as well.

Swetha *et al.* (2005a) has also reported that halquinol did not result in any immunotoxicity in female rats at low (150 mg/kg), intermediate (450 mg/kg) or high dose (1000 mg/kg) in 28 days repeated dose oral toxicity study. However, halquinol administration daily for 90 days at high dose @ 1000 mg/kg body weight (high dose) was found to be thyrotoxic in rats (Swetha *et al.,* 2005b).

Halquinol administered orally to rats @ 200 mg/kg for 97 days did not result in any pathological changes in blood and other organs except for showing only moderate diarrhoea (Freeman and Heseltine, 1963).

2.1.8.2 Mice

The oral LD_{50} of chlorhydroxyquinoline for mice was found to be approximately 800 mg/kg and the intraperitoneal LD_{50} in this species was about 300 mg/kg (Freeman and Heseltine, 1963). LD_{50} (oral) in mice was reported to be 1220 mg/kg in 24 hours and 700 mg/kg in 48 hours by Clarke (1978).

2.1.8.3 Rabbit

Freeman and Heseltine (1963) have reported that when halquinol administered orally and i.v. to rabbits at dose rate of 300 mg/kg body weight for seven days and 2.5-10 mg/kg body weight for four days respectively did not show any signs of toxicity.

2.1.8.4 Guinea pig

Freeman and Heseltine (1963) have reported that seven out of eight guinea pigs were died when halquinol was administered orally @ 300 mg/kg body weight for three days and those animals had gastric lesions and enteritis upon post-mortem examination. Further, three out of nine guinea pigs administered with halquinol orally @ 150 mg/kg body weight for a period of seven days died showing gastric and intestinal lesions upon post-mortem examinations, while guinea pigs administered orally with halquinol @ 100 mg/kg body weight for five days did not show any signs of toxicity.

2.1.8.5 Poultry broilers

The oral median lethal dose (LD_{50}) of halquinol in poultry broilers was reported to be 2183.33 mg/kg with clinical signs of toxicity *viz.* inappetence, ruffling of feathers and sleepy nature of birds which further manifested into anorexia, leg weakness and

weight loss. The post-mortem findings revealed evidence of death due to rupture of liver. The other lesions were extensive subcutaneous hemorrhages on thighs, wings, clotted blood in peritoneal cavity, ascites, rickets, pale musculature and fragile skeletal structures (Shivakumar *et al.,* 2004). A study by Kavitharani *et al.* (2004) in broiler birds has indicated that halquinol administered as single dose @ 2100 ppm was non lethal, while the dose at which 50 per cent mortality occurred was found to be between 1500 and 1700 ppm in 14 days feeding study.

2.1.8.6 Fish

A limit test performed with 200 mg/L concentration of halquinol as per OECD guidelines revealed that halquinol was non toxic to fish at concentration used in the study (Mushigeri *et al.,* 2008).

2.1.8.7 Pig

Pigs administered with halquinol @ 200 mg/kg and 500 mg/kg body weight orally for a period of three and seven days respectively did not show any signs of toxicity (Freeman and Heseltine, 1963).

2.1.8.8 Human

Halquinol was reported to cause neurotoxicity in man (Hanson and Herxheimer, 1981). Halquinol administered to humans @ 70 mg/kg body weight orally for a period of 10 days did not result in any signs of toxicity (Freeman and Heseltine, 1963). Burrows (1975) have reported three cases of contact dermatitis in animal feed workers due to ethoxyquin and halquinol when used as feed additives.

2.2 Growth promoters in poultry industry

Growth promoters in general constitute a large group of substances that are added to feed to improve feed conversion efficiency and increase the lean to fat ratio in animals. Feed additive is defined as any substance or agent added to the basic feed mix for continuous long term administration to livestock for specific purposes, for example, enhancing production or maintenance of health above the levels obtained from the basic feed, improvement of storage qualities and/or the palatability of the basic feed mix.

Antimicrobials and antibiotics are used by the poultry industry as feed additives to enhance growth and feed efficiency and reduce disease. Antibiotic usage has facilitated the efficient production of poultry, allowing the consumer to purchase at a reasonable cost, high quality meat and eggs. Antibiotic usage has also enhanced the health and well being of poultry by reducing the incidence of disease (Donoghue, 2003). Most countries have banned sulfonamides and nitrofurans (furazolidone) as growth promotants because of problems with tissue residue and suspected carcinogenicity (Swick, 1996).

The major benefit of feeding an antimicrobial is cost savings through improved feed conversion. There are also savings from faster growth, reduced mortality, greater resistance to disease challenge, improved pigmentation and improved manure or litter quality. A review of literature on feeding studies by Rosen (1996) has indicated that antimicrobial growth promoters gave positive responses 72 per cent of the time. The magnitude of response depends on animal management, disinfection procedures, age of the farm buildings and feed quality. Under normal practical conditions, savings from improved FCR can be expected to return product cost between two and twelve fold.

Antimicrobial feed additives promote growth by altering the gut microflora to the benefit of intestinal tissue. Swick (1996) has reported that feeding of sub therapeutic levels of antibiotics and antimicrobials result in:

- Increased production of vitamins and other nutrients by beneficial microflora
- Reduced production of growth depressing toxins from microflora
- Lower immune stress resulting in a shift of protein synthesis toward muscle and away from antibody production
- Suppression of bacteria responsible for mild but unrecognized infections
- A thinner gut wall capable of enhanced nutrient absorption
- Lower nutrient use by microflora leaving more for the animal
- Lower production of ammonia in the gut which reduces turnover of mucosal cells and results in less energy consumption by the animal.

Since the mid-eighties of twentieth century, there has been much criticism and concern over the use of growth promoting substances in feed. Regulation has increased over time in most countries. Doses and specific products are now controlled. Use of drugs important to human medicine is limited, as are those known to cause rapid development of resistance in bacteria. USFDA has recently classified growth promoters into two categories. “Category I” products require no withdrawal from feed when used at growth promotant levels and include bacitracin, flavomycin, chlortetracycline, lincomycin, penicillin, tiamulin, tylosin and virginiamycin. “Category II” products do require withdrawal and include arsanilic acid, roxarsone (3-nitro), carbadox and oxytetracycline.

Although growth promoters benefit all involved, consumer's perception is that edible poultry tissues are contaminated with harmful concentrations of drug residues. In a consumer survey, Resurreccion and Galvez (1999) reported that 77 per cent of the consumers responding felt animal drug residues in meat to be an extreme health concern.

The use of antimicrobials is strictly regulated by the Food and Drug Administration (FDA) and USDA to warrant their safety and efficacy. Prior to regulatory approval, the pharmacokinetics and tissue tolerances of an antimicrobial are determined to set the proper dosage. To ensure proper use both the FDA and USDA have research, surveillance and compliance programs to develop detection methods and monitor antimicrobials in poultry meat (Donoghue, 2003).

The selection of a growth promoter in poultry industry must be based on safety and probability to give high economic returns. The most widely used are the antimicrobial agents, including antibiotics. In recent years, there has been a flurry of interest in the use of other non-nutritive substances such as acidifiers, probiotics, enzymes, herbal products, beta-agonists, microflora enhancers and immune-modulators.

2.2.1 Halquinol as growth promoter

In tests on isolated smooth muscles, Kaul and Lewis (1965) noted that halquinol markedly reduced peristalsis. They also observed that halquinol caused slowing of the movement of the intestinal contents of intact animals. Halquinol reduces the tone and motility of smooth muscle of intestine, thus help in enhancing nutrient absorption (Swick, 1996). These properties provide partial explanation of the improved feed conversion and

growth rates which occur from halquinol supplementation of the diet and for prompt alleviation of the symptoms of diarrhoea.

2.2.1.1 Swine

Head (1974) has reported that pigs housed in a uncleaned pig house (after rearing previous batch of pigs) and given feed containing 120 g halquinol/ton had a live weight gain of 19.42 kg over a six week period which was higher than the pigs given untreated feed (16.85 kg). Further, feed conversion efficiency was also higher with halquinol treated feed. Halquinol is added to swine feeds @ 100 to 600 ppm for growth promotion (Botsoglou and Fletouris, 2001).

2.2.1.2 Fishes

Fishes treated with 0.1 per cent halquinol in water showed a higher weight gain compared to untreated control group. Synergistic increment in the free amino acid (FAA) level and total protein concentration of fish musculature suggests enhanced anabolic metabolism resulting in increased weight gain (Mushigeri *et al*., 2008).

2.2.1.3 Poultry

Halquinol is added to poultry feeds at 30 and 60 ppm for growth promotion purpose (Botsoglou and Fletouris, 2001).

2.2.1.4 Lambs

Welch *et al.* (1965) has reported that there was a progressive decrease in feed required per pound of body weight gain with increasing levels of chlorhydroxyquinoline in feed of fattening lambs.

2.3 Drug residues in foods of animal origin

Administration of drugs to food producing animals requires not only consideration of effects on the animal but also the effects on humans who ingest food from these animals. In short, after food producing animals have been exposed to drugs in order to cure or prevent disease or to promote growth, the effects of the residues of such treatment may have on humans should be considered. These residues consist of the parent compound and or compounds derived from the parent drug (or both) including metabolites, and residues bound to macromolecules (Webber, 1979). Black (1984) has opined that drugs used as growth promoters in food animals may cause harmful effects on humans through several mechanisms, such as:

1. Increased microbial drug resistance,
2. Drug residues in food
3. Allergic reactions and sensitization to antimicrobials and
4. Drug toxicity

The important issue of growth promoters is that they may remain in all animal treated derived foods and can constitute an important health risk for consumers. Some of the reported effects in animals include genotoxic, immunotoxic, carcinogenic or adverse

endocrine effects and therefore pose a risk to consumers. Furthermore, when antibiotics are used as growth promoters, they may give rise to allergic reactions and emergence of drug resistant bacteria (Cinquina *et al*., 2003) and or selection of resistant bacteria in the gastrointestinal tract and disruption of the colonization barrier of resident intestinal microflora (Cerniglia and Kotarski, 2005), which can increase its susceptibility to infection by pathogens like *Salmonella spp*. and *Escherichia coli* (Cerniglia and Kotarski, 1998). Thus, the residues of all growth promoters must be monitored in foods of animal origin (Croubles *et al*., 2004).

2.4 Tissue distribution of halquinol

The 8-hydroxyquinolines and their derivatives like clioquinol, halquinol are stated to be absorbed to very little extent from the gastrointestinal tract (Anon., 2005). Up to five per cent of the total dosage of 5,7-dichloroquinolin-8-ol and 5-chloroquinoline-8-ol and seven per cent of the total dosage of 7-chloroquinoline-8-ol were only absorbed in laboratory animals (Clarke, 1978).

Studies in rats indicated slight absorption after oral administration and further investigations in man and other species showed that substantial portions of oral doses of the drug excreted through urine (Heseltine and Freeman, 1959).

Bories and Tulliez (1972) reported that the urinary and faecal elimination of halquinol was rapid, with more than 90 per cent being eliminated within 48 h in the rat. They reported that urinary elimination of halquinol was predominant in calves and faecal elimination in rats.

In rabbits, administered with halquinol, orally at total dose of 200 mg, 39 mg that is 19.5 per cent of the administered dose was found in urine collected at 0-24 h after dosing (Freeman and Heseltine, 1963).

Following a total oral dose of 250 mg of halquinol in man, 26.0 mg was recovered in urine which amounts to 10.4 per cent of dose administered (Freeman and Heseltine, 1963).

Further, Freeman and Heseltine (1963) also observed that substantial portions of halquinol were found in urine in calves following oral doses. They recovered 2071 mg of halquinol (25.9 %) in urine after a total dose of 8000 mg (p.o) in calves.

MATERIALS & METHODS

III. MATERIALS AND METHODS

The oxyquinoline derivative halquinol -HALQUINOL BP 80, composed of 71.39 % of 5,7-dichloroquinolin-8-ol, 26.61 % of 5-chloroquinolin-8-ol and 0 % of 7-chloroquinolin-8-ol, the total content of the two components being 98 % w/w, manufactured and supplied by M/s. Provimi Animal Nutrition India Pvt. Ltd., Bangalore was used in the present experimental study. It was planned to examine the purity or authentication of the procured halquinol before carrying out the tissue distribution study of halquinol in broiler chickens. Therefore, various analytical techniques such as Ultraviolet-visible (UV-Vis) spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Thin Layer Chromatography (TLC), Nuclear magnetic resonance (NMR), High Performance Liquid Chromatography (HPLC) and Liquid chromatography mass spectroscopy (LCMS) were performed. The genuineness of the procured halquinol was assessed by UV-Vis double beam spectrophotometer and FTIR. The purity or presence of any impurities in the procured halquinol was tested and confirmed by TLC method. All chemicals or reagents used for UV-Vis spectroscopy, FTIR and TLC were of analytical grade procured from Merck (E-Merck, India).

3.1 Spectroscopy

Most of what we know about the structure of atoms and molecules comes from studying their interaction with light (electromagnetic radiation). Different regions of the electromagnetic spectrum provide different kinds of information as a result of such interactions. Realizing that light may be considered to have both wave-like and particle-like characteristics, it is useful to consider that a given frequency or wavelength of light is

associated with a "light quanta" of energy we now call a photon. As noted in the following equations, frequency and energy change proportionally, but wavelength has an inverse relationship to these quantities.

$\nu = c/\lambda$ where ν = frequency; λ = wavelength and c = velocity of light ($c = 3 \times 10^{10}$ cm/sec)

$\Delta E = h\nu$ where E = energy; h = planks constant and ν = frequency ($h = 6.6 \times 10^{-27}$ erg sec)

In order to yield information about certain characteristic features of molecules, interaction of light photons, energy levels, frequencies, wavelengths and magnetic resonance are studied in a particular way, known as spectroscopy. Some of the major spectroscopic techniques, their working principles and what do we decipher from that are discussed here.

1. Ultra-violet and visible (UV-vis) spectroscopy

2. Infra-red (IR) spectroscopy

3. Nuclear magnetic resonance (NMR) spectroscopy

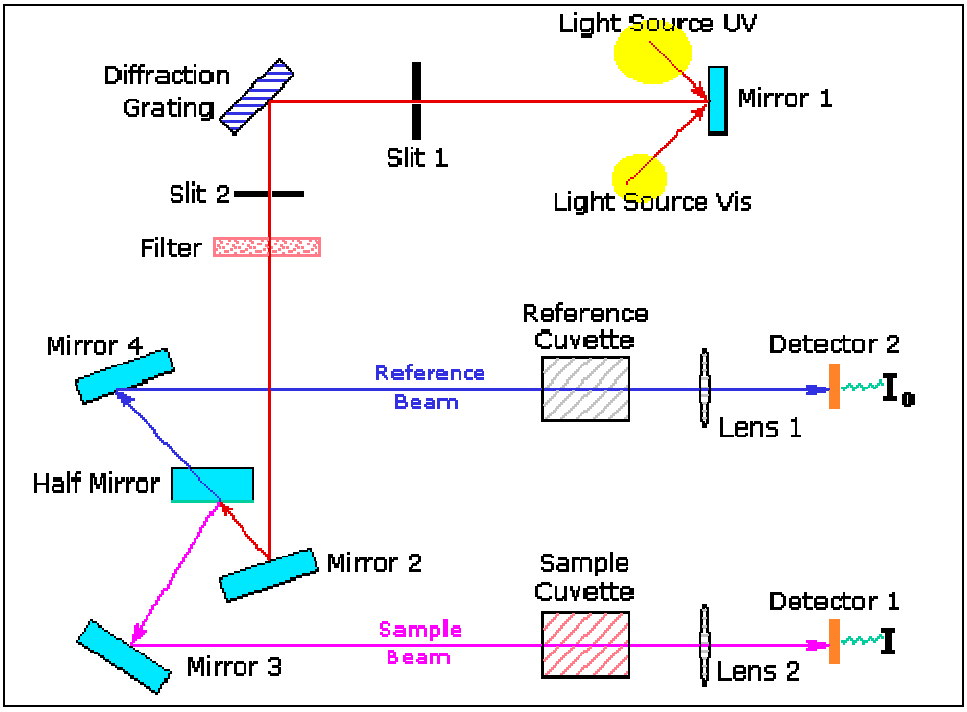
3.1.1 Ultraviolet visible spectroscopy

3.1.1.1 Principle

The visible region of the spectrum comprises photon energies of 36 to 72 kcal / mole, and the near ultraviolet region, extends this energy range to 143 kcal / mole. The energies noted above are sufficient to promote or excite a molecular electron to a higher energy orbital. Consequently, absorption spectroscopy carried out in this region is sometimes called "electronic spectroscopy". When sample molecules are exposed to light

having an energy that matches a possible electronic transition within the molecule, some of the light energy will be absorbed as the electron is promoted to a higher energy orbital. An optical spectrometer records the wavelengths at which absorption occurs, together with the degree of absorption at each wavelength. The resulting spectrum would be presented as a graph of absorbance (A) versus wavelength.

Fig.1 Functioning of Ultra-Violet Spectroscopy



If the sample compound does not absorb light of a given wavelength, $I = I_0$. However, if the sample compound absorbs light then I is less than I_0, and this difference may be plotted on a graph versus wavelength, as shown here. Absorption may be presented as transmittance ($T = I/I_0$) or absorbance ($A = \log I_0/I$). If no absorption has occurred, T = 1.0 and $A = 0$. Most spectrometers display absorbance on the vertical axis, and the commonly observed range is from 0 (100% transmittance) to 2 (1%

transmittance). The wavelength of maximum absorbance is a characteristic value, designated as λ_{max}.

The UV visible absorption spectra (200 to 400 nm) of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol standards and of halquinol were recorded using a double beam UV-visible spectrophotometer (Owen, 2000), Evolution 300 (Thermo Fisher Scientific, Madisson, WI, USA) operated by Vision Pro® software. Individual stock (1mg/ml) solutions of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol and halquinol in methanol were prepared. To find out the extent of the chemical solubility of halquinol in various organic and aqueous solvents spectroscopy was performed on halquinol solution of halquinol and acetonitrile (Table 1 to 5). Spectra were analysed for the solubility of halquinol by considering 100% solubility of halquinol in DMSO. Six solvents were used to determine the maximum solubility of halquinol. These stock solutions were diluted in HCl (0.1M), NaOH (0.1N) and methanol and UV-visible spectra were recorded (Fig 6 to 10).

Table 1. List of solvents used to dissolve Halquinol

Formulation	Abs.	λmax.	Fold Solubility
HAL-Acetonitrile	0.6934	338.5	5.70
HAL-DCM	0.6119	338.5	5.00
HAL-Hexane	0.5234	338.5	4.32
HAL-Methanol	0.6689	338.5	5.53
HAL-Ethanol	0.6940	338.5	5.60
HAL-Water	0.1209	338.5	1.00

Table 2. Ultraviolet-visible parameters of 5,7-dichloroquinolin-8-ol in water

Formulation	Abs.	λmax.	Fold Solubility
5-HAL-Water	0.3367	247	1.00
7-HAL-Water	0.2412	247	0.71
HAL-Water	0.5621	247	1.70

Table 3. Ultraviolet-visible parameters of 5,7-dichloroquinolin-8-ol in acidic medium

Formulation	Abs.	λmax.	Fold Solubility
5-HAL-ACID	0.2739	379.5	1.00
7-HAL-ACID	0.0346	368	0.13
HAL-ACID	0.3709	379.5	1.35

Table 4. Ultraviolet-visible parameters of 5,7-dichloroquinolin-8-ol in basic medium

Formulation	Abs.	λmax.	Fold solubility
5-HAL-Base	0.6397	383.5	1.00
7-HAL-Base	0.6158	383.5	0.96
HAL-Base	0.5847	383.5	0.91

Table 5. Ultraviolet-visible parameters of 5,7-dichloroquinolin-8-ol in DMSO

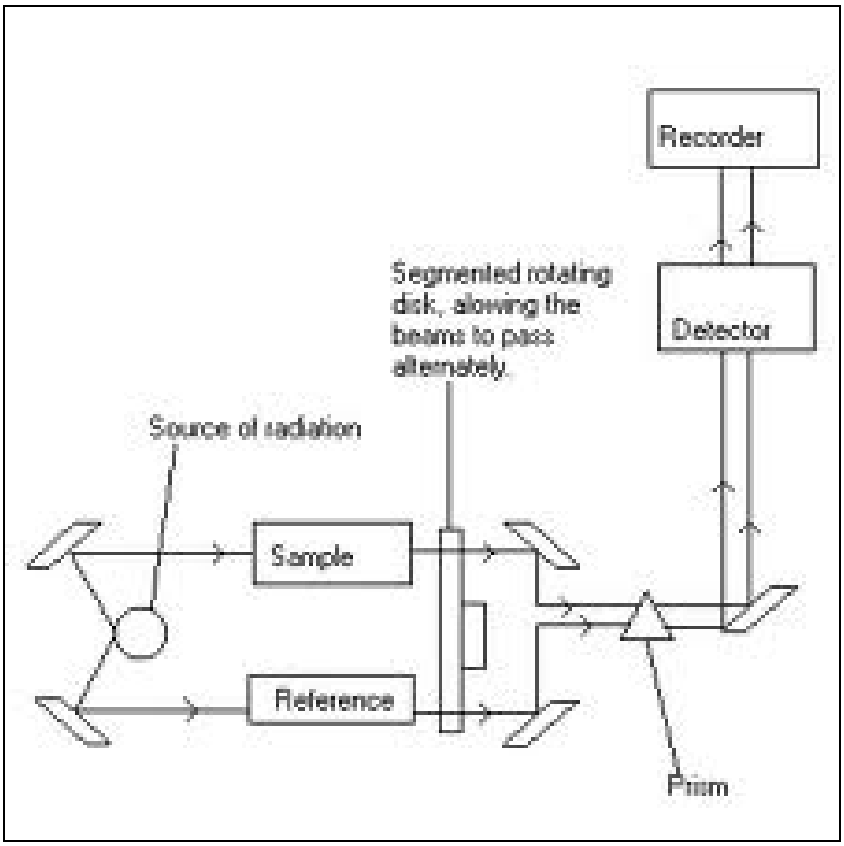
Formulation	Abs.	λmax.	Fold Solubility
5-HAL-DMSO	0.6831	341	1.00
7-HAL-DMSO	0.6657	341	0.97
HAL-DMSO	0.6589	341	0.96

3.1.2 Infrared spectroscopy

3.1.2.1 Principle

Photon energies associated with the part of the infrared (from 1 to 15 kcal/mole) are not large enough to excite electrons, but may induce vibrational excitation of covalently bonded atoms and groups. Consequently, virtually all organic compounds will absorb infrared radiation that corresponds in energy to these vibrations. The exact frequency at which a given vibration occurs is determined by the strengths of the bonds involved and the mass of the component atoms.

Fig. 2: Functioning of Infrared spectroscopy



IR spectroscopy is generally used for structural elucidation and compound identification. It has been found that infrared spectra in the 1450 to 600 cm^{-1} region would be very complex and often called the fingerprint region, assigned for basic backbone of the molecule while bands in the 4000 to 1450 cm^{-1} region are usually due to stretching vibrations of diatomic units in functional groups of the molecules and sometimes called the group frequency region. By combining the information obtained in both the regions reveals the structural characteristics of the compound.

The procedure involving sample preparation and spectral recordings were carried out according to Stuart (2004). Samples were prepared in acetonitrile (1mg/ml) and run on FTIR Nicolet 6700 (Thermo Fisher Scientific, Madisson, WI, USA) operated by

Omnic software 8.1.Data was analysed by mentioning by describing various vibrational bands appeared in spectra because of the chemical nature of the halquinol and its constituents .The infrared spectra of 5,7-dichloroquinolin-8-ol, 5-chloroquinolin-8-ol and halquinol were recorded using two different modes:

1. A direct transmission mode using a KBr disk method and
2. By attenuated total reflectance (ATR) method using smart orbit diamond ATR.

Approximately one mg each of 5,7-dichloroquinolin-8-ol, 5-chloroquinolin-8-ol and halquinol was grounded in potassium bromide (spectroscopy grade) using mortar and pestle and pressed to a transparent disk . The KBr disks were then placed in the sample holder to record infrared (IR) spectra. Similarly, few milligrams of aforesaid compounds were placed individually on the sample plate of the smart orbit and screwed lightly to record IR spectra in ATR mode. The IR spectra of 5,7-dichloroquinolin-8-ol, 5-chloroquinolin-8-ol and halquinol mix are depicted in Fig 11,12 and 13 respectively The details of functional groups obtained by IR spectra are given in Table 9, 10 and 11.

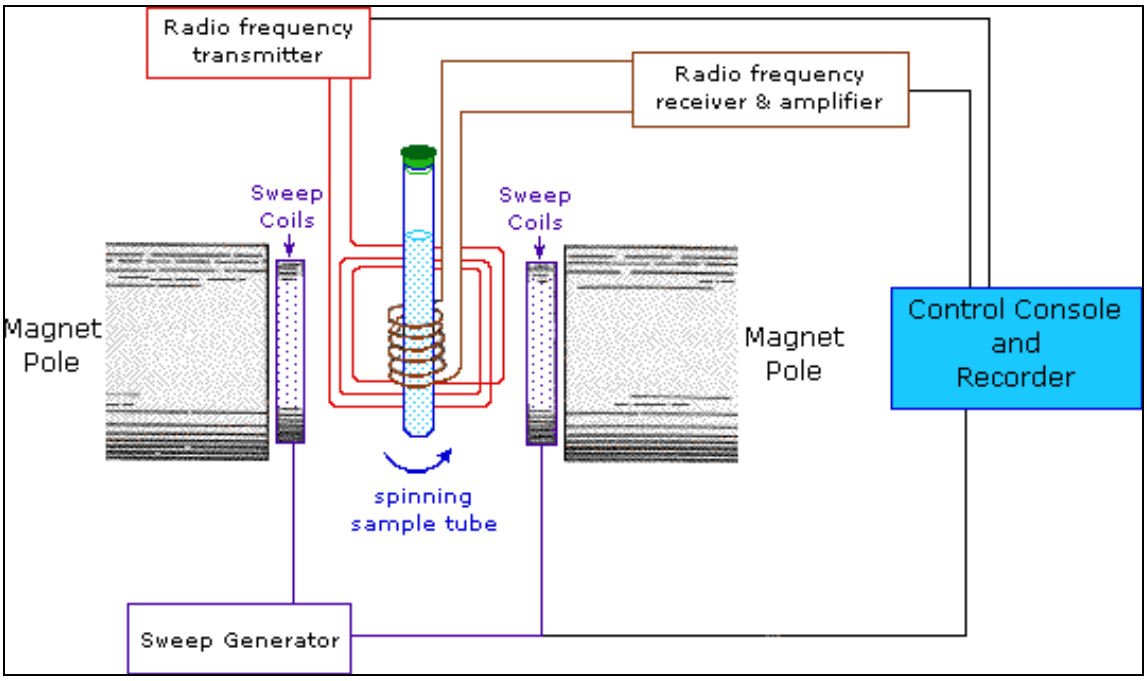
3.1.3 Nuclear Magnetic Resonance (NMR)

3.1.3.1 Principle

The principle behind NMR is that many nuclei have spin and all nuclei are electrically charged. If an external magnetic field is applied, an energy transfer is possible between the base energy to a higher energy level (generally a single energy gap). The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy is emitted at the same frequency. The

signal that matches this transfer is measured in many ways and processed in order to yield an NMR spectrum for the nucleus concerned.

Fig. 3: Functioning of NMR



The precise resonant frequency of the energy transition is dependent on the effective magnetic field at the nucleus. This field is affected by electron shielding which is in turn dependent on the chemical environment. As a result, information about the nucleus' chemical environment can be derived from its resonant frequency. In general, the more electronegative the nucleus is, the higher the resonant frequency. Other factors such as ring currents (anisotropy) and bond strain affect the frequency shift. On the basis of type of nuclei under consideration, NMR spectrum generally recorded are ^{1}H-NMR and ^{13}C-NMR.

In ^{1}H-NMR various parts of spectra reveal different information about the compound as 1) number of signals indicates how many "different kinds" of protons are present; 2) positions of the signals indicates something about magnetic (electronic) environment of protons; 3) intensities (areas) of the signals proportional to number of protons present; 4) splitting of a signal into several peaks indicates the number of nearby nuclei having magnetic moments (usually protons, sometimes fluorine) and 5) The area under a particular signal is proportional to the number of protons giving rise to the signal.

For looking at carbon using NMR, we have to look at carbon-13, which does have a magnetic moment, but which is only about 1% of carbon as it occurs in nature. So, the instrumentation required is more complex. But the analysis of ^{13}C-NMR spectra is considerably easier because the analysis of a C-13 spectrum involves noting the number of signals and their chemical shift. Each carbon gives rise to a signal, but if two or more carbons are equivalent, their signals will exactly overlap. Similar to proton NMR, chemical shift may suggest the type of carbon that is giving rise to a particular signal. Signal splitting and peak areas are not seen in these spectra. Furthermore, signal overlap from non-equivalent atoms is much less common in C-13 spectra than in proton spectra.

It has since been used in an almost unlimited variety of ways in physics, chemistry and biology. For the investigation of biological systems it is convenient to distinguish between three types of applications:

(1) To study structure and function of macromolecules,

(2) To study metabolism, and

(3) To obtain *in vivo* images of anatomical structure and functional (physiological) states.

The use of 1H NMR for metabolic studies was described as early as 1977 by (Gadian,1995) when it was shown that 1H signals could be observed from a range of compounds in a suspension of red blood cells, including lactate, pyruvate, alanine and creatine. A great deal of metabolic information can be derived from such metabolic studies and it was soon recognized that 1H NMR of body fluids has a considerable role to play in areas of pharmacology, toxicology and the investigations of inborn errors of metabolism.

To find out the purity and composition of starting material halquinol and its individual components 5-chloro-8-quinolinol and 5,7-dichloro-8-quinolinol ^{1}H-NMR (Proton nuclear magnetic resonance) and ^{13}C-NMR (Proton nuclear magnetic resonance) spectra were acquired. Tetramethylsilane (TMS) was used as internal standard representing nuclear magnetic resonance shifts (δ) value 0.00.^{1}H-NMR and ^{13}C-NMR were acquired at 400 and 100 MHz frequency, respectively. Scans were performed on 1 mg/mL concentration of halquinol and its components in $CDCl_3$ (Deuterated chloroform). Samples were acquired at 200 scans. Compounds were characterized by magnetic resonance shifts (δ) of various protons (As shown and assigned in NMR spectra Fig (Spectra 14, 15, 16, 17, 18 and 19).

3.1.4 Liquid Chromatography Mass Spectroscopy (LCMS)

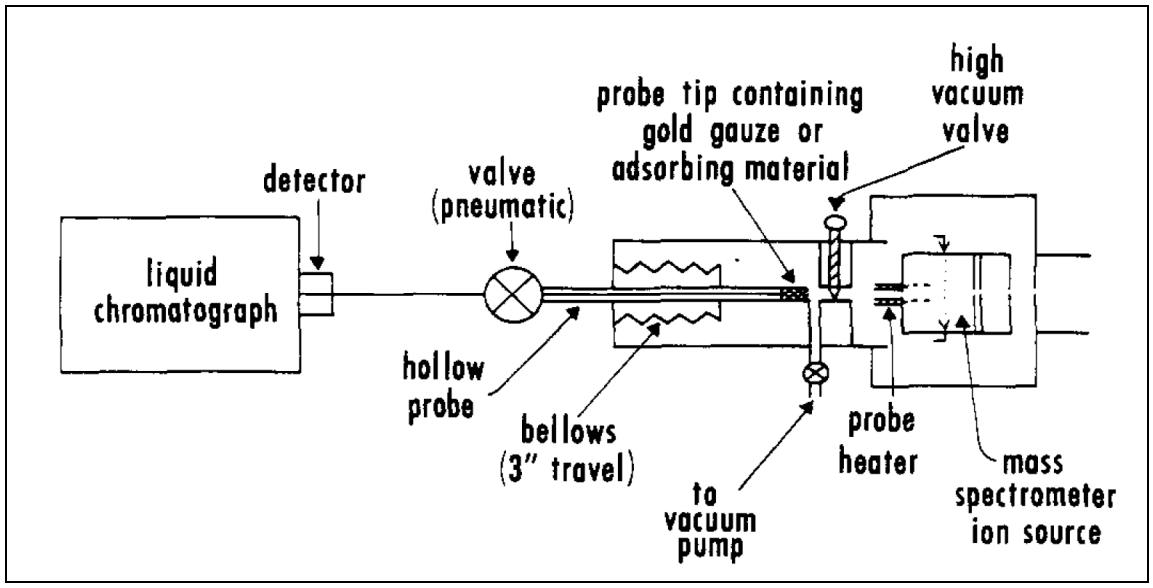
3.1.4.1 Principle

Chromatography is the ability to separate molecules using partitioning characteristics of molecule to remain in a stationary phase versus a mobile phase. High performance liquid chromatography (HPLC) is about solvent being forced through under

high pressures of up to 400 atmospheres. That makes it much faster. It also allows to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture.

HPLC separates things, but provides little extra information about what a chemical might be. In fact, it is hard in HPLC to be certain that a particular peak is pure, and contains only a single chemical. Adding a mass spectrometer to this tells the masses of all the chemicals present in the peak, which can be a very good starting point for identifying them, and an excellent method to check for purity.

Fig.4 Functioning of LC-MS



LC-MS provides superior specificity and sensitivity and can be used to develop highly accurate and reproducible assays. The primary advantage LC-MS has that it is capable of analysing a much wider range of components. Compounds that are thermally labile, exhibit high polarity or have a high molecular mass may all be analysed using LC-

MS. Compounds are separated on the basis of their relative interaction with the chemical coating of these particles (stationary phase) and the solvent eluting through the column (mobile phase) and introduced to the mass spectrometer via a specialised interface to find the accurate mass of the chemical. It gives the clear idea about the presence of the chemical in starting mixture.

3.1.4.2 Chemicals and Materials

Halquinol and its constituents (5,7-dichloroquinolin-8-ol, 5-chloroquinolin-8-ol) were obtained from Sigma Chemicals ltd. LC grade acetonitrile and methanol were purchased. Reagent grade anhydrous disodium phosphate, citric acid, trifluoroacetic acid (TFA), trichloroaceticacid (TCA), disodium ethylene diaminetetraacetate (Na_2EDTA), and sodium hydroxide were obtained from Merck. Distillation and passage were through Milli-Q System (Millipore, Bedfore, United States) purified water. All solvents and solutions used in this study were filtered through a 0.2 mm filter under vacuum and were degassed by ultrasonication before LC analysis. LCMS assay was carried out by the procedure as reported by (Jane *et al* ., 2005). The image of LCMS is depicted in Plate 1. The representative LCMS graphs of halquinol and its metabolites are depicted in Fig. 20 to 37.

3.1.4 .3 HPLC-MS experimental details

3.1.4.4 Instrument:

HPLC: Thermo Finnigan Surveyor

MS: Thermo LCQ Deca XP MAX

Software: Xcalibur

3.1.4.5 Experimental Conditions:

Column: BDS HYPERSIL C18 (Reverse Phase)

Length (mm): 250

I.D. (mm): 4.6

Particle Size (um): 5

Detection: UV @ 220nm-Channel C for Samples GF.

Detector details: HPLC PDA / UV detector

Temperature: Ambient

Injection Volume: 10uL

3.1.4 .6 Solvents

A: Acetonitrile

B: Ethyl Acetate

C: Water

D: Methanol

Flow rate: 1 mL/min

Run Time (min): 30.00

3.1.4.7 MS Experimental Conditions:

Probe/ source voltage: 4.5kV

Mode of Ionization: +ve mode, -ve mode

Mass range: 100m/z to 700m/z

Source type: ESI (Electro Spray Ionization)

Nebulization gas flow: Helium at 1mL/min approx

Plate 1: LCMS instrument (Thermo Finnigan)

3.1.4.8 Chemicals and solvents

The metabolites 5-chloro-quinolin-8-ol sulfate, 5-chloro-quinolin-8-ol glucuronide, 5,7, dichloro-quinolin-8-ol sulfate, 5,7, dichloro-quinolin-8-ol glucuronide, the internal standards diazepam and caffeine were synthesized by Provimi Animal Nutrition Ltd., Bangalore. Methanol (HPLC grade) and ethyl acetate (HPLC grade) were obtained from Sigma (Aldrich Chemical Company, Germany). Stock solutions were prepared in methanol and stored at 4 °C for a maximum of one month.Water was purified, using the Milli-Q or Elga UHQ systems, prior to use.

3.1.4.9 LC–MS analysis

The LC–MS–MS system consisted of Liquid Chromatograph Thermo LCQ Deca XP MAX Thermo TSQ Quantum connected to a triple quadruple mass spectrometer (PE Sciex, Toronto, Canada) in electrospray positive ionization mode. ACTCHTSPAL auto injector was connected to the system. The chromatography was performed in C18 (100mm×2.1 mm) Jones®, connected to a C18 (1 cm×4 mm) Jones® pre-column. The mobile phase was composed by two solutions: A (water and 0.1% acetic acid) and B (90% acetonitrile, 10% water and 0.1% acetic acid). The column was operated at room temperature at a flow rate of 1ml / min.The ions were monitored by Multiple Reaction Monitoring (MRM) according to the method described by Leitner *et al*. (2001).

3.1.4.10 Sample preparation

Breast muscle and liver samples were obtained from poultry shed. Samples were homogenized using a domestic food blender and were stored at -20°C until analysis. To 5.0 g amount of homogenized sample, weighed into 50 ml polypropylene centrifuge tube was added 20 mL of pH 4.0 Na_2EDTA buffer solution, which was vortex-mixed for two min. The tubes were then shaken for 10 min on a flatbed shaker at high speed and were sonicated for 15 min in an ultra-sonication bath. The samples were immersed in an ice/salt bath during ultrasonic processing. To this 10 ml of 0.1 M HCl was added and sonicated for ten min in sonicator. Twenty five ml of ethyl acetate was added and again sonicated for ten min and centrifuged at 4000 g for five min and the supernatants were decanted carefully into a clean second centrifuge tube. Two more extractions were effectuated with 20 mL and 10 mL of pH 4.0 Na_2EDTA buffer solution, and all the steps

were repeated until the supernatants from all three extractions were collected in the second tube.

The cartridges Oasis HLB were conditioned with methanol (3 mL) and water (2 mL). After extraction, the cartridges were flushed with 2 mL of 5% aqueous methanol and were eluted using 2 mL of 1% TFA in methanol. The elutes were evaporated to 0.5 mL under gentle nitrogen stream in a water bath at 30°C and were kept at 4°C just before injection into the chromatographic system to avoid degradation. A blank and one fortification assay were included in each analytical run to check for interferences (*e.g.*, coeluting substances) and to control the accuracy.

Phosphate disodium EDTA (3 %) buffer was added to each sample solutions to prevent the metal ions Cu^{2+}, Fe^{3+}, Zn^{2+} being extracted into the organic phase (acetonitrile fraction), thus a sharp chromatogram of 5,7-dichloroquinolin-8-ol and 5-chloroqunolin-8-ol was obtained. Similar technique was adopted by Hayakawa *et al.* (1982) for chromatography of clioquinol, a derivate of 8-hydroxyquinoline. Further, previous report (Bondiolotti *et al.,* 2006) indicates that addition of phosphate disodium ethylendiaminotetraacetate (Na_2EDTA) buffer prevents the formation of complexes that may reduce recovery of clioquinol in plasma and tissue samples of hamsters quantified by HPLC.

3.1.4.11 Standards, Buffer, and Mobile-Phase Preparation

Individual methanolic stock standard solutions of 5,7-dichloroquinolin-8-ol, 5-chloroquinolin-8-ol and halquinol were prepared at a concentration of 1 mg/mL, into a volumetric flask, and were stored at -20 °C in glass vials for a maximum period of one

month. The working solutions were a mixture of the halquinol prepared by serial dilutions of the stock solution in methanol and were stored in glass vials at 4 °C. These solutions were prepared daily immediately before use and were always kept at 4 °C protected from light. The mobile phases used for spectrofluorometric and mass spectrometric analysis were a mixture of acetonitrile 0.01 M and ethyl acetate (aq pH 2.0) 30:70 and acetonitrile 0.01 M and trifluoroacetic acid (aq pH 2.0) 20:80, respectively. The buffer EDTA solution was prepared weekly by the method described by Mala *et al*. (2010).

3.1.5 Experimental Conditions

3.1.5.1 Liquid chromatography-mass spectrometer.

Thermo TSQ Quantum triple quadrupole Access mass spectrometer coupled to Thermo Accela LC pump and autosampler. LCQuan(V 2.5.6), Xcalibur Qualbrowser(V 2.0.7) software was used to obtain and process data.

3.1.5.2 Labware.

15 mL disposable, conical, graduated, polypropylene tubes with cap (#352097, Becton Dickinson, Franklin Lakes, NJ) polypropylene LC sample vials 2 mL with conical insert (#9301-0978, Agilent, Santa Clara, CA) with pre-scored snap caps.

3.1.5.3 Centrifuges.

(1) refrigerated to 5º C, capable of accelerating 15 mL tubes to 4,000 g

(2) Micro centrifuge capable of accelerating 1.5 mL tubes to 13,500 rpm

3.1.5.4 Organic solvents.

High purity chromatographic and spectrophotometric grade acetonitrile and methanol, or equivalent.

3.1.5.5 LCMS Parameters.

Positive ion electrospray ionization was used for all residues. Instrument was tuned by infusing a 10 μg mL^{-1} solution of ethyl acetate (10 μL min^{-1}) into a stream of 50:50 0.1% formic acid: ACN (250 μL min^{-1}) to optimize parameters such as spray voltage and gas flows. Solutions were infused of each residue (at 10 μg mL^{-1}) to determine the optimal tube lens values and collision energies for the SRM transitions. Following general MS parameters were used**:** spray voltage, 4 kV; capillary temperature, 220ºC; nitrogen sheath gas, 50 arbitrary units; nitrogen auxiliary gas, LC retention times were adjusted to present experimental conditions.

3.1.5.6 Experimental birds

The study was conducted in unsexed Vencobb 400® broiler birds (M/s Venkateshwara Hatcheries, Bangalore, India). Day old chicks were procured from reputed breeder and were reared under deep litter system. Vaccination of broiler chicks for New Castle disease and infectious bursal disease was carried out on 7^{th} and 14^{th} day respectively. Standard poultry feed mixed with halquinol at different dose levels and free of any other antibiotics and/or antimicrobials was used to feed the birds during the trial and birds had free access to clean potable water. Standard vaccination and husbandry conditions were set, but no anticoccidial or antibacterial drugs were administered.

Necessary approval from the Institutional Animal Ethics Committee (IAEC), Veterinary College, Hebbal, Bangalore, India was obtained (No.38/LPM/IAEC/2010) before conducting the present study.

3.1.5.7 Experimental design

Four hundred day-old broiler chicks obtained from a commercial hatchery were divided into a control group and three treatment groups comprising of 100 chicks each. The experimental design is presented in Table 6.

Table 6: The experimental design

Group	Inclusion level of halquinol in feed (ppm)
Group I (Control C, n=100)	-
Group II (Treated T_1, n=100)	60 ppm
Group III (Treated T_2, n=100)	120 ppm
Group IV (Treated T_3, n=100)	240 ppm

The birds belonging to control group were fed on standard poultry feed *ad libitum* free of halquinol or any other antimicrobials, while experimental birds in the treatment groups (T_1, T_2 and T_3) were fed *ad libitum* on standard poultry feed mixed with halquinol at different levels for a period of 40 days (Table 6).

3.1.5.8 Drugs and chemicals

Pure reference standards of 5,7-dichloroquninolin-8-ol and 5-chloroquinolin-8-ol, the two constituent molecules of halquinol were obtained from Sigma, St. Louis, USA. Halquinol used in the present experimental study was procured from M/s. Provimi

Animal Nutrition India Pvt. Ltd., Bangalore, India. Halquinol and its constituent molecules were kept free from metal contact and were protected from light to avoid chelating and photo degradability respectively. For HPLC grade water, water was obtained by using Milli-Q water purification system (Milli-Q gradient, Millipore). HPLC grade acetonitrile and methanol were obtained from E. Merck, India. All other chemicals used in study *i.e.,* orthophosphoric acid, disodium EDTA were of analytical grade. All the solvents and solutions were filtered through 0.45µ nylon membrane filters before using them during HPLC analysis of halquinol.

3.1.5.9 Feed

Five hundred kilogram each of standard starter broiler feed and finisher broiler feed were manufactured at poultry feed manufacturing facility of M/s Provimi Animal Nutrition India Pvt. Ltd., Bangalore, containing no antimicrobial agents and/or growth promoters. Halquinol was added to feed at different levels of 60, 120 and 240ppm and mixed thoroughly using commercial poultry feed mixer. The halquinol pre-mixed feed was then stored in plastic containers until used. Feed ingredients and nutritional composition of the starter and finisher diet is presented in Table 7 (i) and (ii) respectively.

3.2 Analysis of halquinol in feed samples

3.2.1 Extraction procedure

Three necked round bottomed flask (500 ml-1L) with a reflux condenser in an oil bath was set up. Ten gram of feed sample was weighed, and then transferred into 500 ml-1L round bottomed flask. To this, 100 ml of 1% di-sodium EDTA solution and 200 ml of chloroform was added. Contents of the flask were heated to 40°C and stirred for 180 min

(3 hours) at a speed of 300-400 rpm. Contents of the flask were cooled to 35°C, and then filtered using muslin cloth under vacuum. Filtrate transferred into a separating funnel of 500 ml capacity. Residue in the flask was washed using 25 ml x 2 times of chloroform. Washings were pooled and added to separating funnel. Organic layer from bottom was collected. Aqueous layer was washed with 20 ml x 2 times chloroform and washings were pooled and pooled organic layer was evaporated in a rotary evaporator (Heidolph, Germany) under vacuum at 45°C. Residues was dissolved in one milliliter of HPLC grade methanol and predetermined amount of it was injected into HPLC using auto sampler unit.

Table 7: Feed Ingredients and nutritional composition of (i) Starter and (ii) Finisher diets

(i) Starter diet (0-21 days)

Feed Ingredients	Quantity (kg)	Nutritional Composition (calculated value)	
Maize	557.68	Crude protein %	22
Soyabean meal	372.52	Metabolizable Energy Kcal / kg	3025
Sunflower oil	31.63	Crude fibre %	3.35
Limestone powder	14.66	Lysine %	1.25
Dicalcium phosphate	14.56	Methionine %	0.58
DL-methionine	2.40	Calcium %	1
Salt	16.67	Amorphous phosphorus %	0.4
Choline chloride 60%	2.22	Methionine + cysteine %	0.94
Broiler vitamin premix	1.00		
Broiler mineral premix	1.00		
Lysine	0.82		
Sodium bicarbonate	0.52		
Total (kg)	**1000.00**		

(ii) Finisher diet (22-42 days)

Feed Ingredients	Quantity (kg)	Nutritional Composition (calculated value)	
Maize	602.34	Crude protein %	19.5
Soyabean meal	310.75	Metabolizable energy Kcal/kg	3200
Sunflower oil	52.68	Crude fibre %	3.07
Limestone powder	11.98	Lysine %	1.03
Dicalcium phosphate	15.15	Methionine %	0.47
DL-methionine	1.62	Calcium %	0.9
Salt	2.48	Amorphous phosphorus %	0.45
Choline chloride 60%	1.00	Methionine+ cysteine %	0.8
Broiler vitamin premix	1.00		
Broiler mineral premix	1.00		
Total (kg)	**1000.00**		

3.2.2 LC-MS analysis

The mobile phase consisted of 0.1% orthophosphoric acid and acetonitrile mixed at a ratio of 1:1 volumes. The flow rate of mobile phase was one mL per minute. The detection wavelength was set at 247 nm and the samples were analyzed for 30 min with column oven temperature set at 40°C. A reverse phase C_{18} column (250 x 4.6 mm, particle size 5µ, Purosper Star, E-Merck) and HPLC-Thermo Finnigan Surveyor was used for analysis of halquinol in feed samples.

Representative feed samples containing halquinol @ 60, 120 and 240 ppm were assayed using HPLC and the halquinol concentration was found to be at desired level.

3.2.3 Feeding halquinol mixed feed to broiler birds

Day old chicks were allocated to control and treatment groups on same day itself, and from day 1 to day 20, standard poultry starter feed mixed with halquinol at 60, 120, 240 ppm was fed to chicks of T_1, T_2 and T_3 treatment groups respectively and standard poultry starter feed free of halquinol was fed to chicks of control group from day 1 to day 20. From day 21 to day 42, standard finisher poultry feed mixed with halquinol at 60, 120 and 240 ppm was fed to birds of T_1, T_2 and T_3 treatment groups respectively and standard finisher feed free of halquinol was fed to birds of control group from day 21 to day 42. Chicks were housed under deep litter system with partitions in between to separate control and different treatment groups apart. Birds had access to clean potable water *ad libitum* throughout the trial. From day 41 both control and treatment groups were fed standard poultry finisher ration free from halquinol for the rest of the period of study.

3.3 Collection of samples

3.3.1 Collection of samples during period of halquinol exposure through diet

In order to determine tissue distribution of halquinol and its metabolites, upon incorporating it with poultry feed in broilers, six birds from each group were sacrificed on 5, 10, 20, 30 and 40 days during treatment and tissue samples *viz.* liver, kidney, breast muscle of each bird were collected in separate labeled colorless self sealing polythene bags and stored in deep freezer (-20 °C) until assayed.

3.3.2 Collection of samples after the withdrawal of halquinol exposure through diet

With a view to determine tissue depletion of halquinol that is tissue concentration of halquinol after withdrawal of treatment in broilers, six birds from each group were sacrificed at 2 h post-withdrawal of halquinol exposure through diet and on 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days after withdrawal of treatment and tissue samples *viz.* liver, kidney, breast muscle of each bird were collected in separate labeled self sealing polythene bags and were kept in deep freezer until analyzed.

Plate 2. A representative photograph of day old broiler chicks on standard poultry diet pre-mixed with halquinol

Plate 3. A representative photograph of twenty days old broiler birds on standard poultry diet pre-mixed with halquinol

Plate 4. A representative photograph of forty days old broiler birds on standard poultry diet pre-mixed with halquinol

Plate 5. Experimental birds maintained in a broiler house under deep litter system.

3.4 Assay of halquinol in tissue samples

The concentration of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol in tissue samples was determined by high performance liquid chromatography (HPLC; Shimadzu Prominence, Japan) with photodiode array detector set at a wavelength of 247 nm. Necessary studies were carried out to confirm the stability of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol under sample storage conditions. The standard procedures were adopted to confirm the recovery per cent, repeatability and assay precision. All the tissue samples were assayed in duplicate.

3.4.1 Assay of halquinol metabolites in tissue samples

The concentration of conjugated metabolites of halquinol *viz*, 5-chloro-quinolin-8-ol sulfate, 5-chloro-quinolin-8-ol glucuronide, 5,7, dichloro-quinolin-8-ol sulfate, 5,7, dichloro-quinolin-8-ol glucuronide were analysed by LCMS and the mass spectra was performed by Microfocal Origin Pro analytical and Xcalibur softwares. The molecular formulas of the metabolites were calculated using chemdraw software.

3.4.2 Experimental conditions

The HPLC system consists of single pump (LC-20AT), an auto sampler (SIL-20A), photo diode array detector (SPD-M20A) and LC solution® software for data analysis. A reverse phase C_{18} column (250 X 4.6 mm, particle size 5µm, pH:1.5-11, Purospher Star RP-18e, Merck, Germany) served as stationary phase. The mobile phase consisted of 0.1% v/v, orthophosphoric acid and acetonitrile mixed at a ratio of 1:1 v/v.

The flow rate of the mobile phase was one mL per minute and the injection volume was 20 µl. The detection wavelength was set at 247 nm with scanning range of 200-400 nm and the samples were analyzed for 30 min with column oven temperature set at 40°C. There were no interfering peaks in the chromatogram at the retention times of 14.8 min for 5,7-dichloroquinolin-8-ol and 5 min. for 5-chloroquinolin-8-ol. Extracted tissue samples were injected into LCMS automatically through auto sampler unit of LCMS system.

Due to the chelating effects of halquinol and its two constituent molecules: 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol with metallic cations, the existence of even trace amount of metals on the packing material will cause peak tailing and/or irreversible adsorption effects. Therefore, the column was treated with Na_2EDTA to remove all the traces of metals before use, following a procedure previously reported for HPLC analysis of tetracycline by Lian *et al.* (1998).

Aqueous solution (0.1 M) of Na_2EDTA was flushed through the column at a flow rate of 1mL/min for 1 h before the first sample run. The column was then rinsed with water for 30 min to remove all Na_2EDTA residues and was reconditioned by flushing with mobile phase for another 30 minute. No further treatment was necessary during the experiments.

3.4.3 Liver

Frozen liver tissue samples were thawed and external fat and fascia was trimmed off. Five gram of liver tissue was cut into two to three small pieces and then homogenized using motor driven tissue homogenizer (Remi,India), equipped with a

ground glass cylinder and teflon pestle. The homogenate was decanted quantitatively into screw capped polypropylene test tubes of 50 ml capacity. To it was added 0.5 ml of 0.02M phosphate disodium EDTA buffer (pH 6.5) and mixed by shaking. The mixture thus obtained was subjected to extraction of 5,7-dichloroquinolin-8-ol and 5-chloroqunolin-8-ol by adding 20 ml of ethyl acetate and then vortexed for three min. After vortexing, the mixture was centrifuged (Eppendorf Centrifuge 5810R, Germany; fixed angle rotor) at 10,000 rpm for 5 min. The supernatant was transferred into 50 ml glass stoppered test tube. The extraction procedure was repeated with 10 ml ethyl acetate and the supernatant was transferred in to same test tube. The pooled organic fraction was evaporated to dryness in a gentle stream of nitrogen under water bath maintained at 50 °C. The residue left was reconstituted with one milliliter of methanol. This solution was then filtered through a 0.45 µm PVDF syringe driven membrane filter into an auto sampler vial. A 20 µl of this solution was injected into HPLC through auto sampler unit.

3.4.4 Kidney

Five gram of kidney tissue was cut into two to three small pieces and homogenized using motor driven tissue homogenizer (Remi, India) equipped with a ground glass cylinder and teflon pestle. Tissue homogenate was then transferred quantitatively into 50 ml polypropylene screw capped centrifuge tube. To it was added 0.5 ml of 0.02 M phosphate Na_2EDTA (3 %) buffer and mixed by shaking. The mixture thus obtained was subjected to extraction of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol by adding 20 ml of ethyl acetate and vortexing for three min. The mixture was then centrifuged (Eppendorf Centrifuge 5810R, Germany; fixed angle rotor) at 10,000 rpm for 5 min and the supernatant (organic phase) obtained was transferred into

50 ml glass stoppered test tubes. The extraction procedure was repeated with 10 ml ethyl acetate to harvest 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol residues left out. The pooled supernatant (organic fraction) was evaporated to dryness by using gentle stream of nitrogen in water bath maintained at 50°C. The residues left behind were immediately reconstituted with one milliliter of methanol. This solution was then filtered through 0.45µm PVDF syringe driven membrane filter into an auto sampler vial and 20 µl of this solution was injected into HPLC through the attached auto sampler unit.

3.4 .5 Muscle

One hundred gram of breast muscle was taken and cut into two to three pieces, later ground using household mixer grinder. Five gram of this ground muscle tissues were transferred to a 50 ml polypropylene screw capped centrifuge tubes. To this three ml of 0.02 M phosphate disodium EDTA buffer (pH adjusted to 6.5) was added and mixed by shaking, mixture was then homogenized with 20 ml of ethyl acetate using tissue homogenizer (Heidolph Silent Crucher M, Germany; dispersion tool is 18 F/M) at 10,000 rpm for 30 seconds which was then centrifuged (Eppendorf Centrifuge 5810 R, Germany; fixed angle rotor) at 10,000 rpm for five min. The supernatant was then transferred into 50 mL glass stoppered test tube. The extraction procedure was repeated with 10 ml ethyl acetate and the supernatant was transferred in to same test tube. The pooled organic fraction was evaporated to dryness in a stream of nitrogen under water bath maintained at 50°C. The residue left behind was immediately reconstituted with one milliliter of methanol. This solution was then filtered through 0.45 µm PVDF syringe driven membrane filters and 20 µl of this was injected into HPLC through auto sampler unit.

3.5 Construction of standard curve

3.5.1 Liver

Drug free liver samples were obtained after sacrificing healthy broiler birds for standardization of extraction procedures and construction of standard curves for 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol in liver matrix. The calibration curves were constructed in the range of 22 to 642 ng/mL as described above. The standard curves were linear in the range of 22 to 642 ng /mL with r^2 value of 0.999 and limit of quantification of 20 ng/mL for a gram of tissue for both the constituent molecules.

3.5.2 Kidney

Drug free kidney tissue samples were obtained after sacrificing healthy birds for standardization of extraction procedures and construction of standard curve for 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol in kidney matrix. The calibration curves were constructed in the range of 22 to 642 ng/ml. The standard curves were linear in the range of 22 to 642 ng/ml with r^2 value of 0.999 for both the spiked molecules. The limit of quantification of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol was 20 ng/ml for a gram of tissue.

3.5.3 Muscle

Drug free breast muscle samples were obtained after sacrificing healthy birds for standardization of extraction procedures and construction of standard curve for 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol in breast muscle matrix. The calibration curves were constructed in the range of 22to 642ng/ml. The standard curves were linear

in the range of 22 to 642 ng/ml with r^2 value of 0.999 for both the spiked molecules. The limit of quantification was 20 ng/ml for a gram of muscle tissue in both the cases.

All the standard curves generated for detection of 5,7-dichloroquinolin-8-ol and and 5-chloroquinolin-8-ol in (i) liver, (ii) kidney and (iii) breast muscle tissues of broiler chicken are shown in Fig. 5A and 5B.

3.6 Quantification

The standard curves for 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol were prepared by plotting peak areas (x-axis) against concentration (y-axis) of respective molecules. The linear regression formula obtained from calibration curves were used to derive unknown concentration of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol in plasma and tissue samples respectively using the equation:

$$X = a' + b'Y$$

Where, X = dependent variable

a' = intercept

b' = regression coefficient 'X' upon 'Y'

Y = independent variable (peak area)

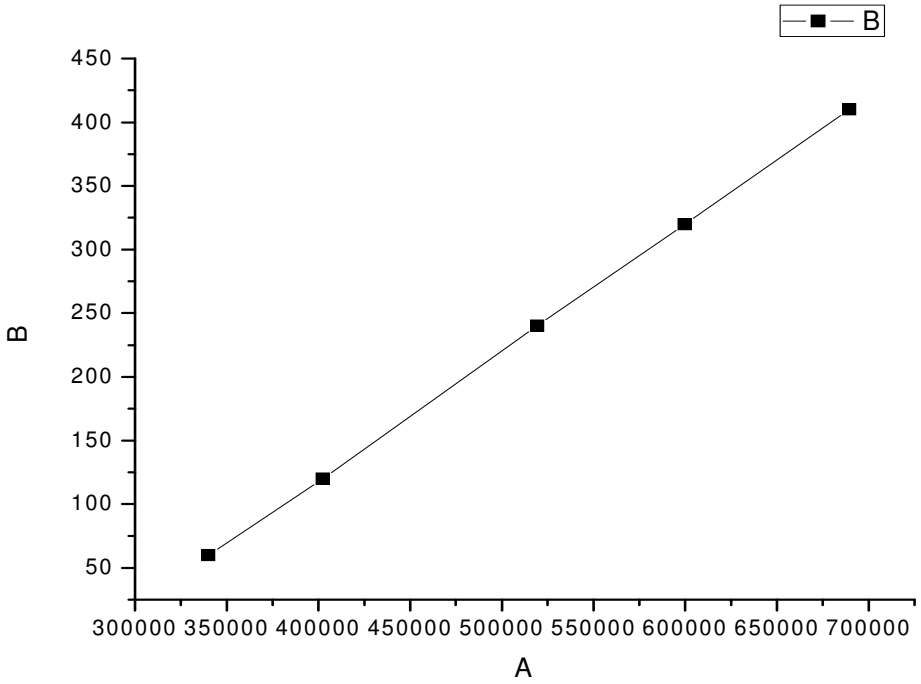
3.6.1 Analytical recovery

Analytical recovery for the method adopted was studied by using external standard technique. Briefly, recovery was determined by adding 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol to drug free tissues *viz:* liver, kidney and muscle (pre-extraction spiking) and to a fluid obtained after extraction from drug free tissue (post-

extraction spiking) at three concentration levels of 20, 80 and 320 ng/ml. Tissue samples were subjected to liquid–liquid extraction. Both pre-extraction and post-extraction spiked plasma or tissue standards were analyzed as described above. Recovery was calculated as ratio of peak areas obtained for pre-extraction spiked plasma or tissue standards and those obtained for post-extraction spiked plasma or tissue standards. For each concentration two determinants were made. Table 8A and 8B respectively depicts per cent recovery of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol from spiked plasma or liver, kidney and breast muscle tissue samples.

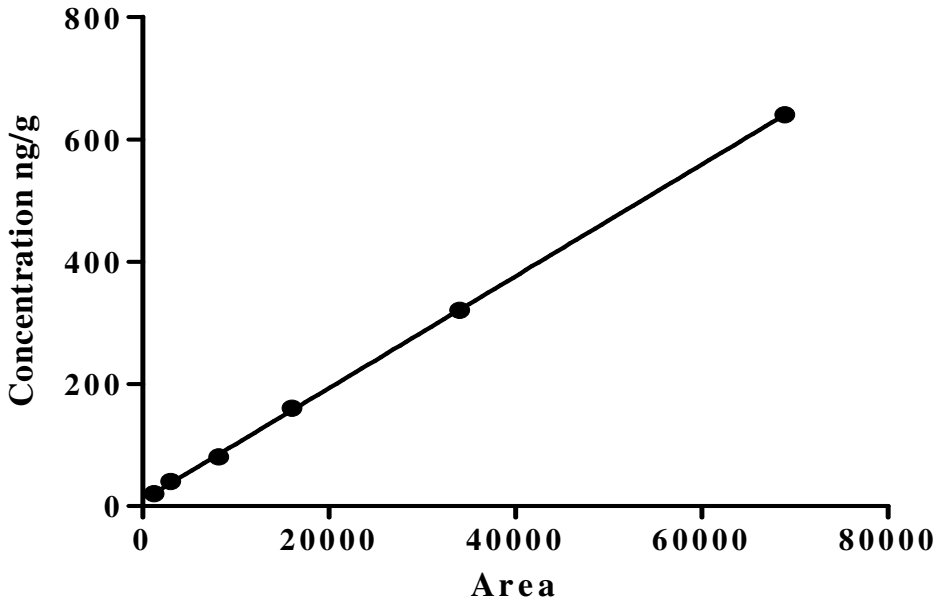
Fig 5A: Standard curve generated for detection of 5,7-dichloroquinolin-8-ol in (i) Liver, (ii) Kidney and (iii) breast muscle tissues of broiler chicken (n= 5)

(i) Liver



A= Area, B= Concentration

(ii) Kidney



(iii) Breast muscle

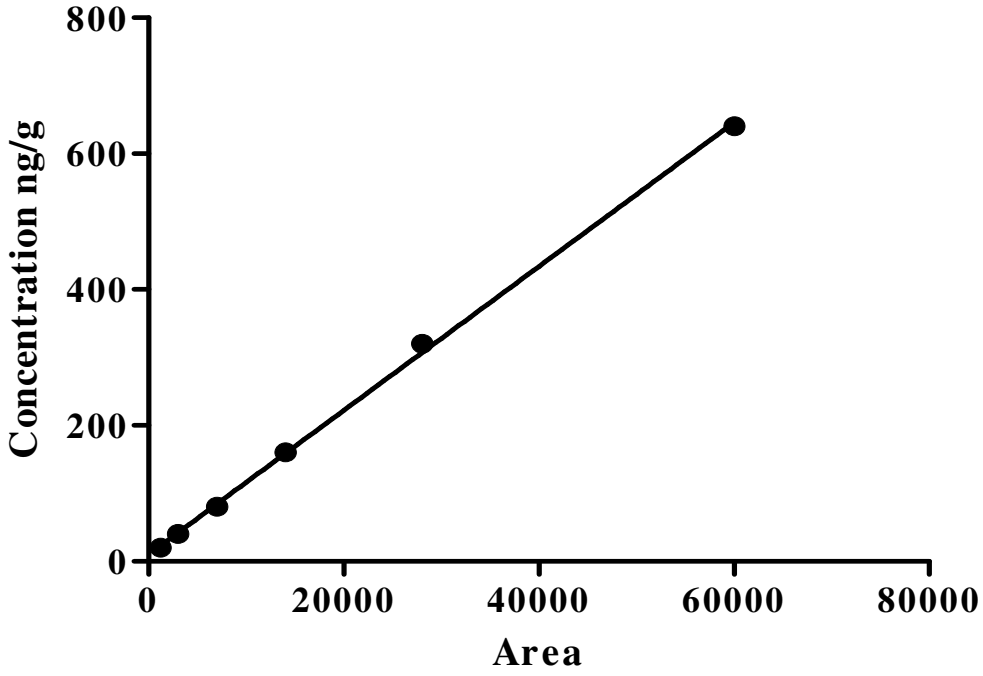
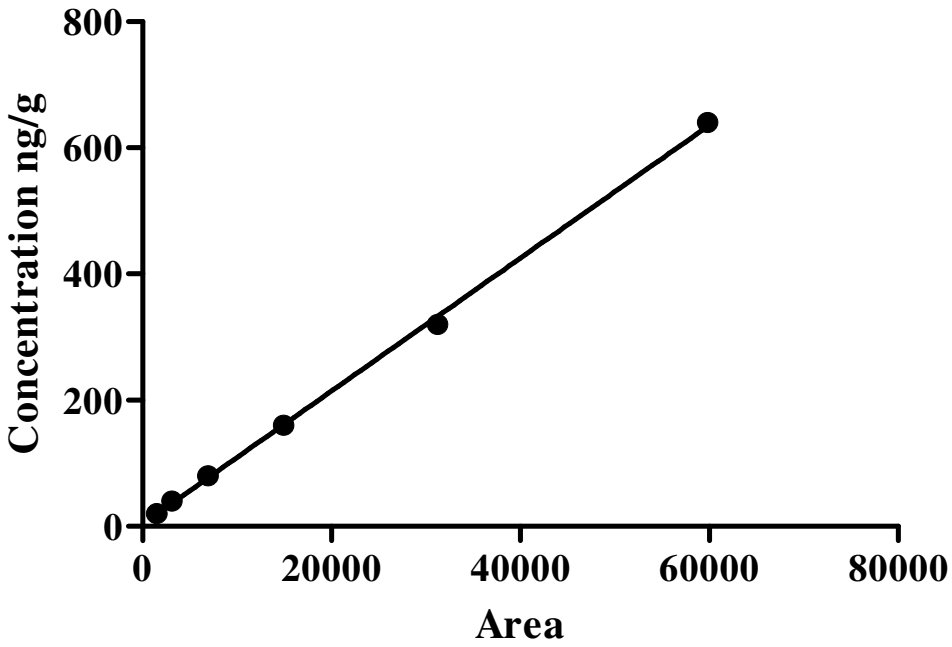
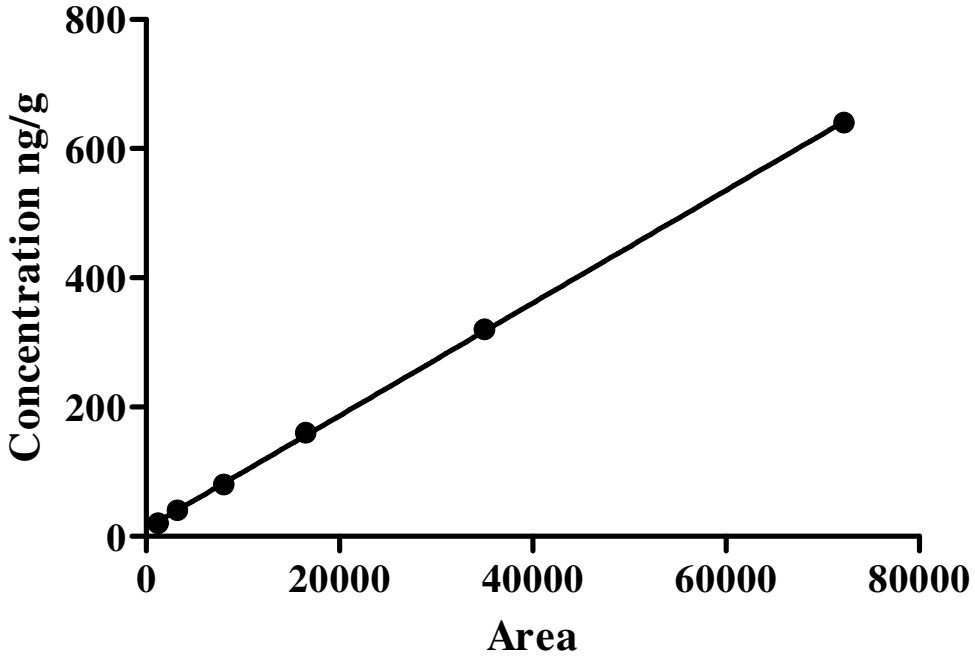


Fig. 5B : Standard curve generated for detection of 5-chloroquinolin-8-ol in (i) liver, (ii) kidney and (iii) breast muscle tissues of broiler chicken (n= 5)

(i) Liver



(ii) Kidney



(iii) Breast muscle

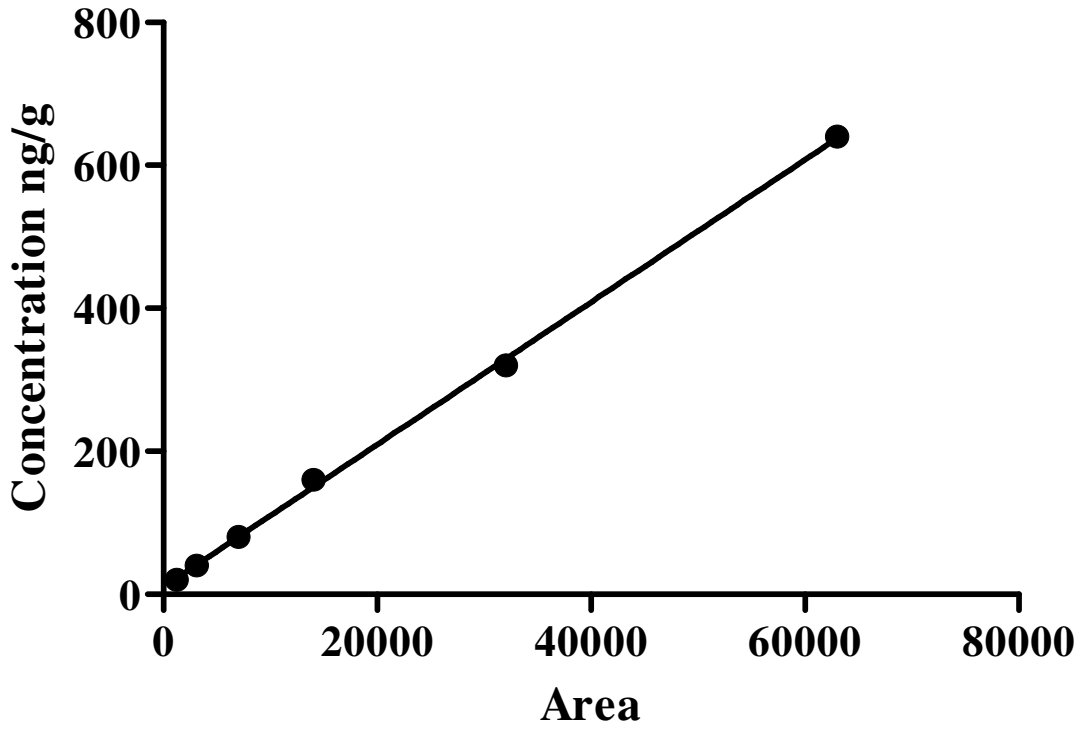


Table 8A: Percent recovery of 5,7-dichloroquinolin-8-ol from spiked (i) liver, (ii) kidney and (iii) breast muscle tissue matrix

(i) Liver

Concentration spiked (ng/g)	Concentration detected (mean±SE; ng/g)	Recovery %
20	14.44±0.35	72.20
80	60.92±0.86	76.15
320	298.14±2.85	93.17
Mean per cent recovery		**85.5**

(ii) Kidney

Concentration spiked (ng/g)	Concentration detected (mean±SE; ng/g)	Recovery %
20	14.80±0.48	74.00
80	63.40±0.87	79.25
320	300.10±1.90	93.78
Mean per cent recovery		**91.9**

(iii) Breast muscle

Concentration spiked (ng/g)	Concentration detected (mean±SE; ng/g)	Recovery %
20	15.0±0.35	75.00
80	70.00±1.0	87.50
320	309.40±1.85	96.69
Mean per cent recovery		**87.64**

Table 8B: Percent recovery of 5-chloroquinolin-8-ol from spiked (i) liver, (ii) kidney and (iii) breast muscle tissue matrix

(i) Liver

Concentration spiked (ng/g)	Concentration detected (mean±SE; ng/g)	Recovery %
20	16.84±0.68	84.20
80	70.15±0.97	87.69
320	308.40±2.61	96.38
Mean per cent recovery		**90.56**

(ii) Kidney

Concentration spiked (ng/g)	Concentration detected (mean±SE; ng/g)	Recovery %
20	16.76±0.52	83.80
80	73.22±0.82	91.53
320	311.0±2.98	97.19
Mean per cent recovery		**92.39**

(iii) Breast muscle

Concentration spiked (ng/g)	Concentration detected (mean±SE; ng/g)	Recovery %
20	15.67±0.44	78.35
80	73.05±1.15	91.31
320	312.4±2.67	97.62
Mean per cent recovery		**89.28**

3.7 Statistical analysis

All the experimental data obtained were expressed as mean±SE. The significant differences between the means of two groups were determined by XCALIBUR, ORIGIN PRO 8 analytical softwares, USA and ONE WAY ANOVA followed by Tukeys Multiple Comparison Test.

RESULTS

IV. RESULTS

The results of various experiments carried using halquinol are described.

4.1 UV Spectroscopy:

The UV visible Spectra indicated that the solubility of halquinol when used in solution form of 1mg/ml was found to be 100% in DMSO. Fold solubility of halquinol in all these solvents was 5.7, 5.6, 5, 4.32, 5.53 and 1 for acetonitrile, ethanol, dichloromethane, hexane, methanol, ethanol and water respectively. Six solvents were used to determine the maximum solubility of halquinol. The maximum solubility of halquinol was found in acetonitrile *i.e,* 5.7. Since the cytoxicity assay of DMSO was high in animal healthy cells, it was not used in the present study. Thus, acetonitrile (LCMS grade) was used throughout the experiment to dissolve halquinol for all the experimental work as fold solubility of halquinol was maximum (5.7) in it.

4.1.1 Ultraviolet Visible Spectrum of 5,7-dichloroquinolin-8-ol

Ultraviolet visible spectrum of 5,7-dichloroquinolin-8-ol in acidic (0.1 N HCl) alkaline (0.1N NaOH) and neutral solvent systems are shown in Fig. 6. The maximum ultraviolet absorption of 5,7-dichloroquinolin-8-ol was found 0.694 at 338.5 nm in acetonitrile solvent system. The fold solubility of halquinol was highest in acetonitrile *i.e*, 5.7 among the all solvent systems used.

4.1.2 Ultraviolet Visible spectrum of 5-chloroquinolin-8-ol

The Ultraviolet-visible spectrum (200-400 nm) of 5-chloroquinolin-8-ol in acidic (0.1N HCl), alkaline (0.1N NaOH) and water solvent systems are shown in Fig. 7, 8 and 9. The maximum ultraviolet absorption of 5-chloroquinolin-8-ol was found at 379.5, 383.5 and 247 nm in acidic (0.1N HCl), alkaline (0.1N NaOH) and neutral solvent systems respectively.

4.1.3 Ultraviolet visible spectrum of halquinol

The maximum ultraviolet absorption of halquinol was found at 379.5, 383.5 and 247 nm in acidic (0.1N HCl), alkaline (0.1N NaOH) and water solvent systems respectively. The Ultraviolet-visible spectrum (200-400nm) of halquinol in acidic (0.1N HCl) alkaline (0.1N NaOH) and neutral (methanol) solvent systems are shown in Fig.6,7,8,9 and 10.

Fig. 6: Ultraviolet-visible spectrum of 5,7-dichloroquinolin-8-ol in different solvent systems

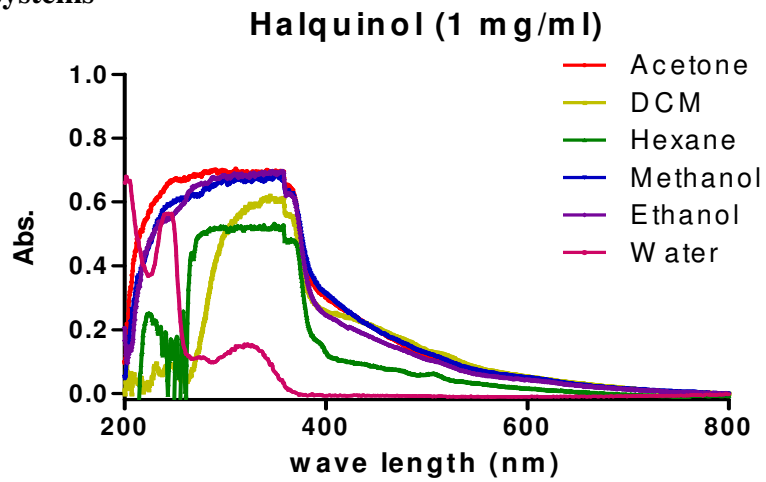


Fig. 7: Ultraviolet visible spectrum of 5,7-dichloroquinolin-8-ol

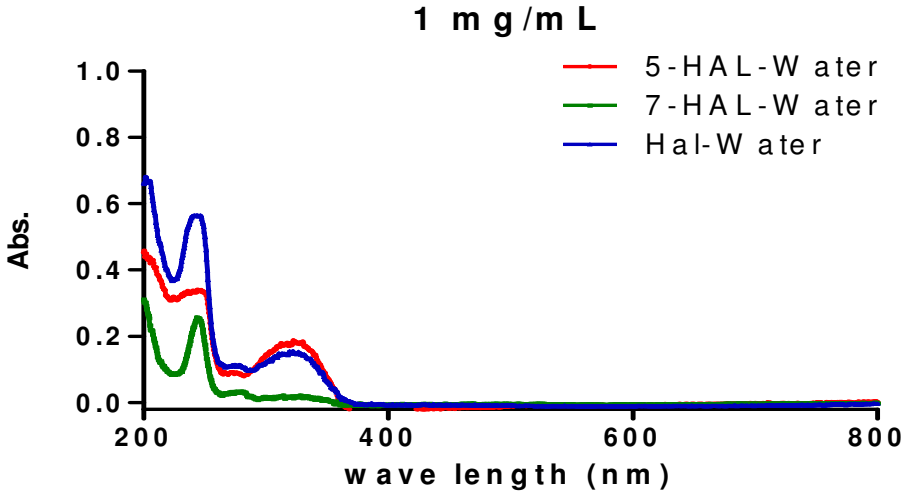


Fig. 8: Ultraviolet-visible spectrum of 5,7-dichloroquinolin-8-ol in acidic medium

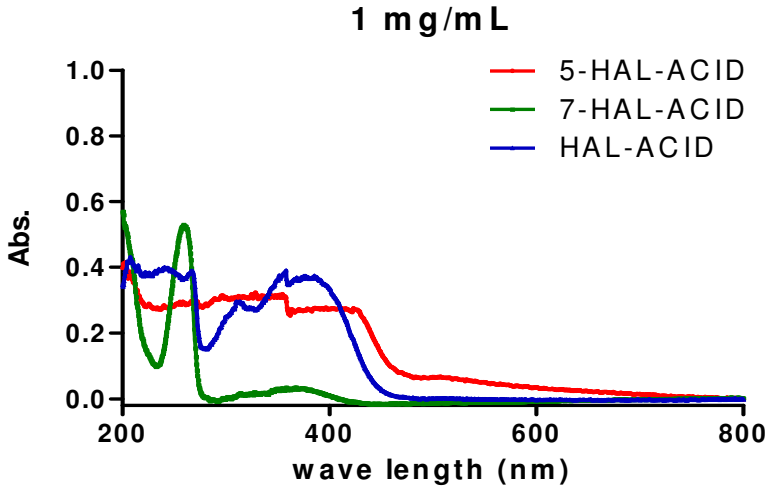


Fig. 9. Ultraviolet-visible spectrum of 5,7-dichloroquinolin-8-ol in basic medium

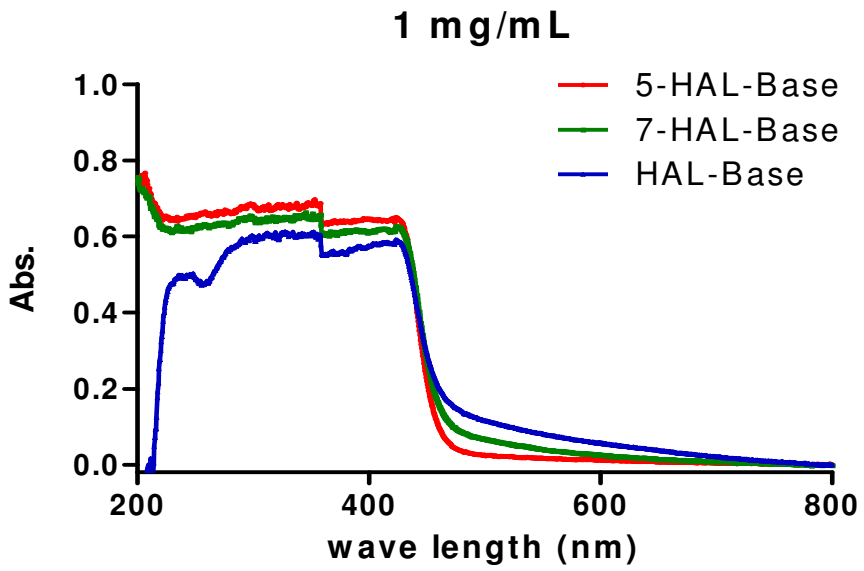
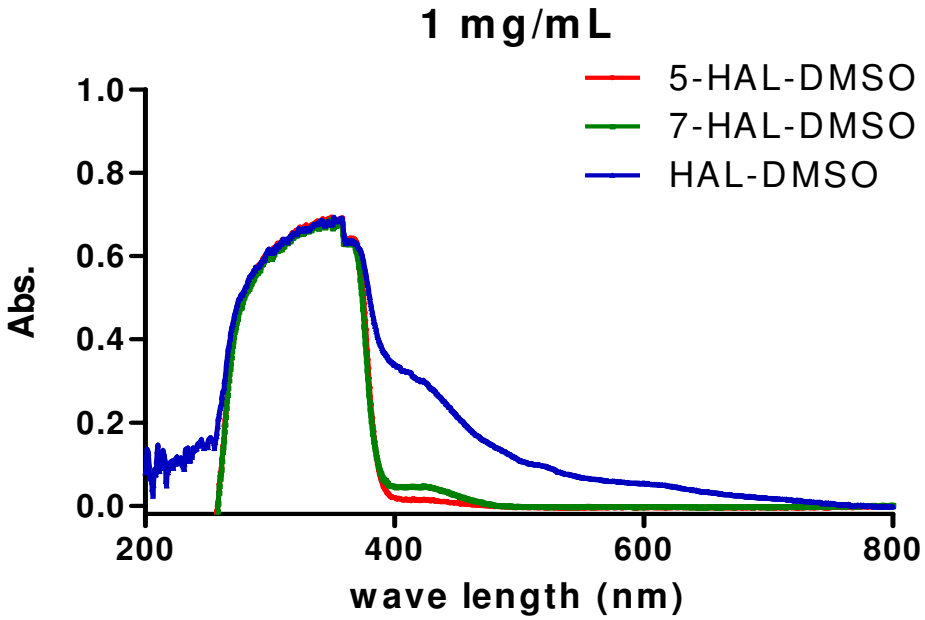


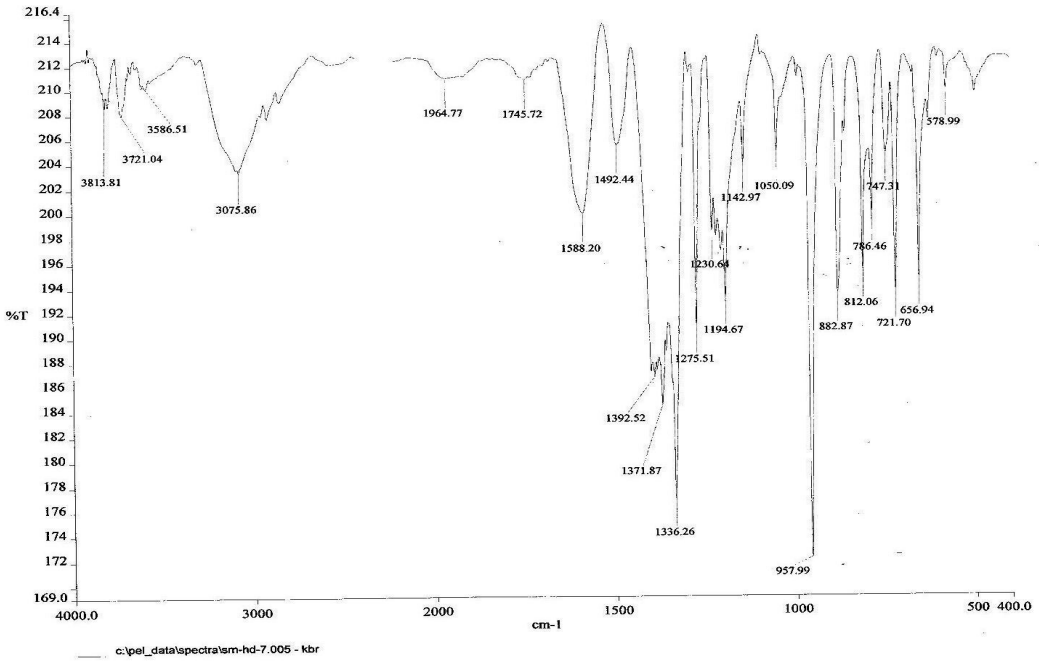
Fig. 10. Ultraviolet-visible spectrum of 5,7-dichloroquinolin-8-ol in DMSO



4.2 IR Spectroscopy:

Samples were prepared in acetonitrile (1 mg/ml) and run on FTIR Nicolet 6700 (Thermo Fisher Scientific, Madisson, WI, USA) operated by Omnic software 8.1.spectroscope. Data was analyzed by describing various vibrational bands appeared in spectra because of the chemical nature of the halquinol and its constituents. The infrared spectra of (Halquinol 5,7) is shown in Fig. 11,12 and 13 respectively. The principal wave numbers obtained in infrared spectrum and their corresponding assignment (bond, compound type and functional groups) were characteristic for 5,7-dichloroquinolin-8-ol as mentioned below:

Fig 11**:** IR Spectrum of 5,7-dichloroquinolin-8-ol.



X axis =Wavenumbers

Y axis=Percentage Transmittance

Fig. 12: IR Spectrum of 5-chloroquinolin-8-ol.

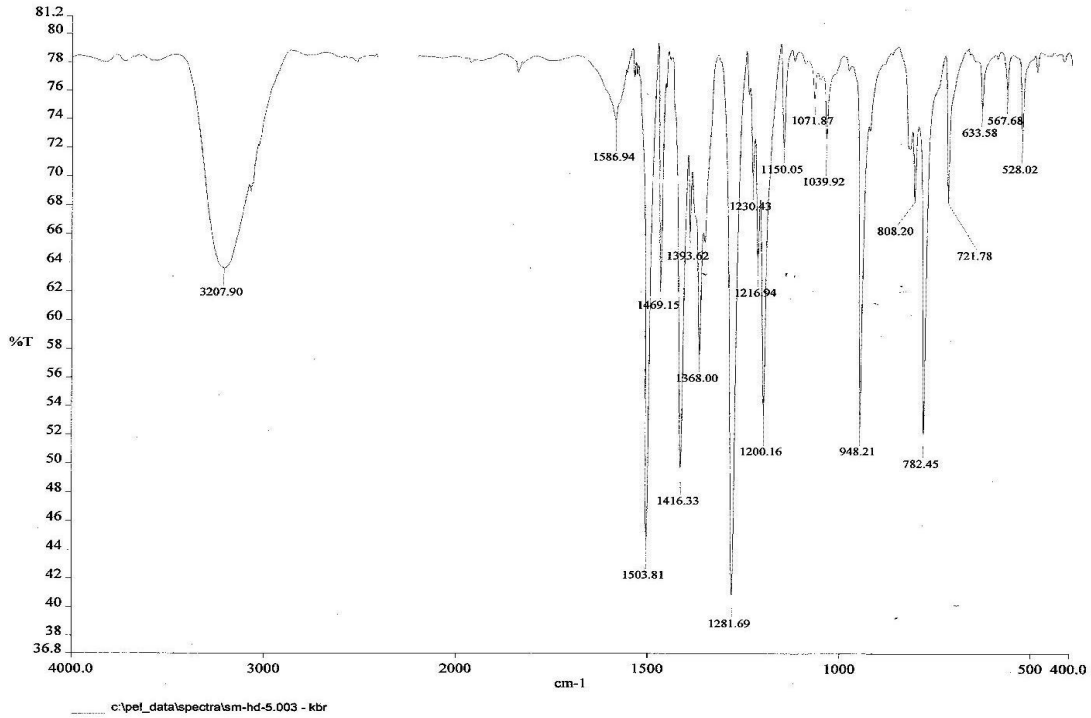
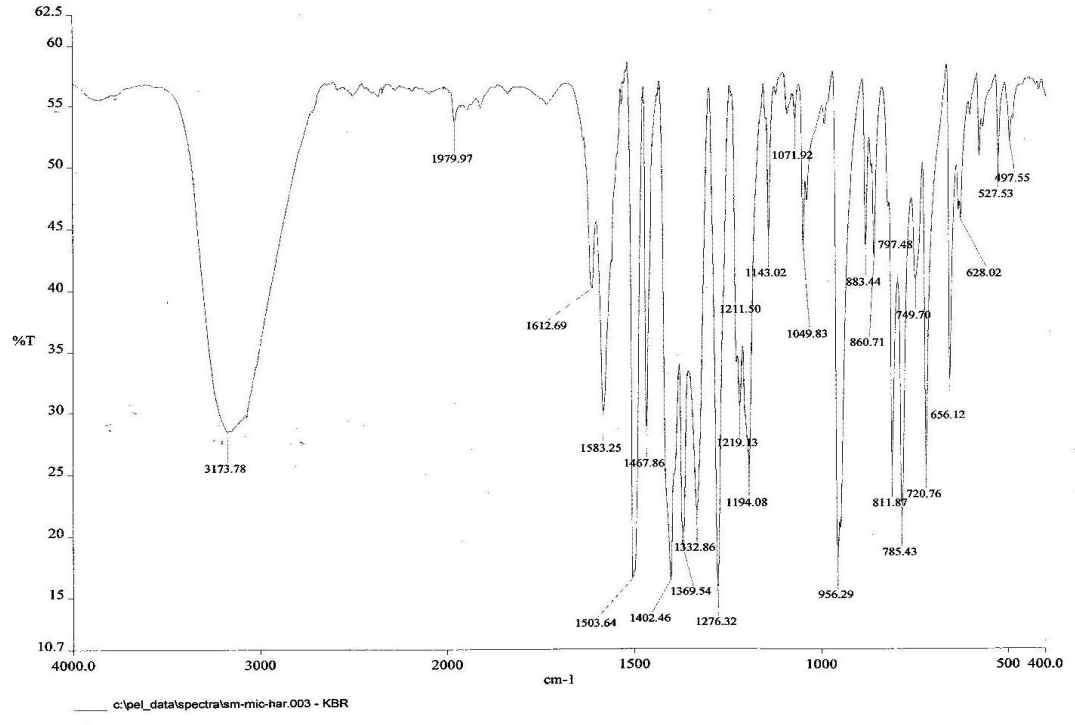


Fig. 13: IR Spectrum of halquinol



X axis =Wavenumbers Y axis=Percentage Transmittance

Table 9: The principal wave numbers obtained in infrared spectrum and their corresponding assignment (bond, compound type and functional group) were characteristic for 5-chloro-8-quinolinol

Wave number(cm^{-1}) KBr disk	Characteristics	Assignment of wave numbers
3207.90	mb	O-H stretch, particularly of phenols
1586.94	wb	C=C stretching (aromatic)
1503.81	s	C=N stretching (aromatic)
1469.15	ms	C=C stretching(aromatic)
1416.33	s	C=C stretching(aromatic)
1368	ms(poor)	C-O stretching(phenolic)
1281.69	st s	C-H stretching(in plane bending)
1200.16	s	C-O stretching(phenolic)
1216.94	s	C-H stretching(in plane bending)
1150.05	ws	C-H stretching(in plane bending)
1071.87	w	C-Cl stretching, C-H stretching(in plane bending)
1039.92	ws	C-H stretching(in plane bending)
948.21	s	C-O stretching(phenolic)
808.20	w	C=C stretching(aromatic)
782.45	s	C-H stretching(in plane bending)
721.78	ws	C-Cl stretching, C-H stretching(in plane bending)
633.58	sts	C=C stretching(aromatic)
567.68	ws	C-O stretching(phenolic)
528.08	st	C-O stretching(phenolic)

Table 10: The principal wave numbers obtained in infrared spectrum and their corresponding assignment (bond, compound type and functional group) were characteristic for 5,7-dichloroquinolin 8-ol

Wave number(cm^{-1}) KBr disk	Characteristics	Assignment of wave numbers
3586.51	wb	O-H stretch, particularly of phenols
3075.86	sb	C- H stretching (aromatic)
1964.77	s	C=C stretching (conjugated)
1745.72	ws	C=C stretching(aromatic)
1588.20	s	C=C stretching(aromatic)
1492.44	ms(poor)	C=C stretching(phenolic),C=N stretching
1392.52	st s	C-O stretching(in plane bending)
1371.87,1376.26,1275.51	s	C-O stretching(phenolic)
1230.64	s	C-H stretching(in plane bending)
1194.67	ws	C-H stretching(in plane bending)
1142.97	w	C-Cl stretching, C-H stretching(in plane bending)
1050.09	ws	C-H stretching(in plane bending)
957.99	s	C-O stretching(phenolic)
882.87	ms	C=C stretching(aromatic)
812.06	s	C-H stretching(in plane bending)
786.46	ws	C-H stretching(in plane bending)
743.31	sts	C=C stretching(aromatic)
721.70	ws	C-O stretching(phenolic)
656.94	st	C-O stretching(phenolic)
578.99	-	C-H out of plane bending

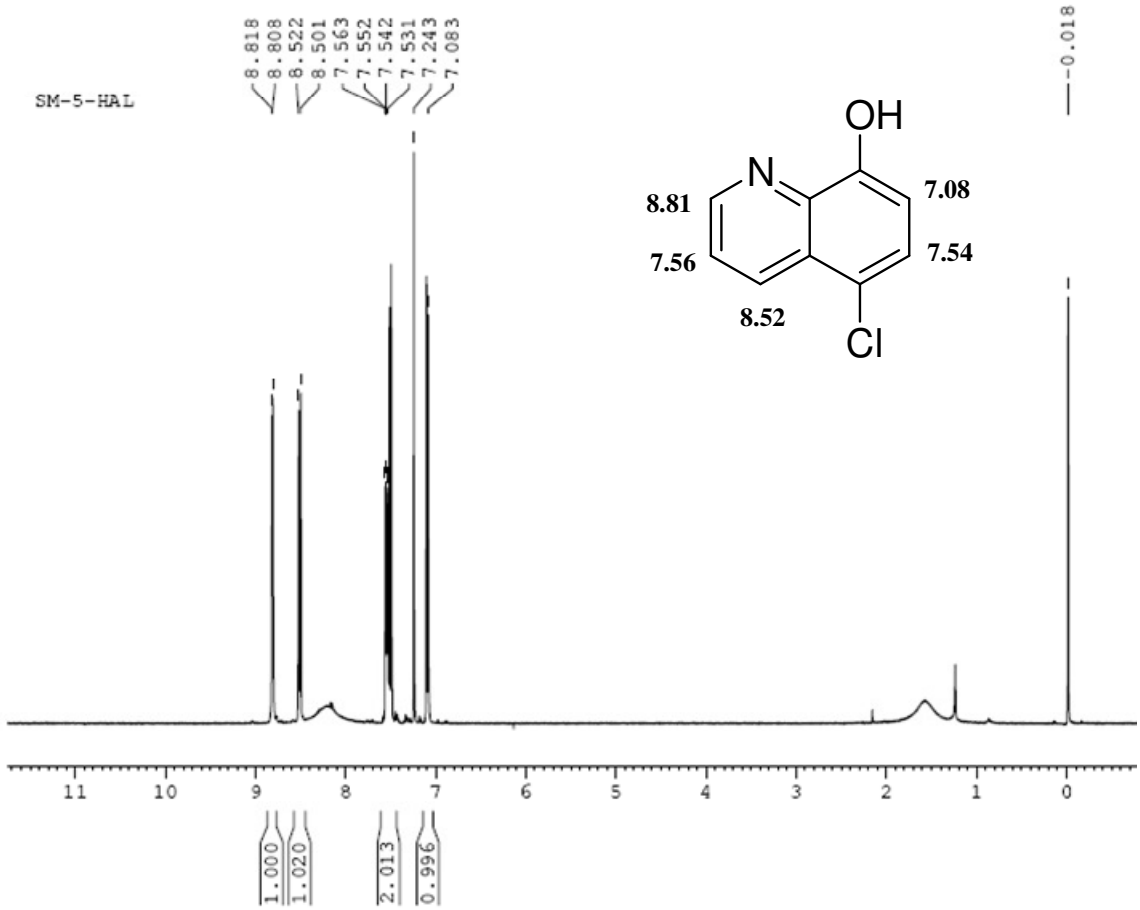
Table 11: The principal wave numbers obtained in infrared spectrum and their corresponding assignment (bond, compound type and functional group) were characteristic for halquinol

Wave number (cm^{-1}) KBr disk	Characteristics	Assignment of wave numbers
3173.78	mb	O-H stretch, particularly of phenols
1979.97	wb	C=C stretching (aromatic)
1612.69	s	C=N stretching (aromatic)
1503.64	ms	C=C stretching(aromatic)
1467.86	s	C=C stretching(aromatic)
1402.46	ms(poor)	C-O stretching(phenolic)
1369.54	st s	C-H stretching(in plane bending)
1332.86	s	C-O stretching(phenolic)
1219.13	s	C-H stretching(in plane bending)
1194.08	ws	C-H stretching(in plane bending)
1143..02	w	C-Cl stretching, C-H stretching(in plane bending)
1071.92	ws	C-H stretching(in plane bending),C-Cl stretching
1049.83	s	C-O stretching(phenolic)
956.29	w	C=C stretching(aromatic)
883.44	s	C-H stretching(in plane bending)
860.71	ws	C-Cl stretching, C-H stretching(in plane bending)
811.87	sts	C=C stretching(aromatic)
785.43	ws	C-O stretching(phenolic)
749.70	st	C-O stretching(phenolic)
720.76	w	------
656.12	b	------

4.3 NMR Spectroscopy: 1H-**NMR of 5-chloro-8-quinolinol (δ)**

7.08 (1H; H next to C-OH); 7.24 (for $CDCl_3$); 7.53-7.56 (2H; 1H next to C-Cl and 1H second next N); 8.52 (1H; H second next to C-Cl); 8.81 (1H; H next to N). NMR data revealed the satisfactory purity of the compound 5-chloro-8-quinolinol Fig.14 (Spectra 14)

Fig. 14. ^{1}H-NMR Spectrum of 5-chloro-8-quinolinol



X axis =Chemical Shift

Fig. 15: ^{1}H-NMR Spectrum of 5,7-dichloro-8-quinolinol

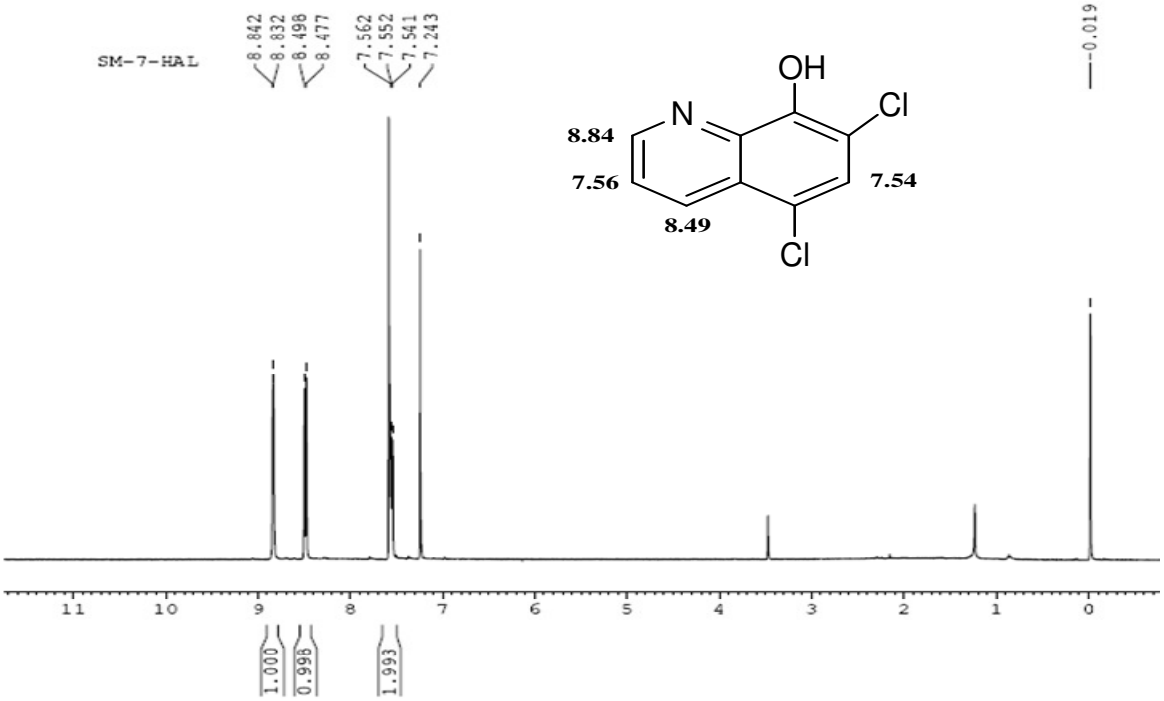
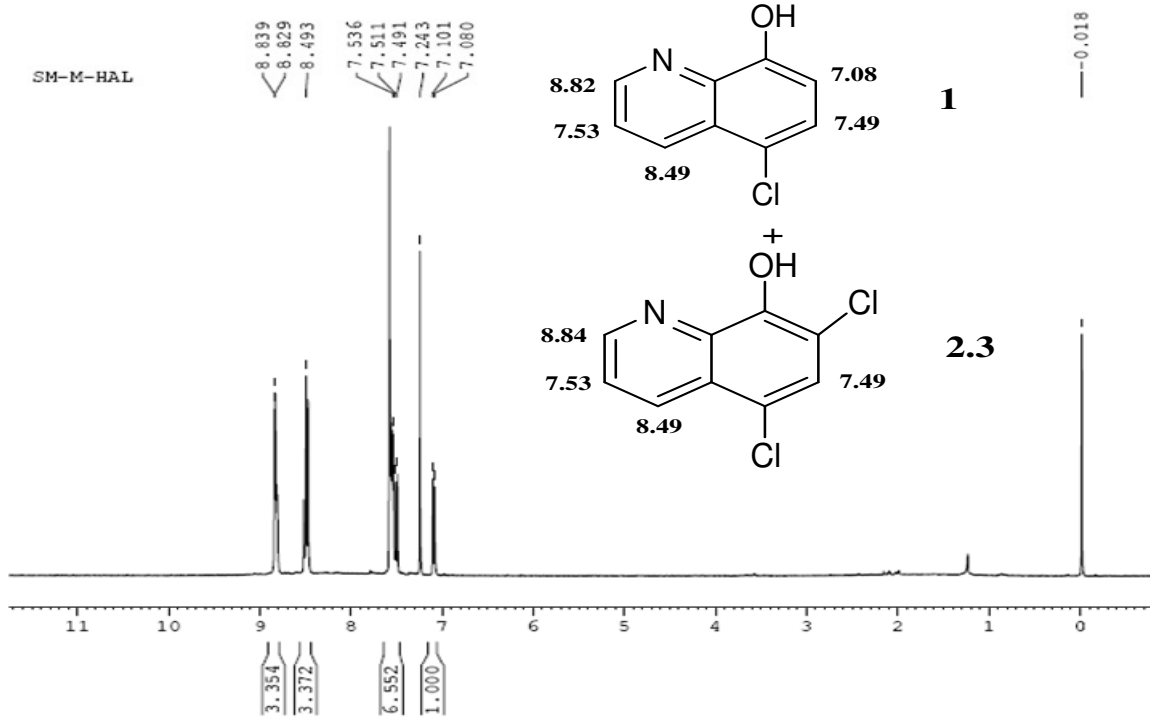


Fig. 16: ^{1}H-NMR Spectrum of halquinol [Mix of 5-chloro-8-quinolinol: 5,7-dichloro-8-quinolinol (1:2.3)]



X axis =Chemical Shift

Fig. 17: ^{13}C-NMR Spectrum of 5-chloro-8-quinolinol

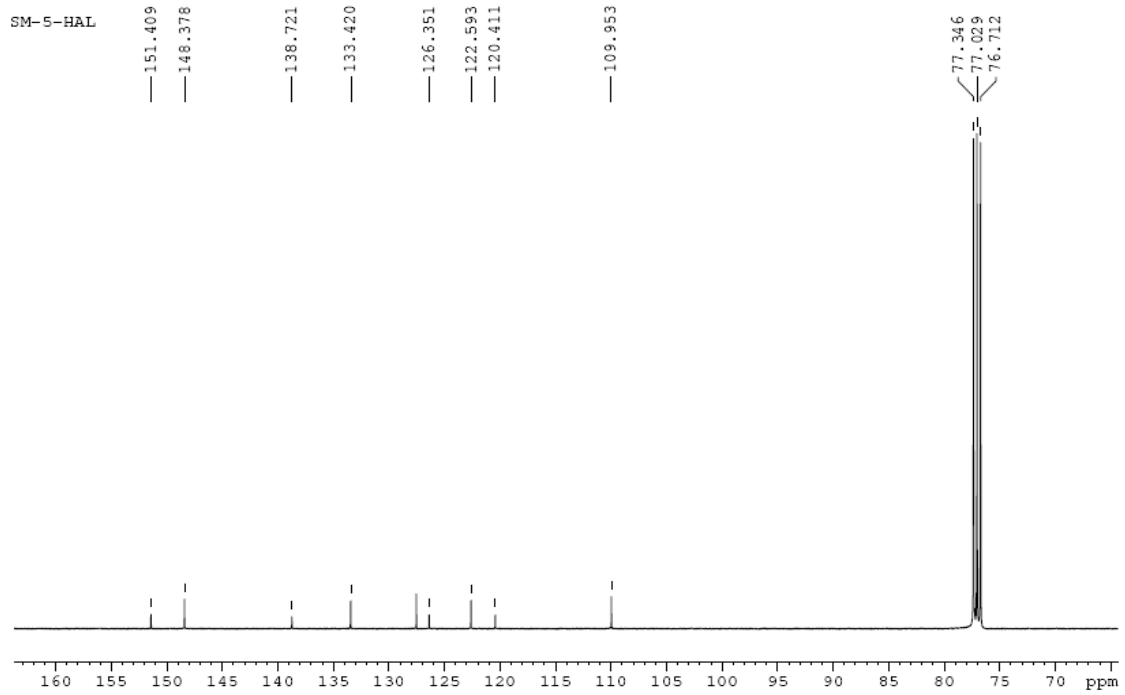
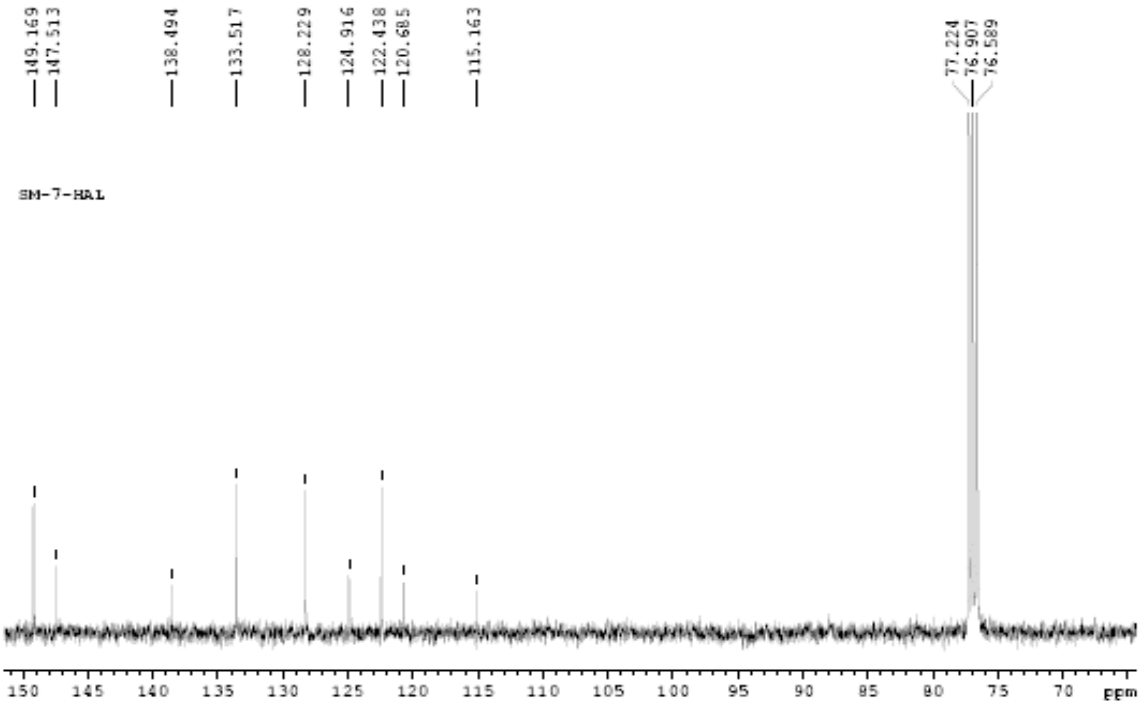
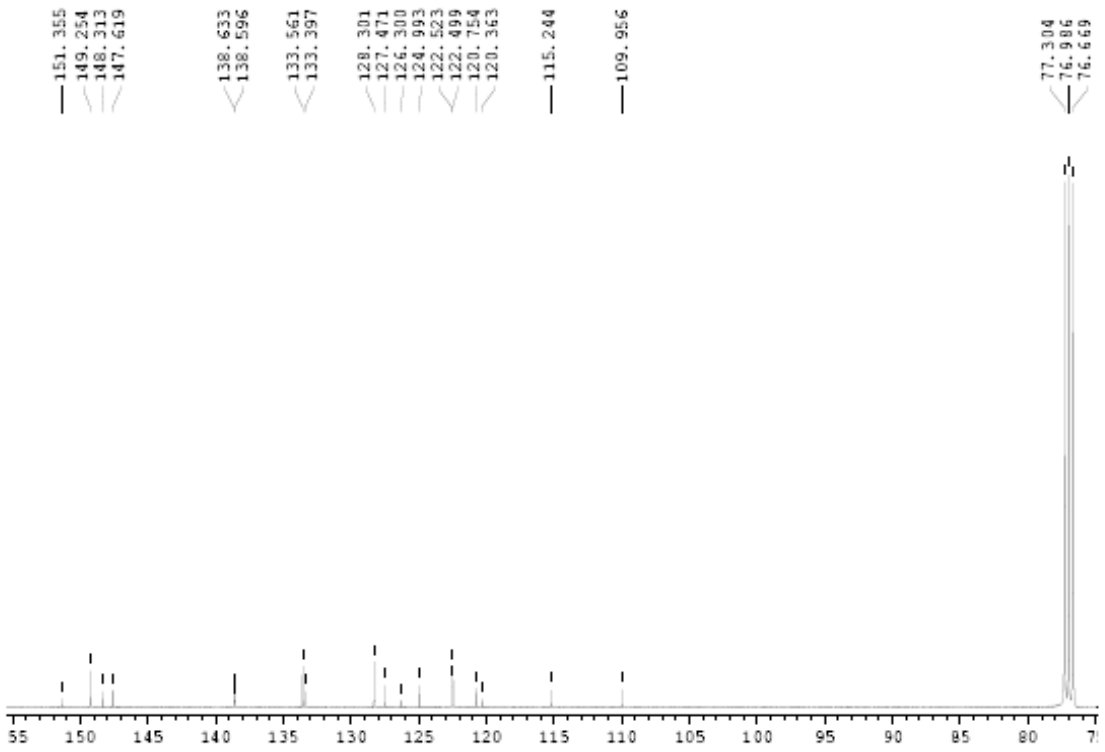


Fig. 18: ^{13}C-NMR Spectrum of 5,7-dichloro-8-quinolinol



X axis =Chemical Shift

Fig. 19: ^{13}C-NMR Spectrum of halquinol [Mix of 5-chloro-8-quinolinol: 5,7-dichloro-8-quinolinol (1:2.3)]



Chemical shift

4.3.1 ^{1}H-NMR of 5,7-dichloro-8-quinolinol (δ).7.24 (for $CDCl_3$); 7.54-7.56 (2H; 1H next to C-Cl and 1H second next N); 8.47-8.49 (1H; H second next to C-Cl); 8.83-8.84 (1H; H next to N). NMR data revealed the satisfactory purity of the compound 5,7-dichloro-8-quinolinol Fig.15 (Spectra 15).

4.3.2 ^{1}H-NMR of halquinol (mixture of 5,7-dichloro-8-quinolinol and 5-chloro-8-quinolinol)(δ).7.08-7.101(H next to C-OH of 5-chloro-8-quinolinol); 7.24 (for $CDCl_3$); 7.49-7.53 (H next to C-Cl and H second next to N of 5-chloro-8-quinolinol; H next to C-Cl and H second next to N of 5,7-dichloro-8-quinolinol); 8.49 (H second next to C-Cl of 5,7-dichloro-8-quinolinol and 5-chloro-8-quinolinol); 8.83-8.84 (H next to N of 5,7-dichloro-8-quinolinol and 5-chloro-8-quinolinol). The analysis of H numbers contributed in NMR spectra of Halquinol revealed that it has the 1:2.3 molar mixture of 5-chloro-8-quinolinol and 5,7-dichloro-8-quinolinol Fig.16 (Spectra16).

4.3.3 ^{13}C-NMR of 5-chloro-8-quinolinol (δ).109.95, 120.41, 122.59, 126.35, 127.83, 133.42, 138.72, 148.37, 151.40 (9 C resonance frequencies supports the presence of only 5-chloro-8-quinolinol in the sample with 9 different chemical environments for C). 76.71, 77.02 and 77.34 are originated due to $CDCl_3$ Fig.17 (Spectra17).

4.3.4 ^{13}C-NMR of 5,7-dichloro-8-quinolinol (δ).115.16, 120.68, 122.43, 124.91, 128.22, 133.51, 138.49, 147.51, 149.16 (9 C resonance frequencies supports the presence of only 5,7-dichloro-8-quinolinol in the sample with 9 different chemical environments for C).76.58, 76.90 and 77.22 are originated due to $CDCl_3$ Fig.18 (Spectra18).

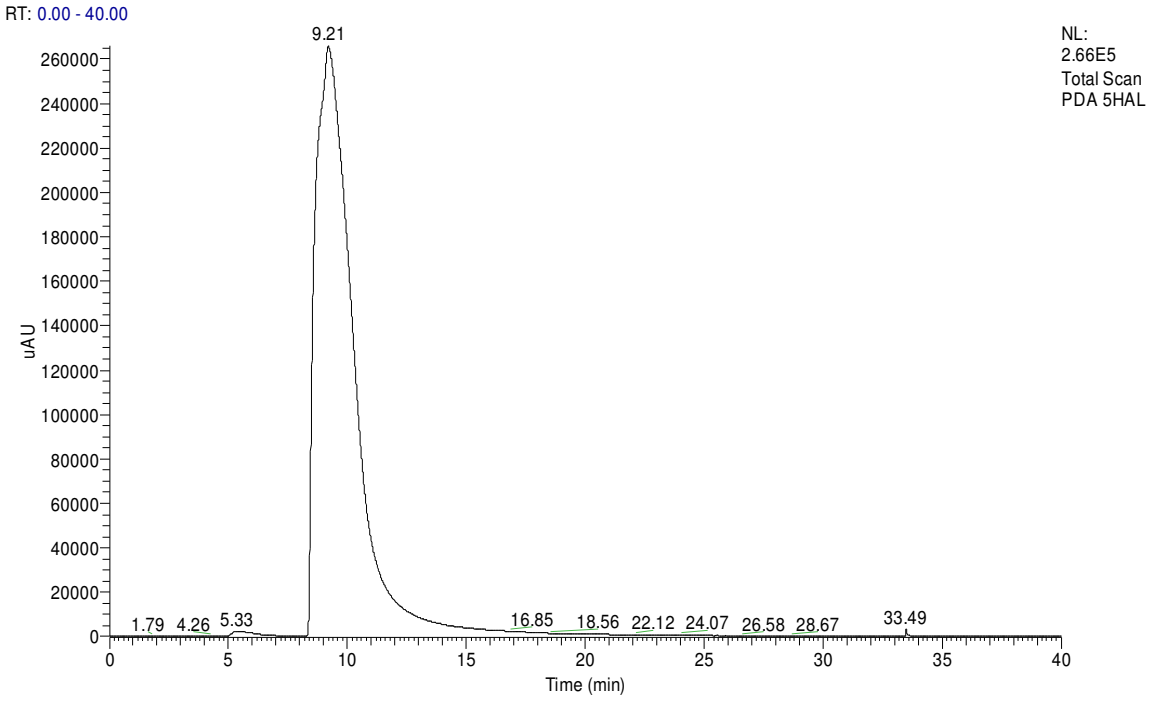
4.3.5 ^{13}C-NMR of halquinol (mixture of 5,7-dichloro-8-quinolinol and 5-chloro-8-quinolinol)(δ).109.95, 115.24,120.36, 120.75, 122.49, 122.52, 124.99, 126.30, 127.47, 128.30, 133.39, 133.56, 138.59, 138.63, 147.61, 148.31, 149.25, 151.35 (18 C resonance frequencies supports the presence of 5,7-dichloro-8-quinolinolwith 9 different chemical environments for C and 5-chloro-8-quinolinolin the sample with 9 other different chemical environments for C). 76.66, 76.98 and 77.30 are originated due to $CDCl_3$ Fig.19 (Spectra19).

4.4 LCMS

The liquid chromatography mass spectroscopy (LCMS) assay system standardized for 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol was used to quantify the constituent molecules of halquinol and its metabolites in tissue samples in the present study. 5,7-dichloroquinolin-8-ol or 5-chloroquinolin-8-ol, were absent in tissue samples (*viz:*liver and breast muscle) of control group (C) collected at each sacrificing intervals in birds throughout the experimental period, upon LCMS analysis. Thus, the chances of any contamination of halquinol by feed or water was eliminated. NMR and IR revealed the purity and molecular characterization of halquinol and its metabolites respectively. The metabolites were conjugated forms of quinoline compounds *viz*., 5-chloro-quinolin-8-ol sulfate,5-chloro-quinolin-8-ol glucuronide, 5,7, dichloro-quinolin-8-ol sulfate and 5,7, dichloro-quinolin-8-ol glucuronide. The LCMS graphs of the various tissue samples of all the test groups are depicted in Fig 20 to 37.

Fig. 20: MS graph of 5HAL standard

5HAL:



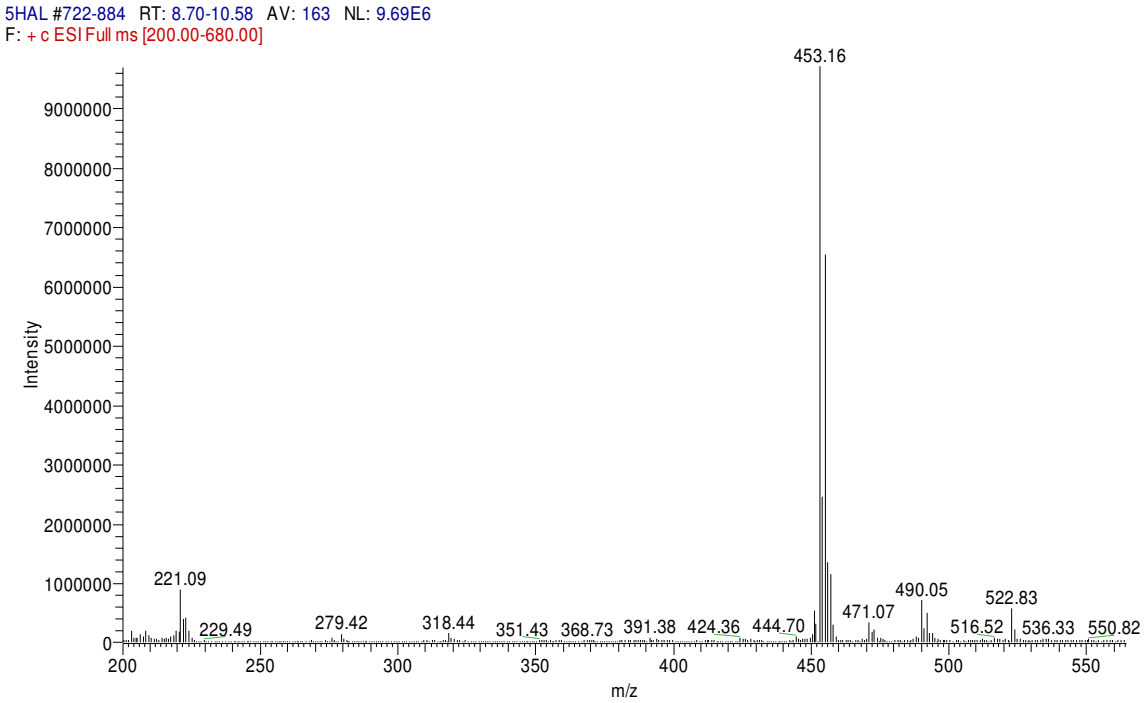


Fig. 21: MS graph of 7HAL Standard

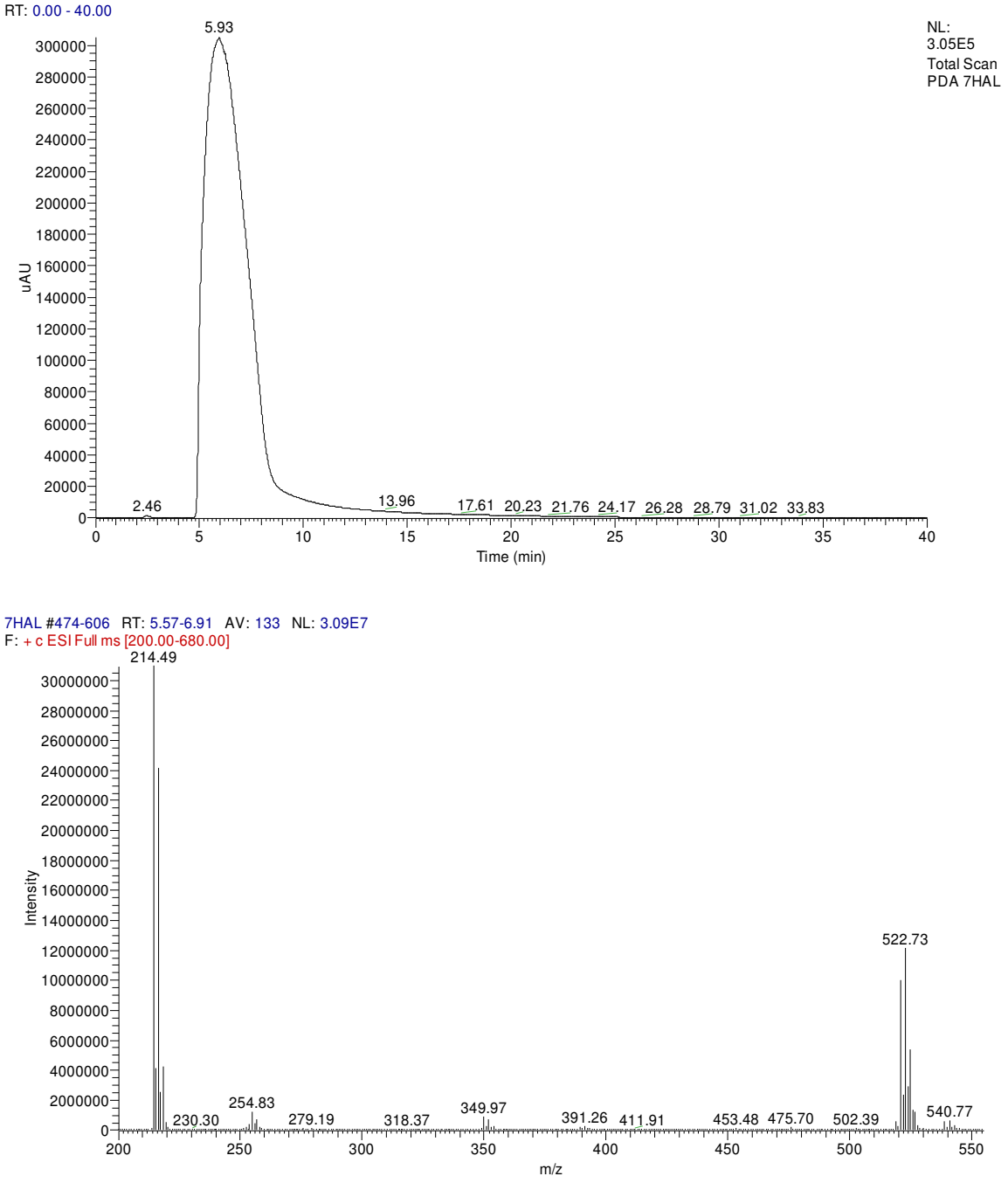


Fig. 22: MS graph of chicken liver tissue samples (T_1)

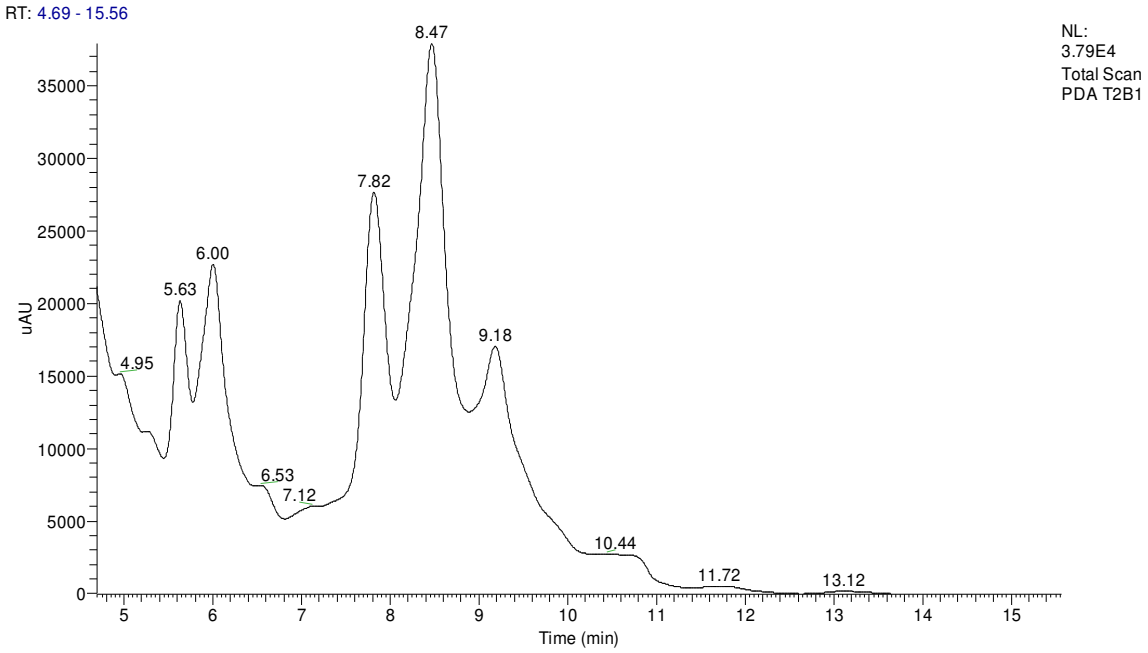


Fig.23: MS graph of chicken breast tissue samples (T_2)

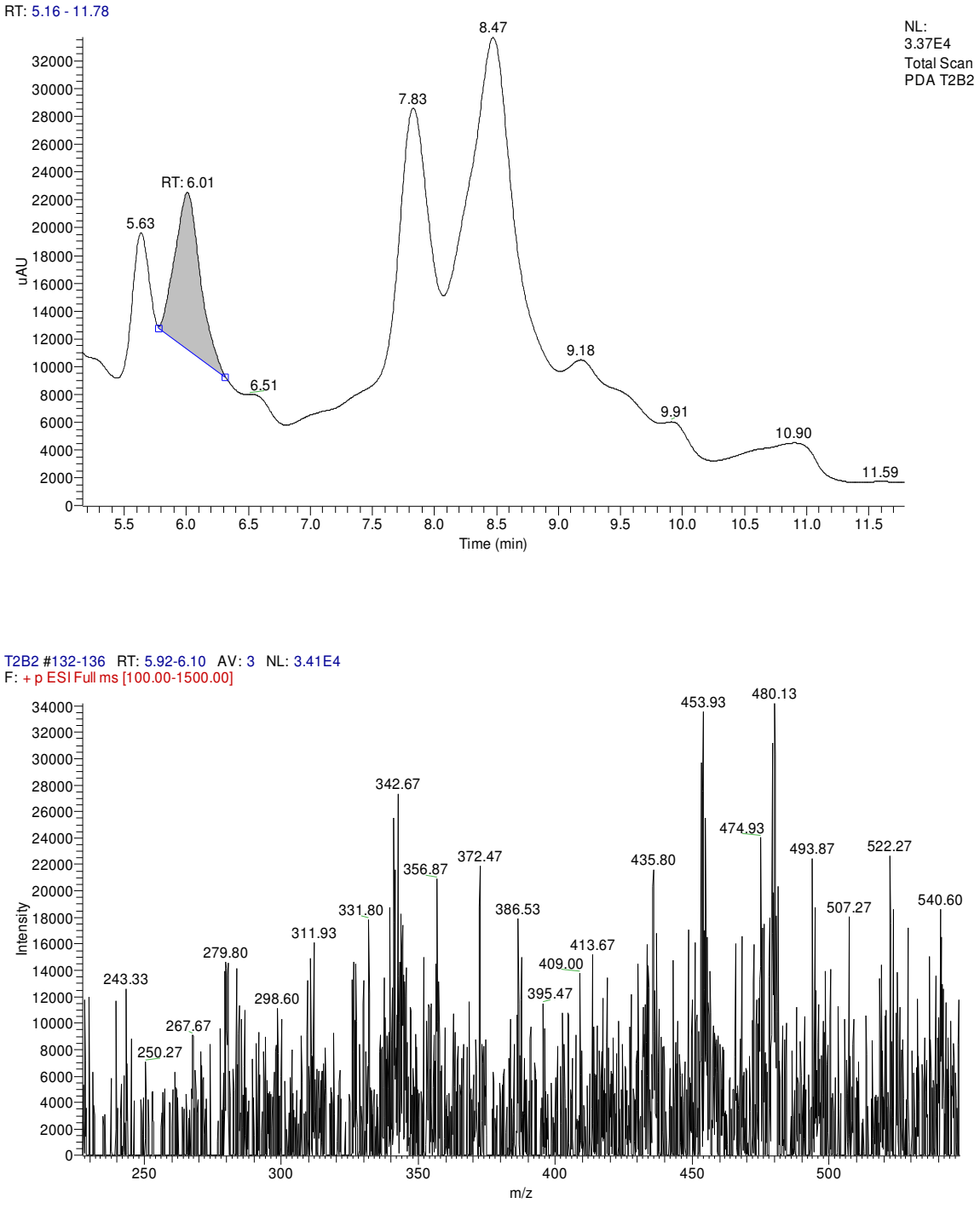


Fig. 24: MS graph of chicken liver tissue samples (T_3)

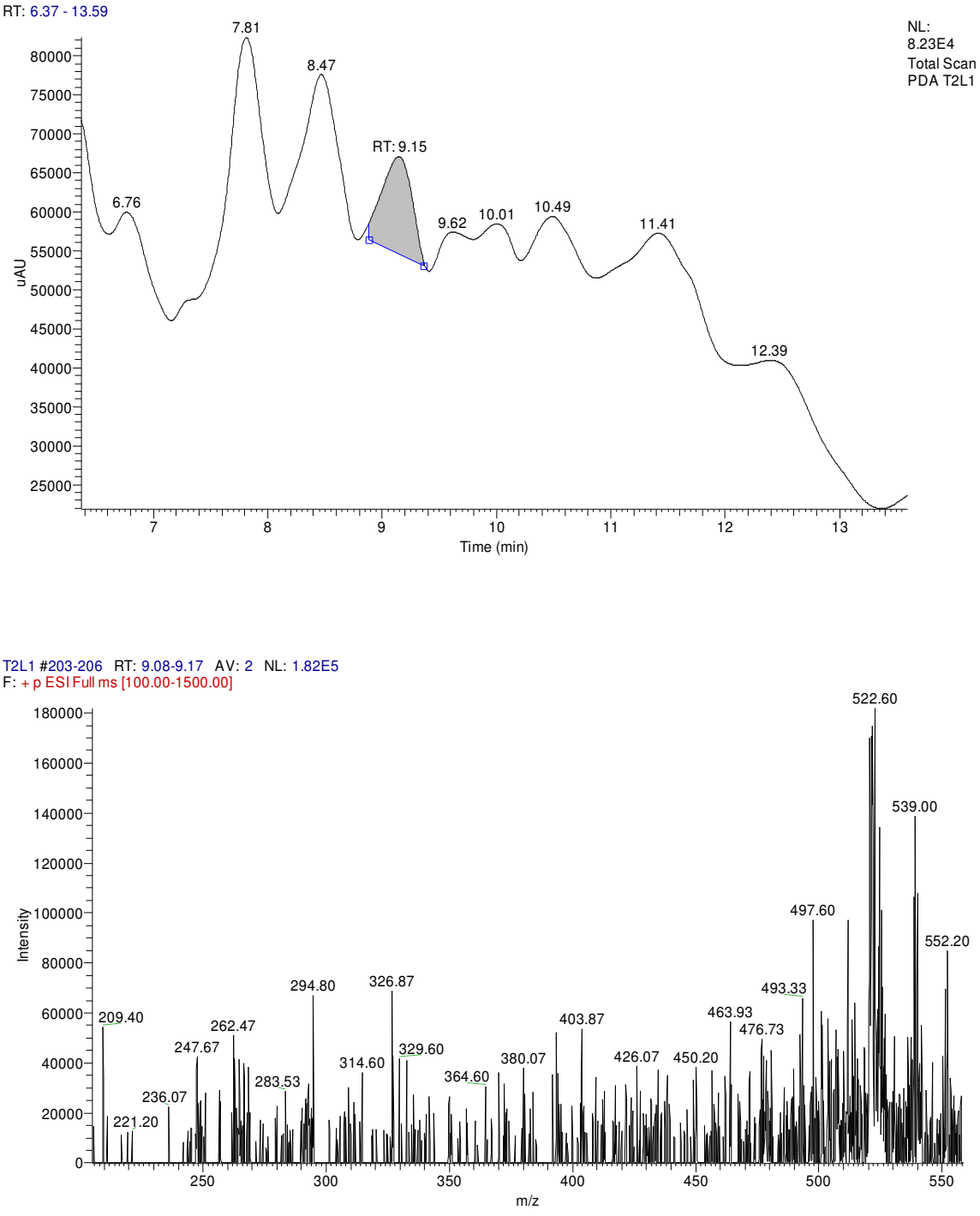


Fig.25: MS graph of chicken breast tissue samples (T_3)

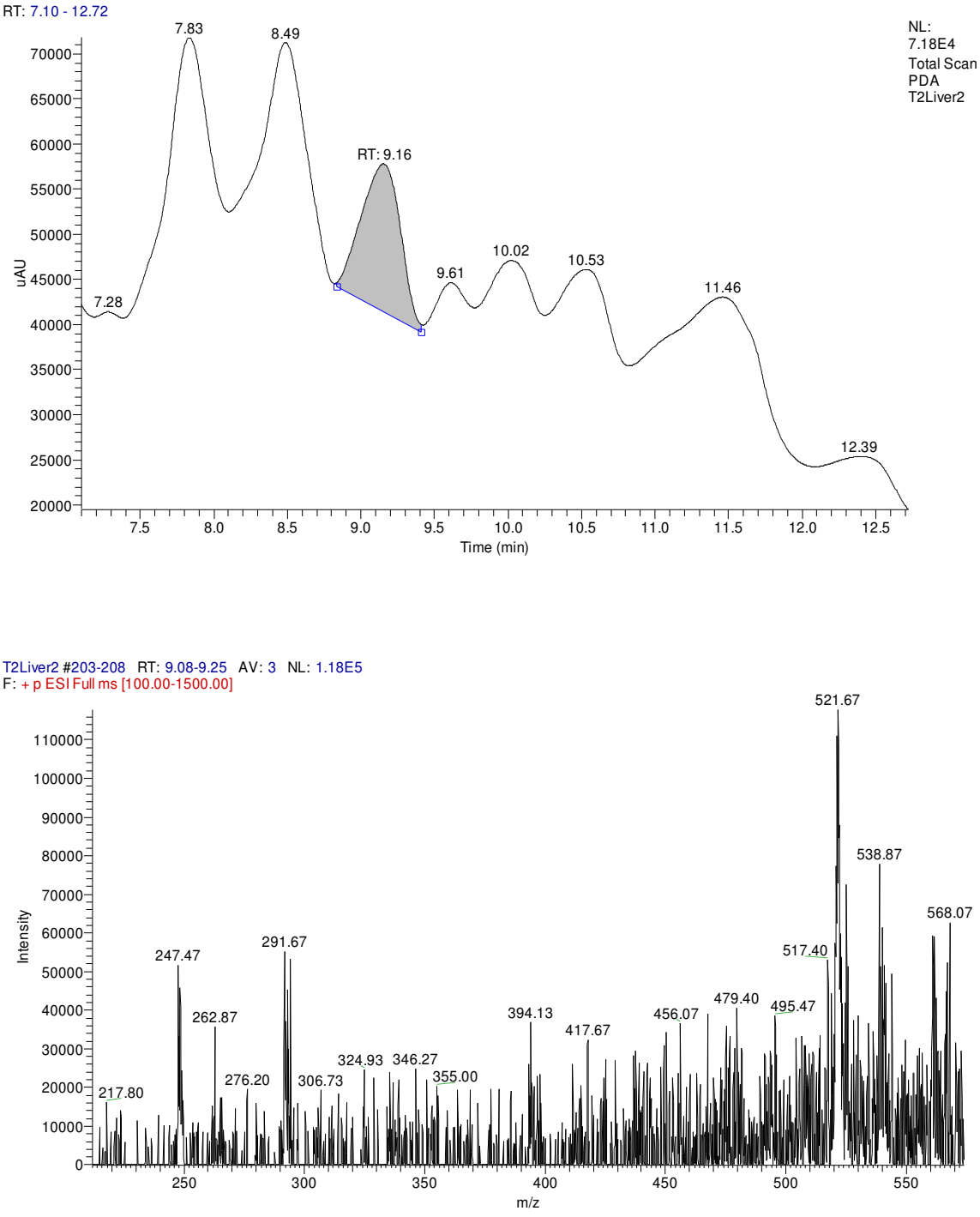


Fig. 26: MS graph of chicken liver tissue samples (T_1)

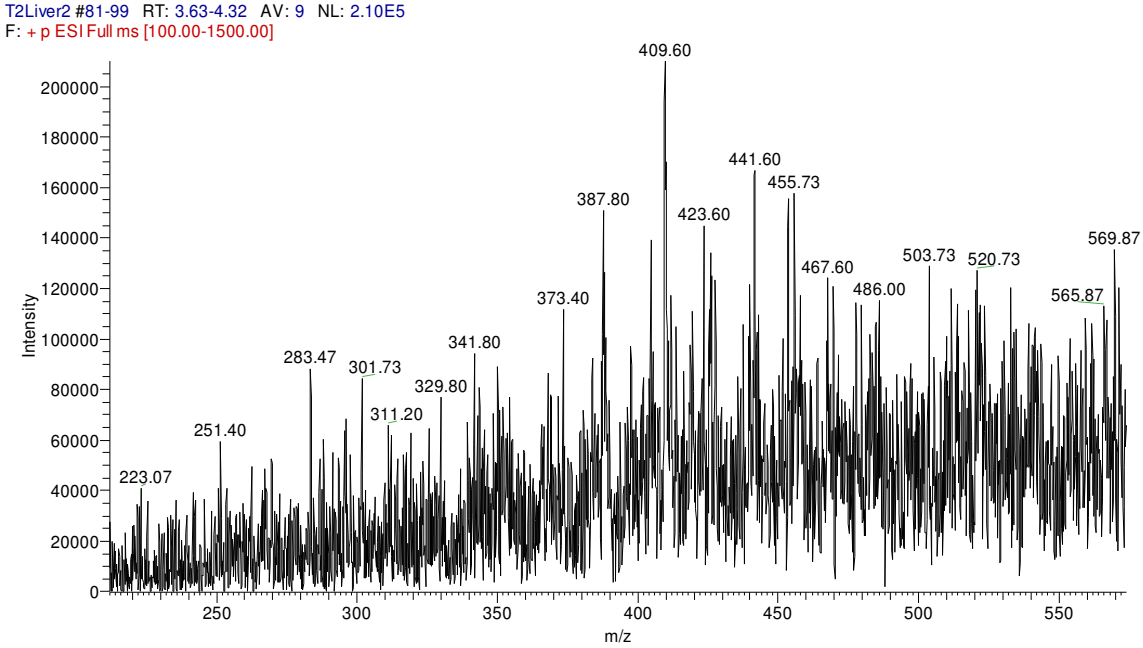


Fig. 27: MS graph of chicken breast tissue samples (T_2)

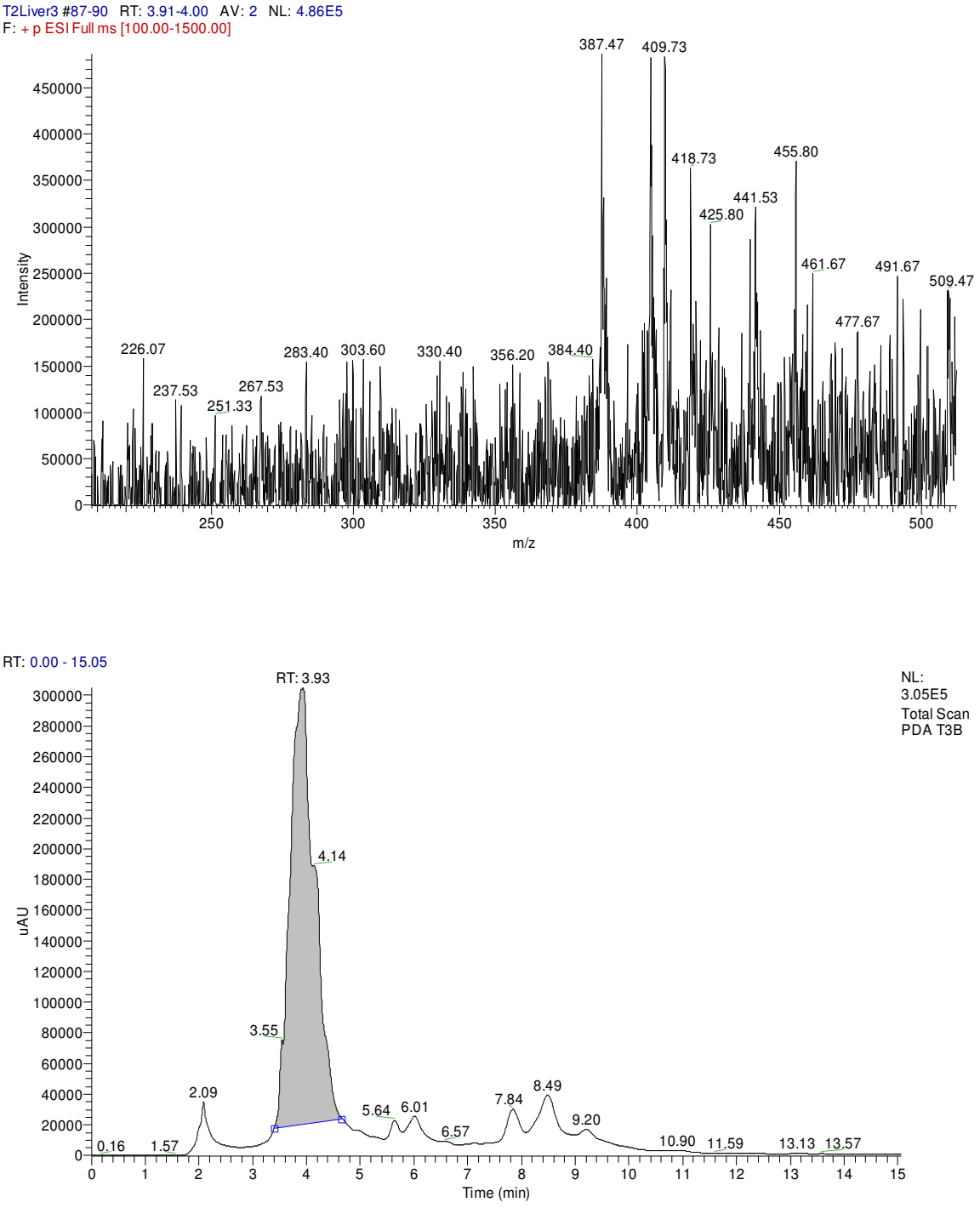


Fig. 28: MS graph of chicken liver samples (T_3)

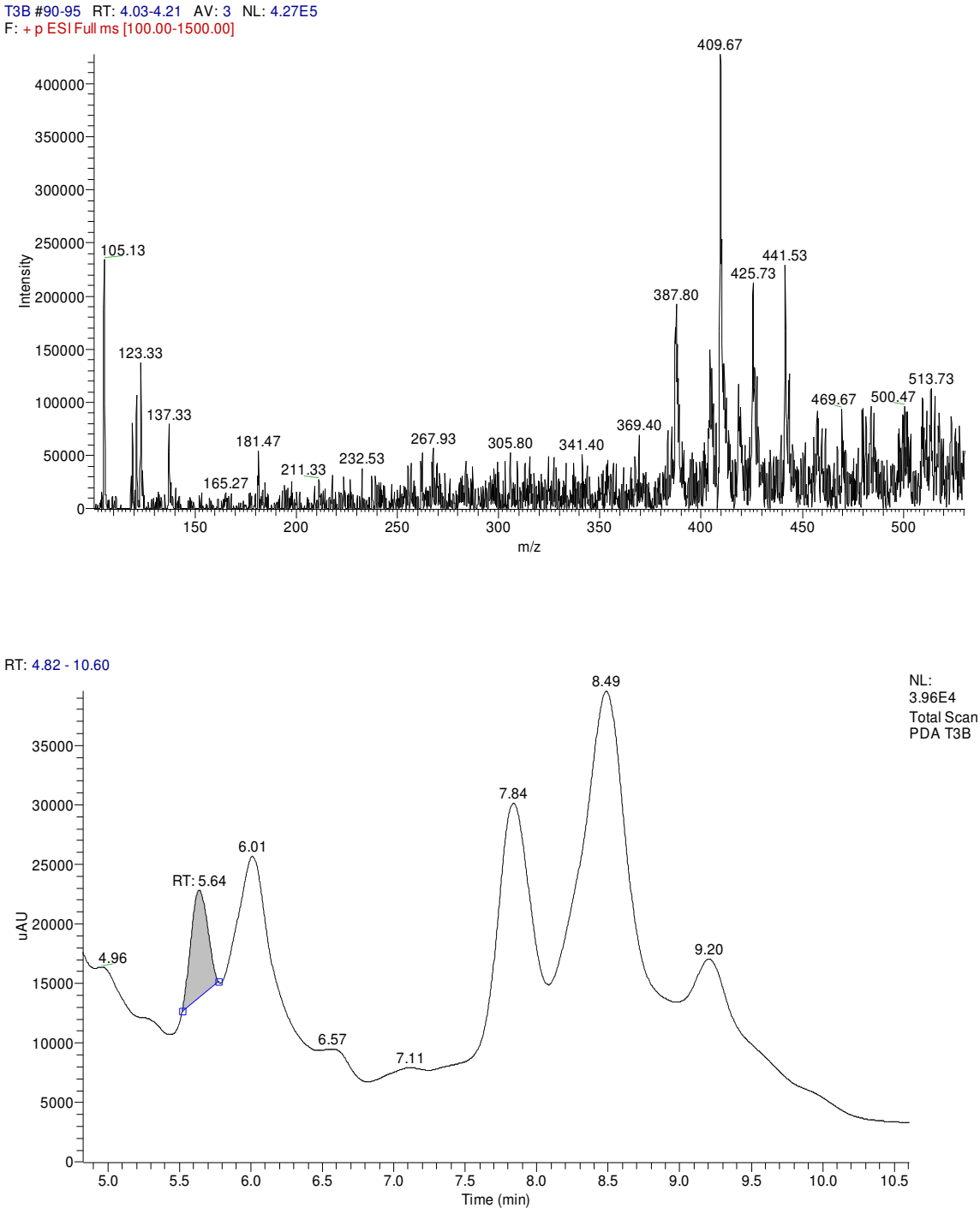


Fig. 29: MS graph of chicken breast tissue samples (T_1)

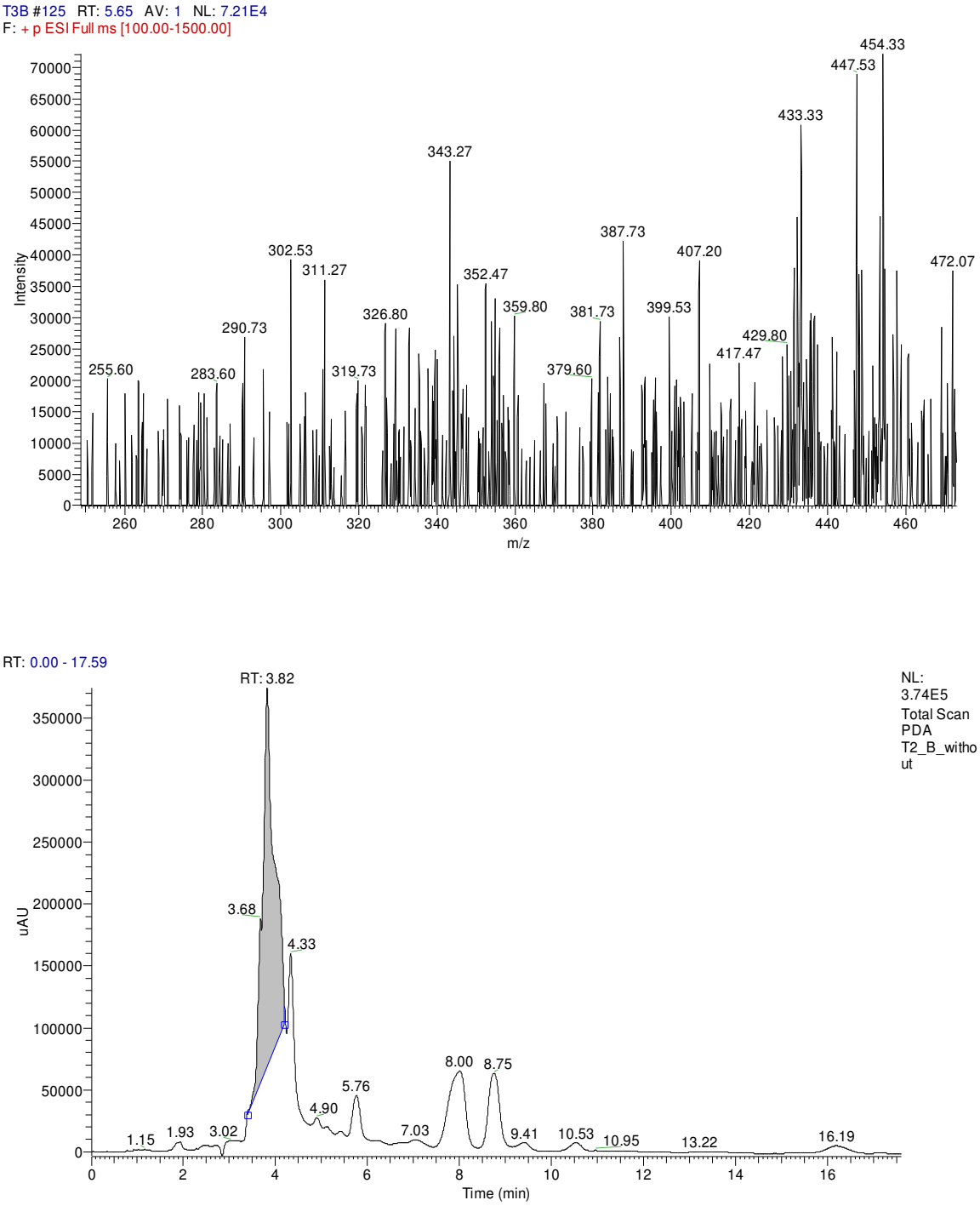


Fig. 30: MS graph of chicken liver tissue samples (T_1)

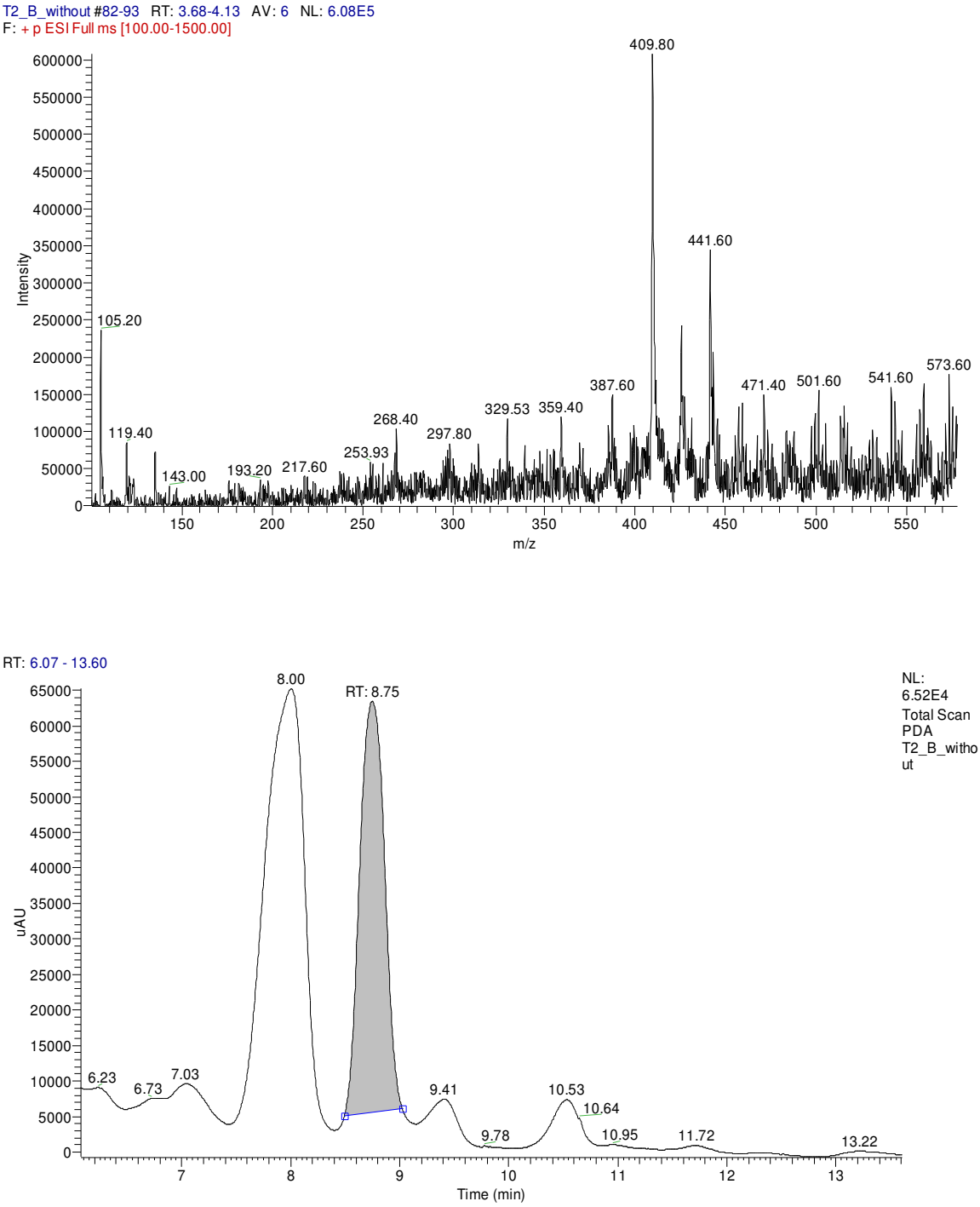


Fig. 31: MS graph of chicken breast tissue samples (T_2)

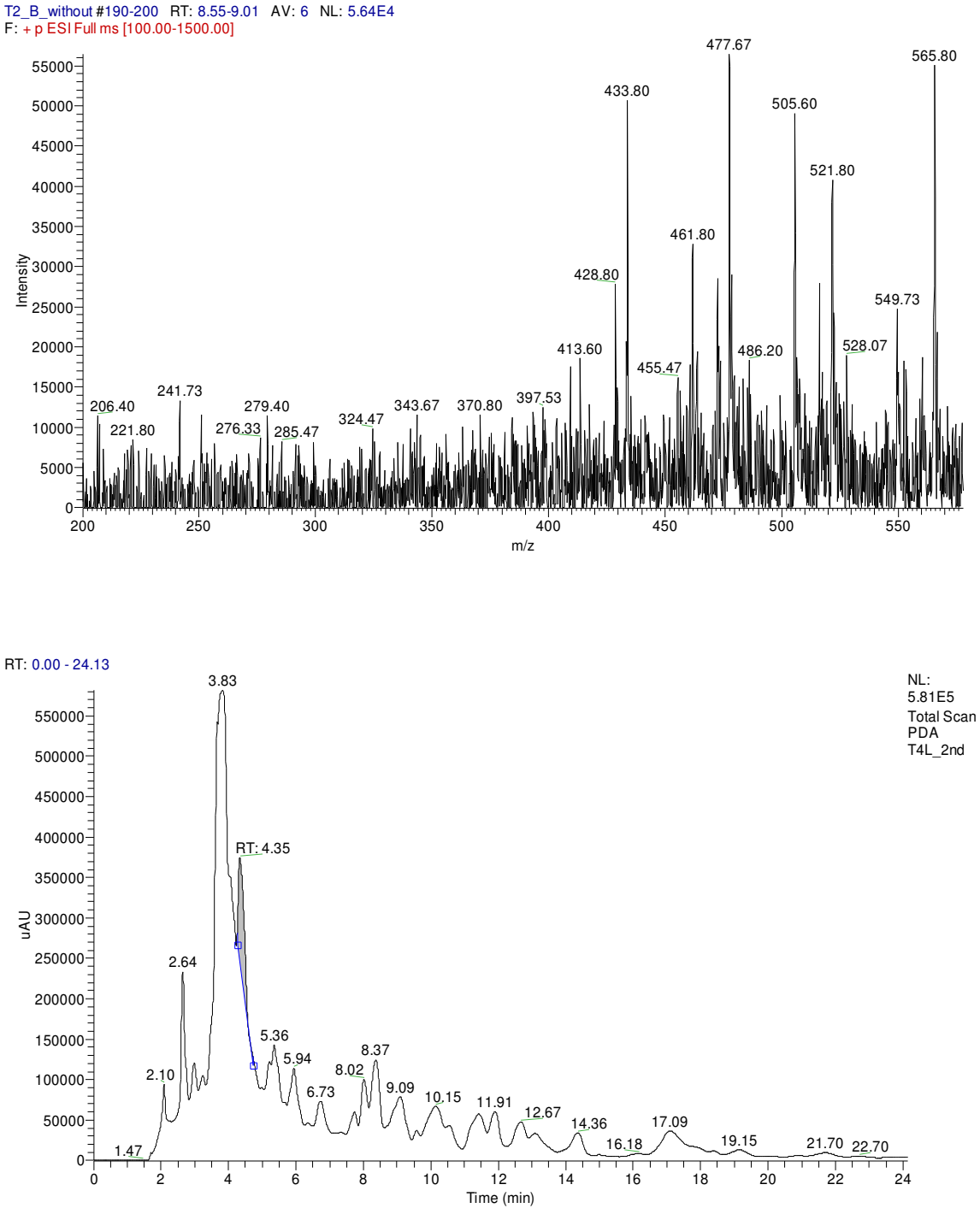


Fig. 32: MS graph of chicken breast tissue samples (T_2)

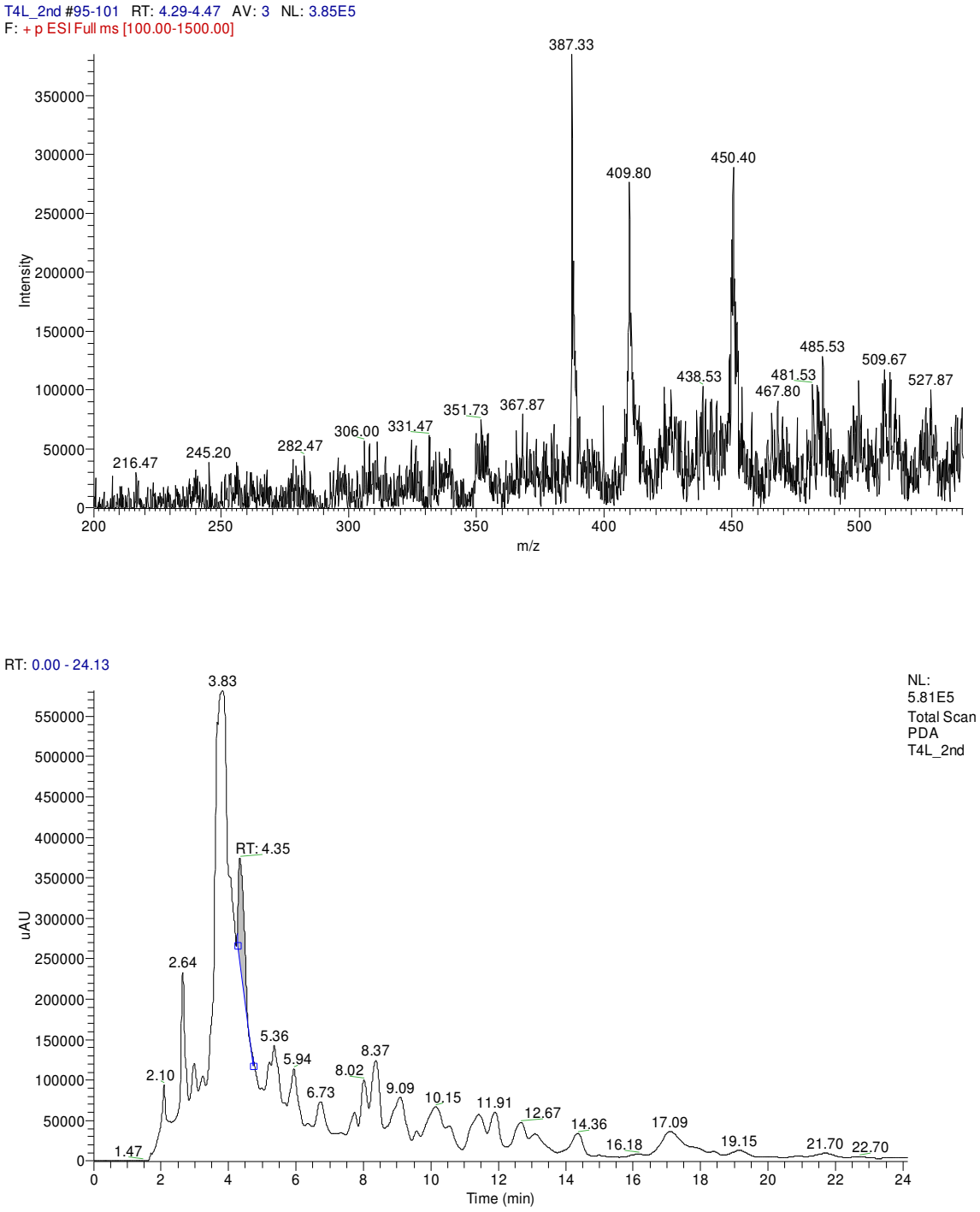


Fig. 33: MS graph of chicken liver tissue samples (T_3)

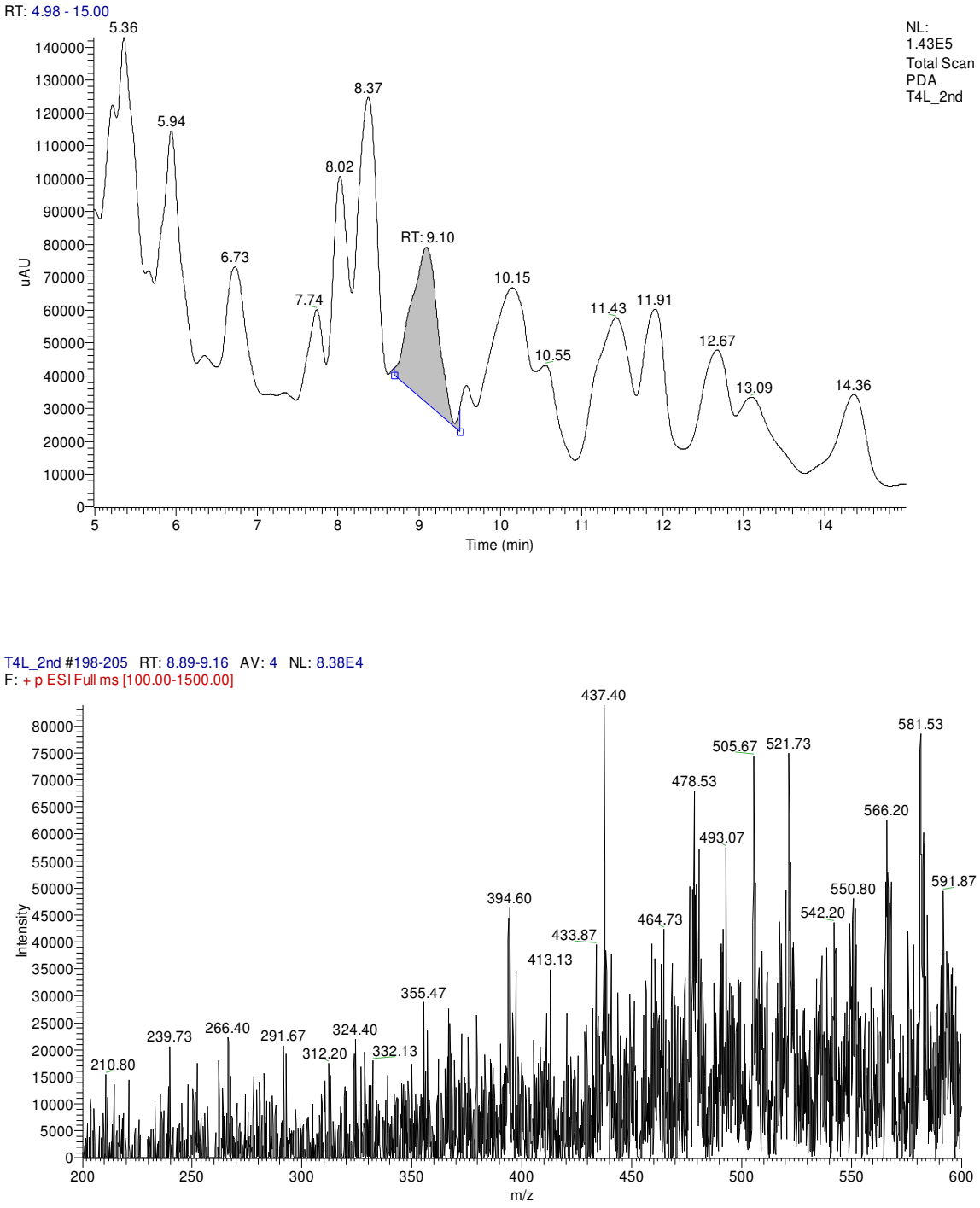


Fig. 34: MS graph of chicken liver tissue samples (T_3)

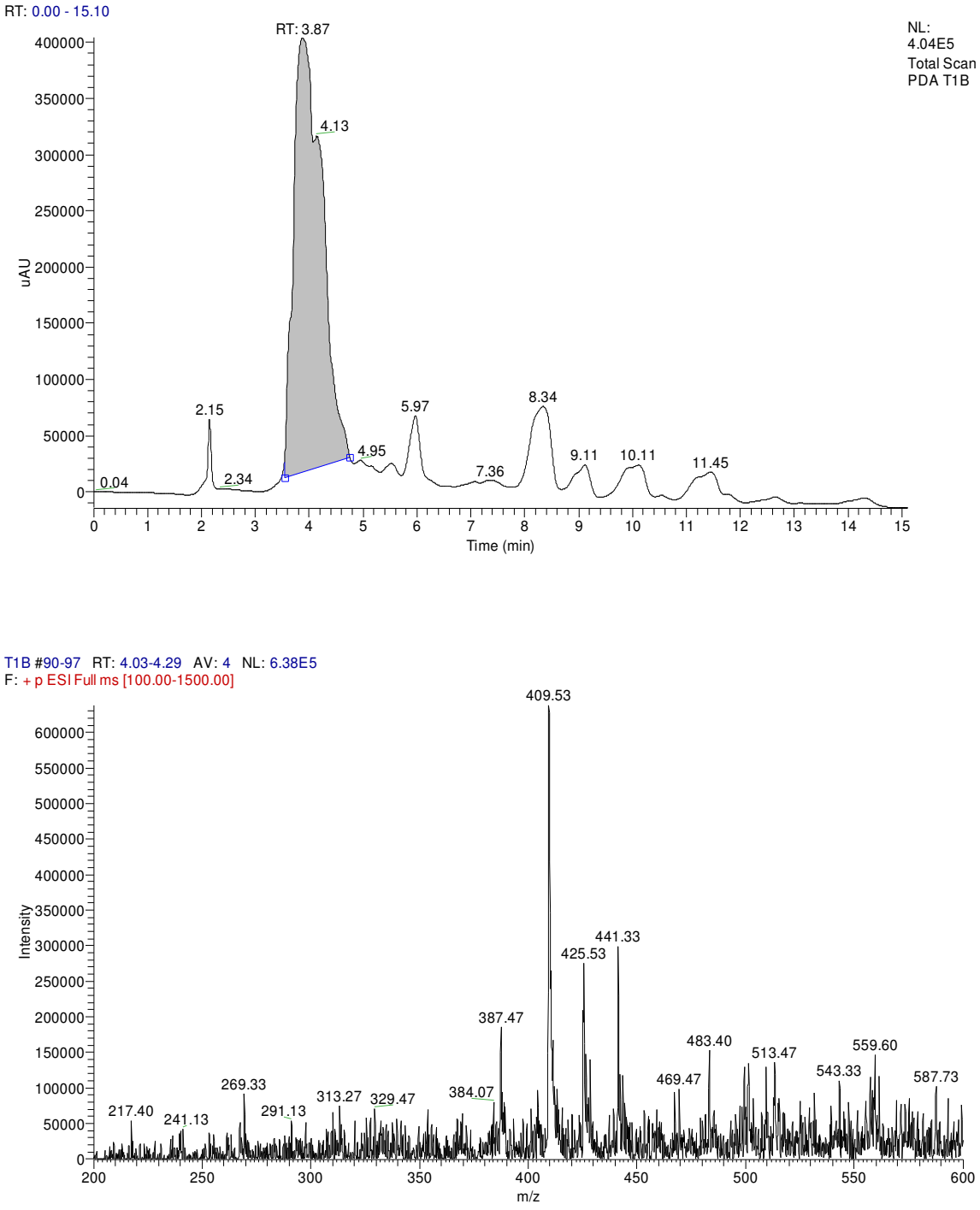


Fig. 35: MS graph of chicken breast tissue samples (T_1)

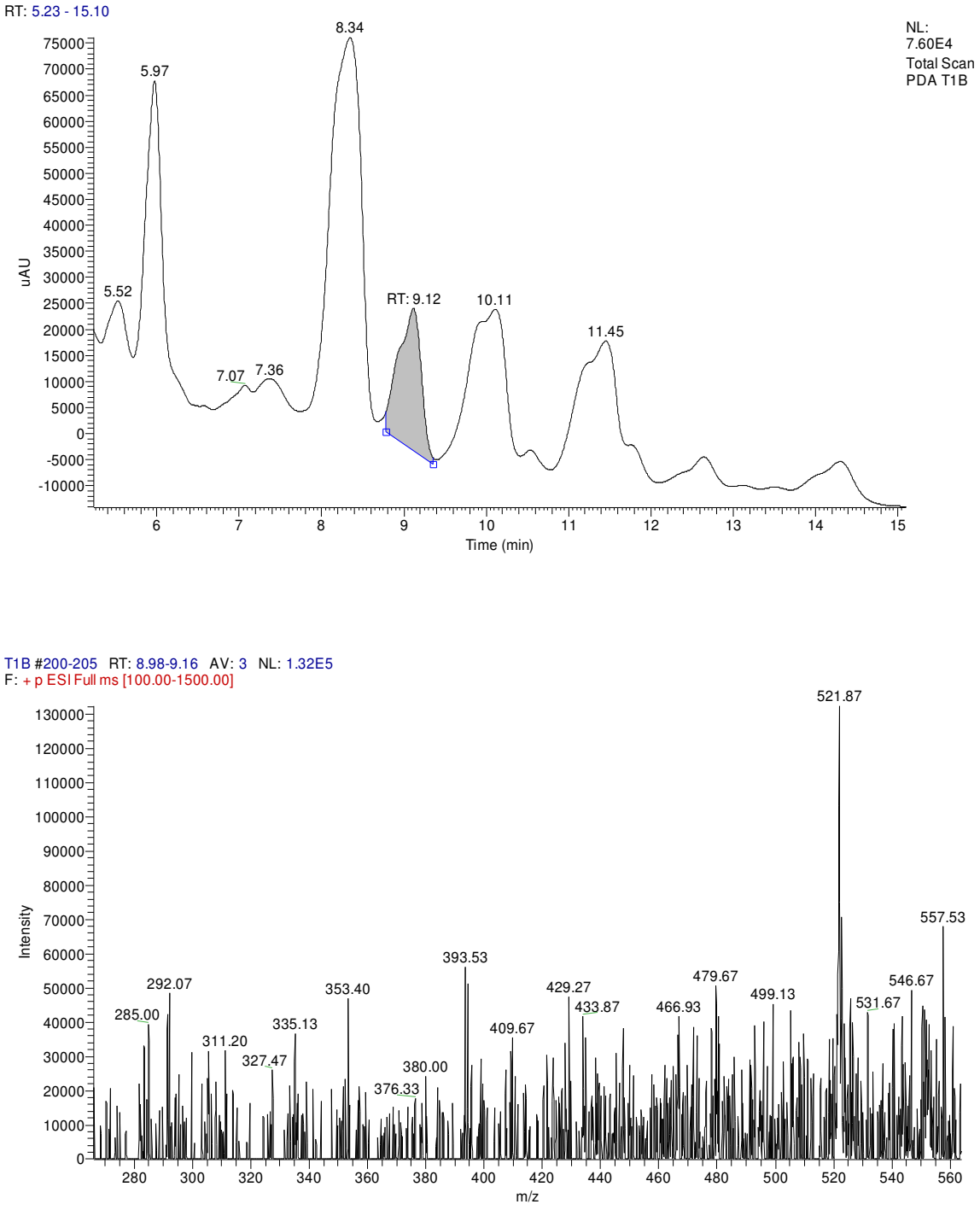


Fig. 36: MS graph of chicken liver tissue samples (T_1)

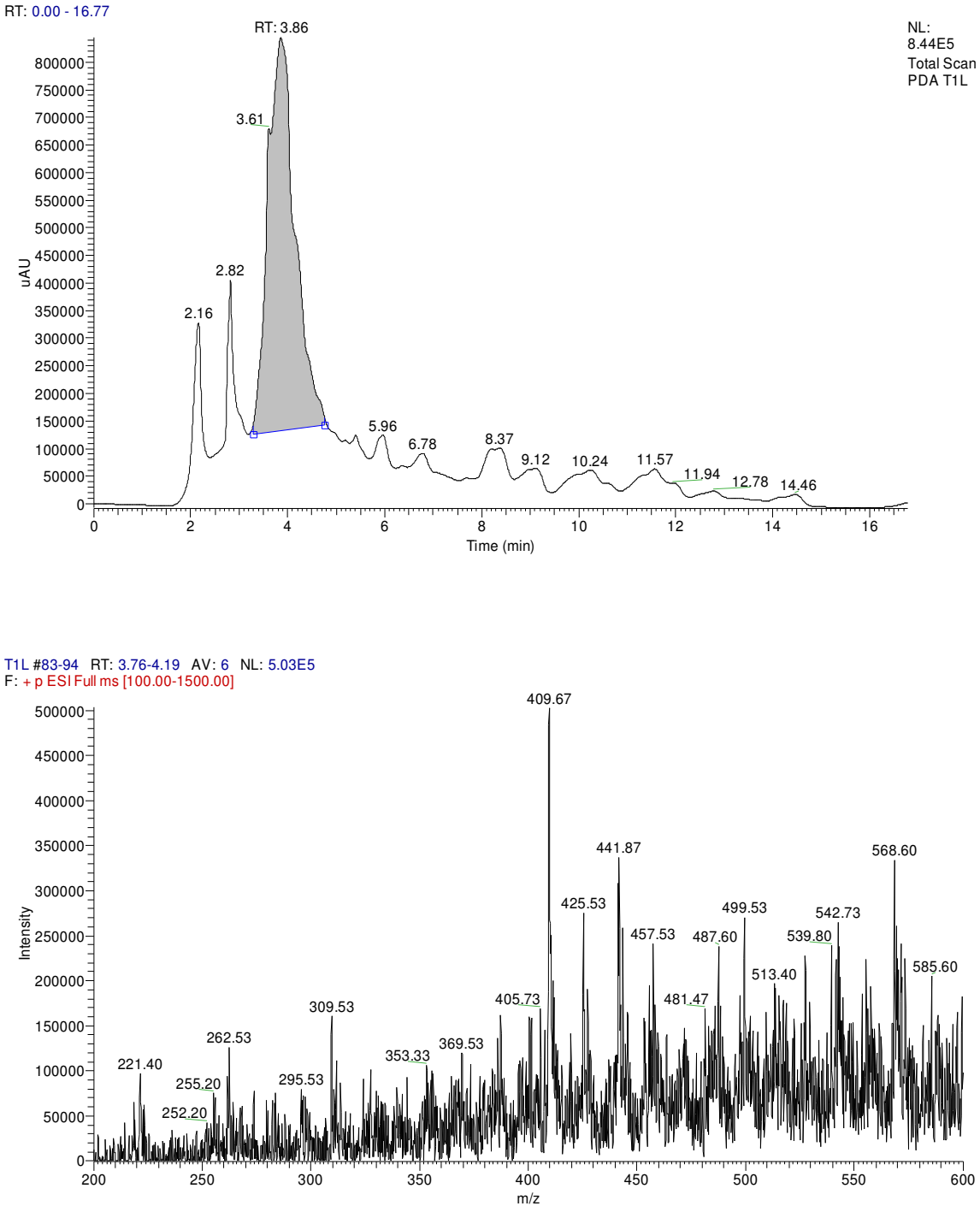
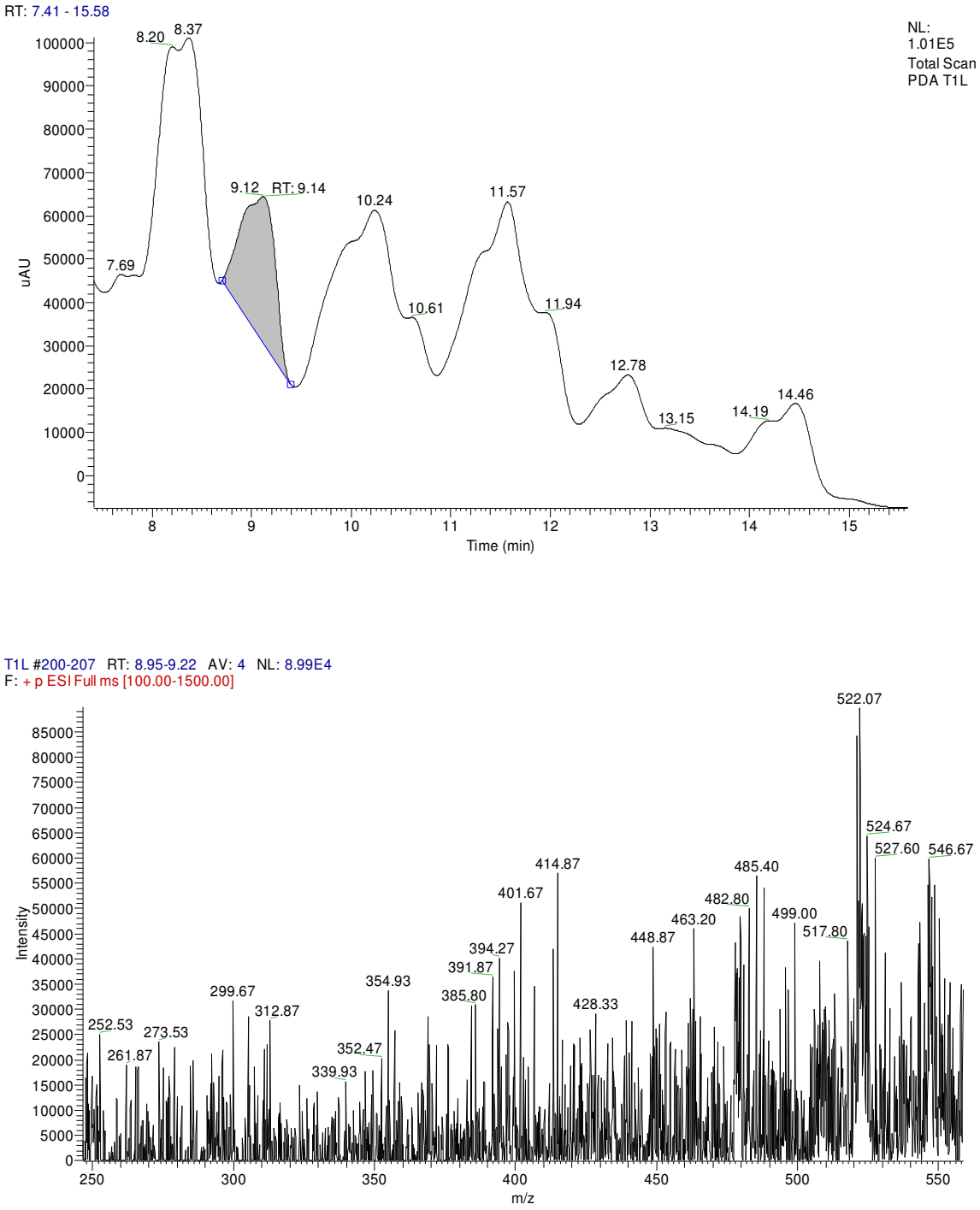


Fig. 37: MS graph of chicken breast tissue samples (T_1)



4.5 Tissue concentration of halquinol and its conjugated metabolites

The concentration of halquinol (5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol) and its conjugated metabolites in various edible tissue samples were in minimal quantities. The concentration of 5,7-dichloroquinolin-8-ol as assayed by LCMS in tissues belonging to various treatment groups are tabulated in Table 12 and 13 respectively.

4.5.1 Tissue distribution of 5,7-dichloroquinolin-8-ol following dietary inclusion of halquinol at 60 ppm as assayed by LCMS

Liver

The concentration of 5,7-dichloroquinolin-8-ol following dietary inclusion of halquinol @ 60 ppm (T_1 group), in liver tissue at different time intervals during 40 days is depicted in Table 12. In LCMS graph the increase in area under curve with successive sacrifice indicated the cumulative behavior of halquinol. The concentration of 5,7-dichloroquinolin-8-ol at 5^{th} day and on 40^{th} day of exposure were 26.09±0.51 $ng.g^{-1}$ tissue and 188.8±3.83 $ng.g^{-1}$ tissue respectively. The increase in concentration of 5,7-dichloroquinolin-8-ol between each successive sampling days of 5, 10, 20, 30 and 40 days during treatment were statistically significant ($p \leq 0.05$) reflecting cumulative behavior of 5,7-dichloroquinolin-8-ol following continuous dietary exposure to halquinol @ 60 ppm in experimental birds (Table 12).

Kidney

The concentration of 5,7-dichloroquinolin-8-ol in kidney tissues at different time intervals during 40 days dietary exposure of halquinol is depicted in Table 12. The

concentration of 5,7-dichloroquinolin-8-ol at 5th day and on 40th day of treatment were 38.11±1.49 ng.g^{-1} tissue and 233.5±5.874 ng.g^{-1} tissue respectively. The increase in concentration of 5,7-dichloroquinolin-8-ol between each successive sampling days of 5, 10, 20, 30 and 40 days during treatment period of halquinol exposure through diet were statistically significant ($p \leq 0.05$) , following continuous dietary exposure to halquinol @ 60 ppm in experimental birds (Table 12).

Muscle

The constituent molecule of halquinol in muscle tissue *viz*: 5,7-dichloroquinolin-8-ol was not detectable at all-time intervals of 5, 10, 20, 30 and 40 days during treatment period, following dietary inclusion of halquinol @ 60 ppm in experimental birds indicating that concentration of 5,7-dichloroquinolin-8-ol was less than 20 ng.g^{-1} at all sacrificing intervals. The LCMS assay did not spot the presence of 5,7-dichloroquinolin-8-ol in muscle tissue throughout the study period .

4.5.2 Tissue distribution of 5,7-dichloroquinolin-8-ol following dietary inclusion of halquinol at 120 ppm

Liver

The concentration of 5,7-dichloroquinolin-8-ol in liver tissues at different time intervals during 40 days dietary exposure of halquinol @ 120 ppm (T_2 group) is depicted in Table 12. In this treatment group, the concentration of 5,7-dichloroquinolin-8-ol at 5th day and on 40th day of treatment were 40.7±0.62 ng.g^{-1} and 411.0±4.22 ng.g^{-1} tissue respectively and the increase in concentration of 5,7-dichloroquinolin-8-ol between each successive sampling days of 5, 10, 20, 30 and 40 days during treatment period (Table 12)

were statistically significant ($p \leq 0.05$) following dietary halquinol exposure @ 120 ppm. The concentration of 5,7-dichloroquinolin-8-ol on all sampling days of 5, 10, 20, 30 and 40 days during 40 days dietary exposure of halquinol at 120 ppm was significantly greater ($p \leq 0.05$) than that compared to corresponding concentrations of 5,7-dichloroquinolin-8-ol on corresponding sampling days at 60 ppm inclusion level (Table 12).

Kidney

The concentration of 5,7-dichloroquinolin-8-ol in kidney following dietary exposure of halquinol at different time intervals is depicted in Table 12. The concentration of 5,7-dichloroquinolin-8-ol at 5^{th} day and on 40^{th} day during treatment were 55.07±0.72 $ng.g^{-1}$ and 364.5±6.05 $ng.g^{-1}$ tissue respectively. There was statistically significant increase ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol between each successive sampling days of 5, 10, 20, 30 and 40 days during treatment which was after halquinol exposure @ 120 ppm for 40 days. The concentration of 5,7-dichloroquinolin-8-ol on all sampling days of 5, 10, 20, 30 and 40 days during treatment period of 40 days dietary exposure of halquinol at 120 ppm is significantly greater ($p \leq 0.05$) when compared to corresponding concentrations of 5,7-dichloroquinolin-8-ol in kidney tissue on corresponding sampling days of 60 ppm inclusion level (Table 12).

Muscle

The LCMS assay could not detect the major constituent of halquinol *viz:* 5,7-dichloroquinolin-8-ol in breast muscle tissue even at 120 ppm inclusion level indicating that the concentration of 5,7-dichloroquinolin-8-ol was less than 20 $ng.g^{-1}$ at all time

intervals during which birds were sacrificed during treatment period of halquinol exposure.

4.5.3 Tissue distribution of 5,7-dichloroquinolin-8-ol following dietary inclusion of halquinol at 240 ppm

Liver

The concentration of 5,7-dichloroquinolin-8-ol in liver tissue at different time intervals during 40 days dietary exposure of halquinol @ 240 ppm (T_3 group) is depicted in Table 12.The concentration of 5,7-dichloroquinolin-8-ol at 5^{th} day and on 40^{th} day during treatment were 64.20±2.78 $ng.g^{-1}$ and 543.7±6.05 $ng.g^{-1}$ tissue respectively and statistically significant increase ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol between each successive sampling days of 5, 10, 20, 30 and 40 days during treatment period was observed. The concentrations of 5,7-dichloroquinolin-8-ol on all sampling days of 5, 10, 20, 30 and 40 days during treatment period of 40 days dietary exposure of halquinol at 240 ppm were significantly greater ($p \leq 0.05$) compared to corresponding concentrations of 5,7-dichloroquinolin-8-ol on corresponding sampling days of both 60 and 120 ppm inclusion levels (Table 12).

Kidney

The concentration of 5,7-dichloroquinolin-8-ol in kidney tissues at different time intervals during 40 days dietary exposure of halquinol is shown in Table 12. The concentration of 5,7-dichloroquinolin-8-ol at 5^{th} day and on 40^{th} day during treatment were 74.15±4.52 $ng.g^{-1}$ and 540.3±10.07 $ng.g^{-1}$ tissue respectively (Table 12). There was significant increase ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol between each

successive sampling days of 5, 10, 20, 30 and 40 days during treatment following continuous dietary exposure of halquinol at 240 ppm. The concentrations of 5,7-dichloroquinolin-8-ol on all sampling days of 5, 10, 20, 30 and 40 days during 40 days dietary exposure of halquinol at 240 ppm was significantly greater ($p \leq 0.05$) compared to corresponding concentrations of 5,7-dichloroquinolin-8-ol on corresponding sampling days of both 60 ppm and 120 ppm dose groups (Table 12).

Muscle

The concentration of 5,7-dichloroquinolin-8-ol in breast muscles at different time intervals during 40 days dietary exposure of halquinol is shown in Table 12, concentration of 5,7-dichloroquinolin-8-ol at 5^{th} day and on 40^{th} day during treatment were 35.13±2.25 $ng.g^{-1}$ and 170.4±5.42 $ng.g^{-1}$ tissue respectively and there was significant increase ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol between each successive sampling days of 5, 10, 20, 30 and 40 days during treatment in breast muscle tissue following dietary exposure @ 240 ppm (Table 12).

4.6 Tissue concentration of conjugated metabolites of halquinol following dietary inclusion of halquinol at 60 ppm

Liver

Liver is the main organ of metabolism of halquinol therefore the conjugated metabolites were detected by LCMS in liver tissue.The tissue concenteration of 5-chloro-quinolin-8-ol glucuronide in liver tissue is depicted in (Table 13). The concentration of 5-chloro-quinolin-8-ol glucuronide in liver tissue at different time intervals during 40 days dietary exposure of halquinol is shown in (Table 13) concentration of 5-chloro-quinolin-

8-ol glucuronide at 5th day and on 40th day during treatment were 25.13±1.25 ng.g^{-1} and 160.4±4.42 ng.g^{-1} tissue respectively and there is significant increase ($p \leq 0.05$) in concentration of 5-chloro-quinolin-8-ol glucuronide between each successive sampling days of 5, 10, 20, 30 and 40 days during treatment in breast muscle tissue following dietary exposure @ 60 ppm (Table 13).

Kidney

The LCMS detected the major metabolite in kidney in minimal levels as it is not the organ of metabolism for halquinol. The concentration of 5-chloro-quinolin-8-ol glucuronide in kidney at different time intervals during 40 days dietary exposure of halquinol is shown in Table 13. The concentration of 5-chloro-quinolin-8-ol glucuronide at 5th day and on 40th day during treatment were 24.12±1.25 ng.g^{-1} and 75.4±4.42 ng.g^{-1} tissue respectively and there is significant increase ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol between each successive sampling days of 5, 10, 20, 30 and 40 days during treatment in kidney tissue following dietary exposure @ 60 ppm (Table 13).

Muscle

The LCMS assay could not detect the major metabolite constituents of halquinol *viz:*5-chloro-quinolin-8-ol glucuronide in muscle at 60 ppm inclusion level indicating that the concentration of 5-chloro-quinolin-8-ol glucuronide was less than 10 ng.g^{-1} at all time intervals during which birds were sacrificed during treatment period of halquinol exposure.

4.6.1 Tissue concentration of conjugated metabolites of halquinol following dietary inclusion of halquinol at 120 ppm

Liver

Liver is the main organ of metabolism of halquinol therefore the conjugated metabolites were detected by LCMS in liver tissue.The tissue concenteration of 5-chloro-quinolin-8-ol glucuronide in liver tissue is depicted in Table 13.The concentration of 5-chloro-quinolin-8-ol glucuronide in liver tissue at different time intervals during 40 days dietary exposure of halquinol is shown in Table 13, concentration of 5-chloro-quinolin-8-ol glucuronide at 5^{th} day and on 40^{th} day during treatment were 45.12±2.15 $ng.g^{-1}$ and 190.4±2.12 $ng.g^{-1}$ tissue respectively and there is significant increase ($p \leq 0.05$) in concentration of 5-chloro-quinolin-8-ol glucuronide between each successive sampling days of 5, 10, 20, 30 and 40 days during treatment in liver tissue following dietary exposure @ 120 ppm (Table 13).

Kidney

The LCMS detected the major metabolite in kidney in minimal levels as it is not the organ of metabolism for halquinol. The concentration of 5-chloro-quinolin-8-ol glucuronide in kidney at different time intervals during 40 days dietary exposure of halquinol is shown in Table 13, concentration of 5-chloro-quinolin-8-ol glucuronide at 5^{th} day and on 40^{th} day during treatment were 34.12±1.35 $ng.g^{-1}$ and 95.4±5.42 $ng.g^{-1}$ tissue respectively and there is significant increase ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol between each successive sampling days of 5, 10, 20, 30 and 40 days during treatment in kidney following dietary exposure @ 120 ppm (Table 13)**.**

Muscle

The LCMS assay could not detect the major metabolite constituent of halquinol *viz:*5-chloro-quinolin-8-ol glucuronide in muscle at 120 ppm inclusion level indicating that the concentration of 5-chloro-quinolin-8-ol glucuronide was less than 10 $ng.g^{-1}$ at all time intervals during which birds were sacrificed during treatment period of halquinol exposure.

4.6.2 Tissue concentration of conjugated metabolites of halquinol following dietary inclusion of halquinol at 240 ppm

Liver

Liver is the main organ of metabolism of halquinol therefore the conjugated metabolites were detected by LCMS in liver tissue.The tissue concenteration of 5-chloro-quinolin-8-ol glucuronide in liver tissue is depicted in Table 13 The concentration of 5-chloro-quinolin-8-ol glucuronide in liver tissue at different time intervals during 40 days dietary exposure of halquinol is shown in Table 13, concentration of 5-chloro-quinolin-8-ol glucuronide at 5^{th} day and on 40^{th} day during treatment were 55.12±2.15 $ng.g^{-1}$ and 243.7±2.12 $ng.g^{-1}$ tissue respectively and there was significant increase ($p \leq 0.05$) in concentration of 5-chloro-quinolin-8-ol glucuronide between each successive sampling days of 5, 10, 20, 30 and 40 days during treatment in liver tissue following dietary exposure @ 240 ppm (Table 13).

Kidney

The LCMS detected the major metabolite in kidney in minimal levels as it is not the organ of metabolism for halquinol. The concentration of 5-chloro-quinolin-8-ol

glucuronide in kidney at different time intervals during 40 days dietary exposure of halquinol is shown in Table 13, concentration of 5-chloro-quinolin-8-ol glucuronide at 5^{th} day and on 40^{th} day during treatment were 44.12±1.35 $ng.g^{-1}$ and 285.3±5.42 $ng.g^{-1}$ tissue respectively and there is significant increase ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol between each successive sampling days of 5, 10, 20, 30 and 40 days during treatment in kidney tissue following dietary exposure @ 240 ppm (Table 13).

Muscle

The LCMS assay could not detect the major metabolite constituent of halquinol *viz:*5-chloro-quinolin-8-ol glucuronide in breast muscle at 240 ppm inclusion level indicating that the concentration of 5-chloro-quinolin-8-ol glucuronide was less than 10 $ng.g^{-1}$ at all time intervals during which birds were sacrificed during treatment period of halquinol exposure.

Table12: Tissue concentration ($ng.g^{-1}$) of halquinol* at different time intervals during 40 days dietary exposure in broiler chickens

Day of halquinol exposure	Halquinol® concentration in diet						
	60 ppm (T_1)		120 ppm (T_2)		240 ppm (T_3)		
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Muscle
5	26.09 ± 0.51^{A}	38.11 ± 1.49^{a}	40.70 ± 0.62^{F}	55.07 ± 0.72^{f}	64.20 ± 2.78^{K}	74.15 ± 4.42^{k}	$35.13\pm2.25^{>}$
10	37.45 ± 1.29^{B}	59.52 ± 1.60^{b}	86.13 ± 1.47^{G}	96.47 ± 1.48^{g}	138.7 ± 2.95^{L}	151.8 ± 4.36^{l}	$52.45\pm1.14^{\#}$
20	109.22 ± 2.34^{C}	119.0 ± 2.62^{c}	170.0 ± 3.65^{H}	185.5 ± 3.06^{h}	329.2 ± 5.83^{M}	322.7 ± 7.55^{m}	$96.47\pm2.33^{\$}$
30	112.4 ± 2.92^{D}	124.2 ± 5.46^{d}	216.6 ± 3.67^{I}	219.3 ± 3.70^{i}	396.7 ± 4.62^{N}	388.3 ± 4.78^{n}	$104.7\pm3.22^{@}$
40	188.8 ± 3.83^{E}	233.5 ± 5.87^{e}	411.0 ± 4.22^{J}	364.7 ± 6.05^{j}	543.7 ± 6.04^{O}	540.3 ± 10.07^{o}	$170.4\pm5.42^{\wedge}$

Note: 1 Values are expressed as mean ± SE; n= 6 birds sacrificed at a time

2. * = represents 5,7-dichloroquinolin-8-ol

3. Tissue concentration data (liver & kidney) between different dose groups and between different days during treatment period were analyzed by xcalibur analytical softwares, while muscle concentration data was analysed by one way ANOVA followed by Tukey's multiple comparison test

4. Values bearing different alphabets as superscripts both between rows (days) and columns (dose levels) for individual tissue varysignificantly ($p\leq0.05$);Capital alphabets indicate comparison within liver tissue and small alphabets indicate comparison within kidney tissue and values bearing symbol, a

Table 13: Tissue concentrations ($ng.g^{-1}$) of 5-chloro-quinolin-8-ol glucuronide* at different time intervals during 40 days dietary exposure in broiler chickens

Day of halquinol exposure	Halquinol® concentration in diet						
	60 ppm (T_1)		120 ppm (T_2)		240 ppm (T_3)		
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Muscle
5	25.13 ± 1.25^{A}	24.12 ± 1.25^{a}	45.12 ± 2.15^{F}	34.12 ± 1.35^{f}	55.12 ± 2.15^{K}	44.12 ± 1.35^{k}	----------
10	27.35 ± 1.29^{B}	39.52 ± 1.60^{b}	76.13 ± 1.47^{G}	46.47 ± 1.48^{g}	68.7 ± 2.95^{L}	81.8 ± 4.36^{l}	----------
20	70.22 ± 2.34^{C}	58.0 ± 2.62^{c}	100.0 ± 3.65^{H}	65.5 ± 3.06^{h}	119.2 ± 5.83^{M}	122.7 ± 7.55^{m}	----------
30	85.4 ± 2.92^{D}	62.2 ± 5.46^{d}	166.6 ± 3.67^{I}	77.3 ± 3.70^{i}	200.7 ± 4.62^{N}	244.3 ± 4.78^{n}	----------
40	160.8 ± 4.42^{E}	75.4 ± 4.42^{e}	190.4 ± 2.12^{E}	$95.4.\pm5.42^{j}$	243.7 ± 2.12^{O}	285.4 ± 5.42^{o}	----------

Note: 1 Values are expressed as mean ± SE; n= 6 birds sacrificed at a time

2. * = 5-chloro-quinolin-8-ol glucuronide*

3. Tissue concentration data (liver & kidney) between different dose groups and between different days during treatment period were analyzed by xcalibur analytical softwares, while muscle concentration data was analysed by one way ANOVA followed by Tukey's multiple comparison test

4. Values bearing different alphabets as superscripts both between rows (days) and columns (dose levels) for individual tissue vary significantly ($p\leq0.05$); Capital alphabets indicate comparison within liver tissue and small alphabets indicate comparison within kidney tissue and values bearing symbol, a

4.7 Tissue concentration of 5.7-dichloroquinolin-8-ol after withdrawal of halquinol exposure at 60 ppm dose level

Liver

After withdrawal of halquinol exposure through diet there was faster decline in concentration of 5,7-dichloroquinolin-8-ol in liver tissue from initial concentration of 188.8±3.83 $ng.g^{-1}$ tissue at 2 h post withdrawal of treatment to 20.14±0.82 $ng.g^{-1}$ tissue at 3rd day after withdrawal of halquinol exposure (Table 14 and Fig. 38). There was significant decrease ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol on each successive sampling days of 1, 2 and 3rd days following withdrawal of treatment and was less than LOQ on day 4 of withdrawal of halquinol (Table 14).

Kidney

After withdrawal of halquinol exposure through diet there was rapid decline in concentration of 5,7-dichloroquinolin-8-ol from initial concentration of 233.5±5.887 $ng.g^{-1}$ tissue at 2 h post withdrawal to 38.80 ± 1.04 $ng.g^{-1}$ tissue at 3rd day after withdrawal of halquinol exposure (Table 14 and Fig. 38). There was significant decrease ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol on each successive sampling days following withdrawal of treatment and becoming not detectable by day 4th after end of treatment (Table 14).

4.7.1 Tissue concentration of 5,7-dichloroquinolin-8-ol after withdrawal of halquinol exposure through diet at 120 ppm inclusion level

Liver

After withdrawal of halquinol exposure through diet there was faster decline in concentration of 5,7-dichloroquinolin-8-ol from initial concentration of 411.0 ± 4.22 $ng.g^{-1}$ at 2 h post withdrawal to 36.33 ± 0.34 $ng.g^{-1}$ at 3rd day after withdrawal of halquinol exposure (Table 14 and Fig.39). Significant decrease ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol on each successive sampling days of 1, 2 and 3 days following withdrawal of halquinol exposure and becoming not detectable by day 4th after end of treatment was observed from Table 14.

Kidney

After withdrawal of halquinol exposure through diet, there was rapid decline in concentration of 5,7-dichloroquinolin-8-ol from initial concentration of 364.7 ± 6.05$ng.g^{-1}$ tissue at 2 h post withdrawal of treatment to 39.83 ± 0.94 $ng.g^{-1}$ tissue at 3 rd day after withdrawal of halquinol exposure (Table 14 and Fig. 39). There was significant decrease ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol on each successive sampling days following withdrawal of treatment and is not detectable by 4thday of halquinol withdrawal (Table 14).

4.7.2 Tissue concentration of 5,7-dichloroquinolin-8-ol after withdrawal of halquinol exposure through diet at 240 ppm inclusion level

Liver

Following withdrawal of halquinol exposure through diet there was rapid decline in tissue concentration of 5,7-dichloroquinolin-8-ol from initial concentration of 543.7±6.04ng.g^{-1} at 2 h post withdrawal of treatment to 39.50±1.18 ng.g^{-1} at 3rd day after withdrawal of halquinol exposure (Table 14 and Fig. 40). There was significant fall ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol on each successive sampling days following withdrawal of treatment and was not detectable by day '4' following halquinol withdrawal (Table 14).

Kidney

Following withdrawal of halquinol exposure through diet there was rapid decline in concentrations of 5,7-dichloroquinolin-8-ol from initial concentration of 540.3±10.07ng.g^{-1} tissue at 2 h post withdrawal of treatment to 37.50±1.29 ng.g^{-1} tissue at 3rd day after withdrawal of halquinol exposure (Table 14 and Fig. 40). There was significant decrease ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol on each successive sampling days following withdrawal of treatment and is not detectable on day 4 following withdrawal of halquinol (Table 14).

Muscle

After withdrawal of halquinol exposure through diet there was rapid decline in concentration of 5,7-dichloroquinolin-8-ol in muscle tissue from initial concentration of 170.4±5.42 ng.g^{-1} tissue at 2 h post withdrawal of treatment to 29.8±1.22 ng.g^{-1} tissue at

2nd day after withdrawal of halquinol exposure (Table 14 and Fig. 40). There was significant decrease ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol on each successive sampling days of 1 and 2 following withdrawal of treatment and not detectable in samples obtained on day '3'(Table 14).

4.8 Tissue concentration of 5-chloro-quinolin-8-ol glucuronide after withdrawal of halquinol exposure at 60 ppm dose level

Liver

After withdrawal of halquinol exposure through diet there was faster decline in concentration of 5,7-dichloroquinolin-8-ol in liver tissue from initial concentration of 160.8±4.42 $ng.g^{-1}$ tissue at 2 h post withdrawal of treatment to 7.14±0.12 $ng.g^{-1}$ tissue at 4th day after withdrawal of halquinol exposure (Table 15). There was significant decrease ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol on each successive sampling days of 1, 2 and 3rd days following withdrawal of treatment and becoming not detectable by day 5th after end of treatment (Table 15).

Kidney

After withdrawal of halquinol exposure through diet there was rapid decline in concentration of 5,7-dichloroquinolin-8-ol from initial concentration of 75.4±4.42 $ng.g^{-1}$ tissue at 2 h post withdrawal to 18.40 ± 2.04^{d} $ng.g^{-1}$ tissue at 4th day after withdrawal of halquinol exposure (Table 15). There was significant decrease ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol on each successive sampling days following withdrawal of treatment and becoming not detectable by day 5th after end of treatment (Table 15).

4.8.1 Tissue concentration of 5,7-dichloroquinolin-8-ol after withdrawal of halquinol exposure through diet at 120 ppm inclusion level

Liver

After withdrawal of halquinol exposure through diet there was faster decline in concentration of 5,7-dichloroquinolin-8-ol from initial concentration of 190.4±2.12 $ng.g^{-1}$ at 2 h post withdrawal to 16.33±0.12 $ng.g^{-1}$ at 4^{th} day after withdrawal of halquinol exposure (Table 15) . Significant decrease ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol on each successive sampling days of 1, 2 and 3 days following withdrawal of halquinol exposure and becoming not detectable by 5^{th} day after end of treatment was observed from Table 15.

Kidney

After withdrawal of halquinol exposure through diet, there was rapid decline in concentration of 5,7-dichloroquinolin-8-ol from initial concentration of 95.4±5.42 $ng.g^{-1}$ tissue at 2 h post withdrawal of treatment to 17.12±1.64 $ng.g^{-1}$ tissue at 4^{th} day after withdrawal of halquinol exposure (Table 15). There is significant decrease ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol on each successive sampling days following withdrawal of treatment and is not detectable by 5^{th} day of halquinol withdrawal (Table 15).

4.8.2 Tissue concentration of 5,7-dichloroquinolin-8-ol after withdrawal of halquinol exposure through diet at 240 ppm inclusion level

Liver

Following withdrawal of halquinol exposure through diet there was rapid decline in tissue concentration of 5,7-dichloroquinolin-8-ol from initial concentration of 243.72±2.12 $ng.g^{-1}$ at 2 h post withdrawal of treatment to 17.40 ± 0.16$ng.g^{-1}$ at 4^{th} day after withdrawal of halquinol exposure (Table 15). There is significant fall ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol on each successive sampling days following withdrawal of treatment and is not detectable by 5^{th} day of halquinol withdrawal (Table 15).

Kidney

Following withdrawal of halquinol exposure through diet there was rapid decline in concentrations of 5,7-dichloroquinolin-8-ol from initial concentration of 285.4±5.42 $ng.g^{-1}$ tissue at 2 h post withdrawal of treatment to 16.40±0.14^{l} $ng.g^{-1}$ tissue at 4^{th} day after withdrawal of halquinol exposure (Table 15). There is significant decrease ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol on each successive sampling days following withdrawal of treatment and is not detectable on day 5 following withdrawal of halquinol (Table 15).

Muscle

After withdrawal of halquinol exposure through diet there were no traces of either halquinol or its conjugated metabolite throughout the study for five days (Table 15).

Table 14: Tissue concentration ($ng.g^{-1}$) of halquinol* at different time intervals following withdrawal of halquinol exposure in feed of broiler chickens

Day following withdrawal	Halquinol® concentration in diet						
	60 ppm (T_1)		120 ppm (T_2)		240 ppm (T_3)		
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Muscle
0.08#	188.8 ± 3.83^{E}	233.5 ± 5.87^{e}	411.0 ± 4.22^{J}	364.7 ± 6.05^{j}	543.7 ± 6.04^{O}	540.3 ± 10.07^{o}	$170.4\pm5.42^{\#}$
1	58.17 ± 1.33^{B}	77.3 ± 2.10^{b}	89.17 ± 1.24^{F}	82.12 ± 3.84^{f}	160.30 ± 3.32^{J}	280.4 ± 3.37^{j}	$54.62\pm3.16^{\wedge}$
2	45.04 ± 1.21^{C}	54.44 ± 1.19^{c}	69.17 ± 1.04^{G}	73.83 ± 1.16^{g}	73.17 ± 2.70^{K}	81.87 ± 2.87^{k}	$29.8\pm1.22^{@}$
3	20.14 ± 0.82^{D}	38.80 ± 1.04^{d}	36.33 ± 0.34^{H}	39.83 ± 0.94^{h}	39.50 ± 1.18^{L}	37.50 ± 1.29^{l}	ND
4	ND	ND	ND	ND	ND	ND	ND

Note:
1. Values are expressed as mean ± SE; n= 6 birds sacrificed at a time ; * = represents 5,7-dichloroquinolin-8-ol
2. # Samples were collected at 2h post-withdrawal of dietary source of halquinol
3. Tissue concentration data between different days within a tissue of different dose groups, following withdrawal of halquinol exposure were analyzed by One Way Anova followed by Tukey's multiple comparison test
4. Values bearing different alphabets as superscripts between rows (days) for individual tissues vary significantly ($p\leq0.05$); Capital alphabets indicate comparison within liver tissue, small alphabets indicate comparison within kidney tissue while values bearing different symbols as superscripts indicate comparison within muscle tissue
5. ND = not detectable

Table 15: Tissue concentration ($ng.g^{-1}$) 5-chloro-quinolin-8-ol glucuronide* at different time intervals following withdrawal of halquinol exposure through feed in broiler chickens

Day following withdrawal	Halquinol® concentration in diet						
	60 ppm (T_1)		120 ppm (T_2)		240 ppm (T_3)		
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Muscle
0.08#	160.8 ± 4.42^{A}	75.4 ± 4.42^{a}	190.4 ± 2.12^{J}	95.4 ± 5.42^{j}	243.72 ± 2.12^{O}	285.4 ± 5.42^{o}	ND
1	48.17 ± 1.33^{B}	67.03 ± 2.10^{b}	69.17 ± 1.24^{F}	82.12 ± 3.84^{f}	160.30 ± 3.32^{J}	280.4 ± 3.37^{j}	ND
2	35.04 ± 1.21^{C}	44.44 ± 1.19^{c}	49.17 ± 1.04^{G}	73.83 ± 1.16^{g}	73.17 ± 2.70^{K}	81.87 ± 2.87^{k}	ND
3	10.14 ± 0.82^{D}	28.80 ± 1.04^{d}	36.33 ± 0.34^{H}	39.83 ± 0.94^{h}	39.50 ± 1.18^{L}	37.50 ± 1.29^{l}	ND
4	7.14±0.12	18.40±2.04	16.33±0.12	17.12±1.64	17.40±0.16	16.40±0.14	ND
5	ND	ND	ND	ND	ND	ND	ND

Note:
1. Values are expressed as mean ± SE; n= 6 birds sacrificed at a time ; * = represents 5-chloro-quinolin-8-ol glucuronide
2. # Samples were collected at 2h post-withdrawal of dietary source of halquinol
3. Tissue concentration data between different days within a tissue of different dose groups, following withdrawal of halquinol exposure were analyzed by one way ANOVA followed by Tukey's multiple comparison test
4. Values bearing different alphabets as superscripts between rows (days) for individual tissues vary significantly ($p\leq0.05$); Capital alphabets indicate comparison within liver tissue, small alphabets indicate comparison within kidney tissue while values bearing different symbols as superscripts indicate comparison within muscle tissue
5. ND = not detectable

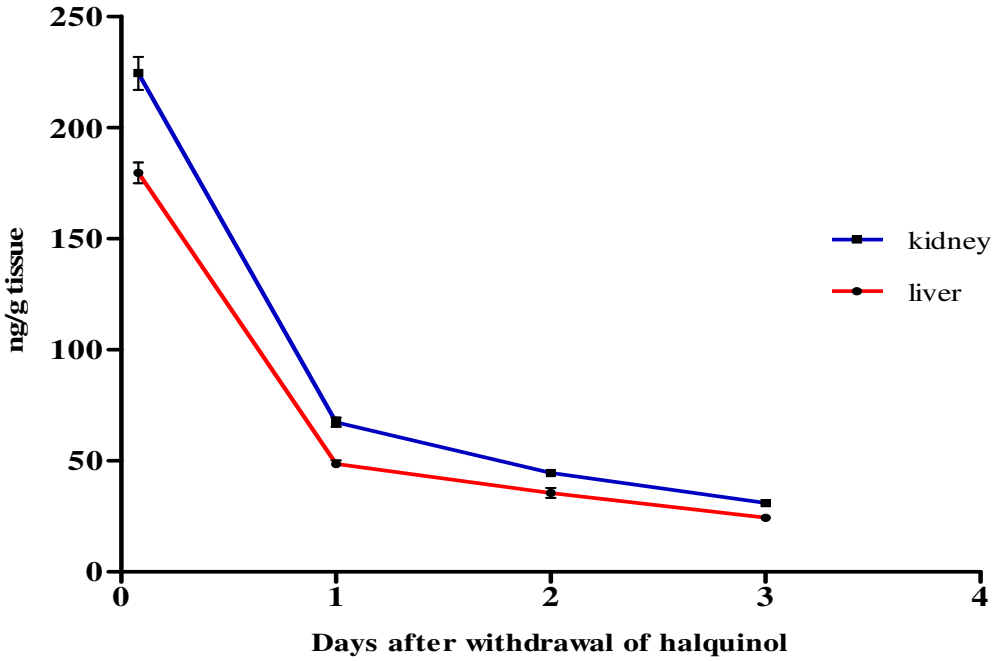


Fig. 38: Mean tissue concentration-time profile of halquinol (5,7-dichloroquinolin-8-ol) in broiler chickens following withdrawal of dietary exposure to halquinol @ 60 ppm x 40 days (n=6 birds sacrificed at each time interval)

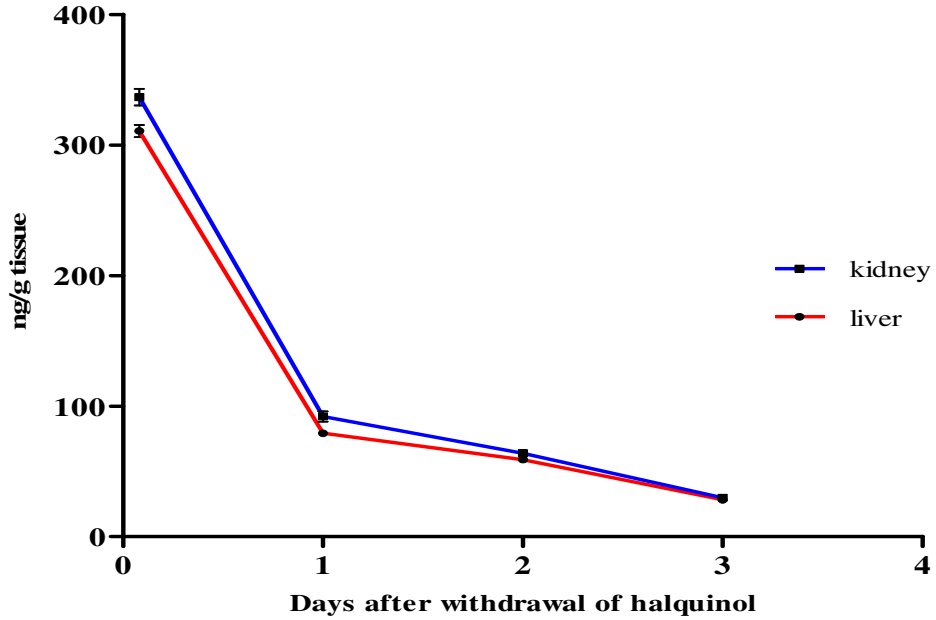


Fig. 39: Mean tissue concentration-time profile of halquinol (5,7-dichloroquinolin-8-ol) in broiler chickens following withdrawal of dietary exposure to halquinol @120 ppm x 40days (n=6 birds sacrificed at each time interval)

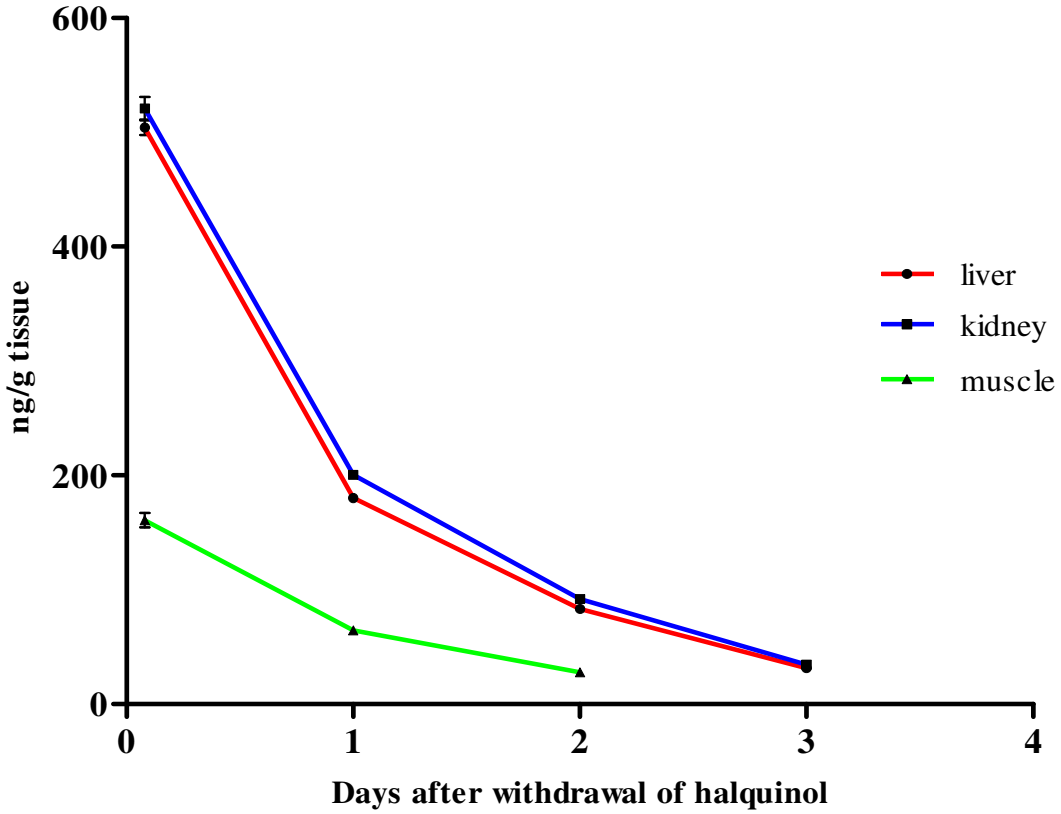


Fig. 40 : Mean tissue concentration-time profile of halquinol (5,7-dichloroquinolin-8-ol) in broiler chickens following withdrawal of dietary exposure to halquinol @ 240 ppm x 40days (n=6 birds sacrificed at each time interval)

DISCUSSION

V. DISCUSSION

The present work was undertaken to evaluate the distribution of halquinol and its metabolites in various tissues of chickens following dietary inclusions using NMR and other advanced techniques and to establish meat withdrawal period of halquinol and its metabolites in chicken.

Use of antimicrobial substances is one of the age old practices to augment growth in food animals and poultry. Quinoline compounds are one among the several antimicrobial agents employed in poultry farming as feed additive. Quinoline derivatives were introduced way back in 1960s for the treatment of amoebic dysentery in man. Historically, an oxyquinoline derivative, iodochlorhydroxyquinoline was banned, since its prolonged usage caused a neurological syndrome described as sub-acute myelo-optic neuropathy in Japanese people (Cooper *et al.,* 1995).

In experimental studies, it was proved that the common pathogenic organisms of poultry did not gain resistance when halquinol (Non antibiotic antimicrobial) was used as a growth promoter in the dose range of 60, 120 and 240 ppm (Shivakumar *et al*., 2004). The present study was aimed to determine the withdrawal period of halquinol and its metabolites in chicken in the same doses. The experiment was conducted to know the metabolic profile of halquinol, the various metabolites of halquinol after it is being fed to the broiler chicks. The method of identification, to characterize the metabolites ,various molecular techniques were used *viz*, NMR, IR and LCMS to know the concentration of halquinol and its metabolites in various edible tissues of chicken.

In the present study, broiler chickens were fed with halquinol mixed feed at different levels of 60, 120 and 240 ppm for a period of 40 days. The dose selected in the present study was based on the earlier studies of Shivakumar *et al*. (2004).

5.1 Halquinol characterization

In the present study, the characterization of the halquinol procured from (Provimi Animal Nutrition Ltd., Bangalore) was done by various analytical techniques such as Ultraviolet-visible (UV-Vis) spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Thin Layer Chromatography (TLC), Nuclear Magnetic Resonance (NMR), High Performance Liquid Chromatography (HPLC) and Liquid Chromatography Mass Spectroscopy (LCMS) were performed. The genuineness of the procured halquinol was assessed by UV-Vis double beam spectrophotometer and FTIR. The purity or presence of any impurities in the procured halquinol was tested and confirmed by NMR and the results were in accordance with the findings of Boison and Keng, (1998).

5.2 Identification of the halquinol in edible tissues of chicken

Halquinol was incorporated in the feed of broiler chickens at different levels *viz*: 60, 120 and 240 ppm for a period of 40 days and few birds (n=6 each) in each treatment group at regular intervals were sacrificed to determine tissue concentration of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol, the metabolites of halquinol. The remaining birds were serially sacrificed (n = 6) at regular intervals from all groups following withdrawal of experimental exposure to halquinol and vital edible tissues *viz:* liver, kidney and breast muscles were collected and subjected to liquid chromatography mass spectroscopy (LCMS).

The detection and quantification of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol and its major metabolites i.e, 5,7-dichloroquinolin-8-ol sulfate and 5,7-dichloro-quinolin-8-ol glucuronide in tissues was carried out by using LCMS fitted with photo diode array detector. Reference standards of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol from Sigma® were used for standardization, recovery and assay precision studies. The tissue clean-up and analytical procedures to quantify 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol, the constituent molecules of halquinol with permissible limits of repeatability, precision and sensitivity were standardized in the present study. However, the tissue concentration of 5-chloroquinolin-8-ol and 5,7-dichloroquinolin-8-ol sulfate was less than the detection limit of the assay system (20 ng/g tissue). Therefore, the interpretations of halquinol residues made in the present study were limited to 5,7-dichloroquinolin-8-ol only and 5-chloro-quinolin-8-ol glucuronide.

The m/z value and area under curve of halquinol and its metabolites indicated the increase in its concentration on successive sampling. The RT value for halquinol was 5.57 to 6.97 and the mass was also known by performing LCMS of test samples. The area under curve was the indicator of increasing concentration of halquinol in the successive sampling. All the constituents of halquinol were identified by above mentioned molecular methods.

In the present study, 5,7-dichloroquinolin-8-ol was detected in the liver and kidney samples belonging to all the three treatment groups throughout the experimental period, while its residue concentration was not found in breast muscles except in those belonging to 240 ppm group. Similarly, the tissue levels of 5,7-dichloroquinolin-8-ol

were below the limit of sensitivity of the developed assay system, except in four samples (out of six) belonging to 240 ppm group. Thus, the absorption and bioavailability of halquinol is poor in the present study at the dose of 60 and 120 ppm. However, the bioavailability of the halquinol and its conjugated metabolites was 70 % at the dose of 240 ppm. This is in contrast to the findings of Botsoglou and Fletouris (2001) who reported that the bioavailability of halquinol was 5 to 10 per cent who used HPLC. The increased extractability of halquinol and its conjugated metabolites in the present study might be attributed to the accurate measurement by the advance techniques like NMR and LCMS used in the present study for the quantification purpose.

The IR characteristics observed for this halquinol and its constituents in the present study were similar to earlier studies of Mushigeri *et al.* (2008) in the Indian major carp *Catla catla* in Hamilton. The result of the present study was also similar to that of the earlier study conducted by (Pavithra *et al*., 2011) who confirmed that the halquinol consisted of two major constituents *i.e*, 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol using TLC method. However, in the present study, the presence of halquinol and its constituents was confirmed by characteristic IR bands and their corresponding functional groups.

5.3 Screening and quantification of metabolites of halquinol

The extracted chicken tissue samples were subjected to metabolic screening and quantification by using LCMS in the SID Analysis laboratory, Indian Institute of Science, Bangalore.

All the analysed samples were positive for the presence of 2 main metabolites viz.,5-chloro-quinolin-8-ol sulfate and 5-chloro-quinolin-8-ol glucuronide. The conjugated metabolites of halquinol were identified by MS chromatogram (LCMS) and the mass of the metabolites were calculated by Chemdraw 4 software. Perusal of the literature revealed a similar report by Alhendi *et al*, (2000) Who worked on clioquinol compound (a member of the same quinoline family which also follow the same metabolic pathway) and found similar conjugated metabolites of clioquinol *viz*, clioquinol sulfate and clioquinolglucuronide. Scanty reports are available regarding the tissue distribution of metabolites of clioquinol i.e, 5-chloro-quinolin-8-ol sulfate, 5-chloro-quinolin-8-ol glucuronide except the report by (Horie *et al*., 1998).

The present finding was in accordance with Borries and Tulliez (1972) who conducted radio labeled studies in rat and found that halquinol is excreted as glucoronide conjugate in the bile similar to that of one of the closely related compound clioquinol which essentially undergo glucuronidation in the intestine (Kotaki *et al*., 1983). In addition , quinolines undergo aromatic hydroxylation and N-oxygenation based on region selectivity among them (Dowers *et al*., 2004).

Some reports are available on the tissue distribution profile of halquinol and its constituents in broiler chicks. The study conducted by Mushigeri *et al*. (2008) confirmed the distribution pattern of the halquinol and its constituents *i.e* ,5,7-dichloroquinolin-8-ol and chloroquinolin-8-ol in broiler chicken tissues.

Two last samples *viz* on days 30^{th} and 40^{th} had very high concentration of halquinol as well as its metabolites mainly the 5-chloro-quinolin-8-ol glucuronide was

found indicating the cumulative behavior of halquinol and its metabolites. The present finding is in accordance with the earlier studies by Swetha *et al*. (2009) who had also reported the cumulative behavior of quinoline compounds in rats.

In the present study, the significant increase ($p \leq 0.05$) in concentrations of 5,7-dichloroquinolin-8-ol in liver and kidney on each successive sampling days of 5, 10, 20, 30 and 40 in the three treatment groups were dose dependent. On day 40 of halquinol exposure the tissue concentration of 5,7-dichloroquinolin-8-ol was relatively high in liver and kidney in experimental birds which received halquinol @ 240 ppm. However, maximum residue concentration of 5,7-dichloroquinolin-8-ol was present in breast muscles ($160.0 \pm 6.31 ng.g^{-1}$) of birds belonging to 240 ppm group on day 40. Bound residues may arise from incorporation of residues of the drug in to endogenous compounds, chemical retention of proven drug or its metabolites in to macromolecules or physical encapsulation in to tissue matrices (Beltran *et al*., 1985).The present finding was in accordance with the findings of (Bories and Tulliez, 1972; Kotaki *et al*., 1983) who also observed the cumulative behavior of quinoline compounds in chicken tissues.

The method developed in the present study was in accordance with earlier study conducted by Jane *et al*. (2005) who had done the determination of nitrofuran metabolites in poultry muscle and eggs by liquid chromatography-tandem mass spectrometry by LC-MS/MS) with the similar method . The method focused on analytes which were known to be produced by quinoline compounds. A simple pH-buffered sample extraction was developed. The limit of detection (LOD) for the quantitatively validated analytes ranged from 1 to 20 ($ng.g^{-1}$).

In breast muscle samples, 5,7-dichloroquinolin-8-ol was not detected throughout the experimental period in birds exposed to halquinol @ 60 and 120ppm in the present study. However, residues were detected in 240 ppm group and the concentration was found to be 160.4 $\pm$ 6.51 ng.g-^{1} (Table 1). When compared to liver and kidney tissue (60 and 120 group), the mean residue concentration of 5,7-dichloroquinolin-8-ol and its metabolite in breast muscles was relatively less despite the experimental birds were fed halquinol @ 240ppm. This may be attributed to less vascular nature of skeletal muscles when compared to highly perfused organs (liver and kidney) in the present study. The results of the present study were supported by the earlier findings of (Ellis, 2004) who carried out the analysis of metabolism of 3,S-Dinitro-otoluamide-C14 in Chickens with slight modification method.

5.4 Tissue depletion of 5,7-dichloroquinolin-8-ol following withdrawal of halquinol

Following 'a day' after withdrawal of halquinol given either therapeutic dose (60 ppm) or extra-label dose (120 and 240 ppm), the concentrations of 5,7-dichloroquinolin-8-ol fell significantly ($p \leq 0.05$) in both liver and kidney tissues and their concentration was not detectable (< 20 ng.g^{-1}) on day '4' in these tissues after withdrawal.

Relatively, the depletion of 5,7-dichloroquinolin-8-ol from breast muscles of experimental birds fed halquinol @ 240 ppm was rapid when compared to other edible tissues subjected to analysis in the present study. There are no reports of either pharmacokinetic profile of halquinol or metabolic site (s) in poultry following extra - vascular administration. Depletion of tissue residues depends on terminal elimination phase of the drug in question. It has been reported that clioquinol, a quinoline compound

mostly metabolised to clioquinolglucuronide and sulfate and these metabolites were excreted in urine and bile (Kotaki *et al*., 1983). Rapid depletion of 5,7-dichloroquinolin-8-ol in all the treated birds might be due to combined effect of intestinal and/or hepatic metabolism and urinary or biliary excretion in the present study.

However the depletion of the major metabolite *viz* 5-chloro-quinolin-8-ol glucuronide was slow when compared to halquinol which supports the fact that the metabolites of drug takes a day or two longer than the parent compound. In the present study the concentration of 5-chloro-quinolin-8-ol glucuronide was not detectable by day 5, a day longer than what required for halquinol.This finding was in accordance with the earlier findings of (Boison and Keng ,1998) who had found similar depletion studies of pencillin and its metabolites in bovine tissues using LCMS.

5.5 Pre-slaughter withdrawal period

Fixation of meat withdrawal period (WP) requires quantification of tissue residues of drug or its metabolites quantified according to specified analytical procedures as specified from time to time by enforcement agencies. However, partly because of complexity in structural chemistry of halquinol, tissue or plasma kinetics of individual constituent molecules of halquinol has not been determined. In the present study, tissue residues of 5,7-dichloroquinolin-8-ol was determined, however the concentration of 5-chloroquinolin-8-ol was below the detection limit of assay system. Hence, the WPs for halquinol in broiler chickens were made based on the tissue depletion profile of 5,7-dichloroquinolin-8-ol and its major metabolite (5-chloro-quinolin-8-ol glucuronide)

obtained in the present study. The present study concords with earlier findings of Kao *et al*. (2001) who had established the WP of nitrofuran in broiler chickens by following the similar method.

By whatever procedures are applied, separate withdrawal periods (WPs) must be estimated from each edible tissue and the longest of these individual tissues ,WPs then becomes overall withdrawal period for the marketed product (Woodward *et al*., 2009). US-FDA requires that log-linear kinetics and least square regression to be used to calculate withdrawal time in edible tissue/s after deriving a statistical tolerance limit (Reeves, 2010). An important feature of the statistical tool is that it takes account of both acceptable daily intake (ADI) and kinetic behavior of the residue in muscle, fat, liver and kidney Kirbi *et al*. (2005).

Generally, MRL and tolerance values are employed to derive WPs for marketed veterinary medicines. Hence it can be derived by examining the time-dependent tissue depletion preferably in key tissues (*viz*: liver, muscle, kidney and fat) against the MRL or tolerance values (Martin *et al*., 2009). Upon extensive literature survey, it was found that there is lack of information on either residue kinetic data or other parameters of regulatory toxicological importance (*viz:* tolerance, ADI, NOAL, NOAEL) with respect to halquinol or its constituent molecules. However, in the absence of MRL, particularly keeping in view of developing countries where veterinary drug assessment and MRL values are difficult to generate, FAO/WHO joint expert committee on food additives (JECFA) has permitted the use of veterinary medicine products and trade at least based on scientific approach or those that develop and use their own national standards.

However, there is no standard for halquinol residues in foods of animal origin set by Agricultural and Processed Food Products Export Development Authority or by Government of India under Prevention of Food Adulteration Act (GOI, 2004), thus, complicating the fixation of WPs.

According to EMEA CVMP, 1996 guidance, regulatory standards require that drugs be regulated on the basis of total residues. Total residues resulting from drug administration to an animal consist of the parent drug and all metabolites, conjugates, and residues bound to endogenous macromolecules. To ensure compliance with the withdrawal period, an assay is needed to monitor total residues in the edible tissues. However, it would be impractical to subject all known metabolites of a drug to analysis, since some drugs can give rise to numerous metabolites (Woodward, 1992). Maximum residue limits (MRLs) for a commodity are set for residues measured by a valid method of analysis. This method may measure the chemical or a derivative of the chemical and may include metabolites originating from the parent compound or other chemicals. In some cases, the nominal concentration of the parent compound is calculated from the measured concentration of a metabolite, but in other cases a derivative or metabolite is used as a measure of the residue (Ellis, 2008). Therefore, the residue to which the MRL applies to each chemical compound (Webber, 1979). Admittedly, all the indirect ways employed for establishing pre-slaughter WP cannot guarantee absolutely that the poultry meat is free from antimicrobial substance.

Halquinol is derived from a mixture of compounds obtained by the chlorination of 8-quinolinol and it is composed of 5,7-dichloroquinolin-8-ol (57–74%), 5-chloro-8-

quinolinol (23–40%), and 7-chloro-uinolinol-8-ol (not more than 3%), however the stability of the third component is weak. In the present study, commercial product of halquinol (Provimi Animal Nutrition India Pvt. Ltd. Bangalore) which was certified to contain respectively 71.39 and 26.21 per cent of 5,7-dichloroquinolin-8-ol and 5-chloro-quinolin-8-ol was used (Kaul and Lewis ,1965).

SUMMARY

VI. SUMMARY

Halquinol, a quinoline derivative is a mixture of 5,7-dichloroquinolin-8-ol, 5-chloroquinolin-8-ol and 7-chloroquinolin-8-ol in the ratio of 57-74 : 23-40 : 3 per cent. It possesses broad spectrum antibacterial, antifungal and antiprotozoal activity. It is used extensively in India and other Asian countries to overcome common challenges of modern poultry and swine farming like microbial infections and growth promotion aspects. The present study was conducted in broiler chickens with the objectives (a) To determine the distribution of halquinol and its metabolites in various tissues following dietary inclusion in chickens (b) to study the halquinol as well as its metabolites in various tissues of using NMR and other advanced techniques (c) to establish meat withdrawal period of halquinol and its metabolites in chicken.

Four hundred day old Vencobb-400® broiler chicks were divided into four groups of 100 chicks each. Group I served as untreated control and received standard poultry diet without any antimicrobial substances in it, while experimental birds in group II (T_1), III (T_2) and IV (T_3) received standard poultry diet containing halquinol (Provimi Animal Nutrition India Pvt. Ltd., Bangalore) @ 60, 120 and 240 ppm respectively, for a period of 40 days. Edible tissues *viz*: liver, kidney and breast muscles were collected by sacrificing experimental birds (n=6 each) on day 5, 10, 20, 30 and 40 of halquinol exposure to determine tissue distribution. The tissue depletion of halquinol and its metabolites from the aforesaid edible tissues were determined by sacrificing the remaining birds (n=6 each) on day 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 following withdrawal of dietary exposure of halquinol.

The tissue samples were subjected to liquid-liquid extraction using acetonitrile and ethyl acetate respectively and the tissue clean-up and analytical procedures were standardised to quantify 5,7-dichloroquinolin-8-ol, 5-chloroquinolin-8-ol and its main metabolite 5-chloro-quinolin-8-ol glucuronide, with permissible limits of repeatability, precision and sensitivity. However, the tissue concentration of 5-chloroquinolin-8-ol and the other three metabolites (5-chloro-quinolin-8-ol sulfate, 5,7, dichloro-quinolin-8-ol sulfate, 5,7, dichloro-quinolin-8-ol glucuronide) below the lower limit of detection. The extraction recovery of 5,7-dichloroquinilin-8-ol was 89.5, 91.9 and 87.64 per cent for liver, kidney and breast muscle tissues respectively.

High performance liquid chromatography (HPLC, Shimadzu Prominence, Japan) system equipped with a reverse phase C_{18} column (250 x 4.6mm , particle size 5µm, pH:1.5-11, Purospher Star RP-18e, Merck, Germany) as stationary phase and detection was measured at 247 nm with scanning range of 200 to 400 nm. The mobile phase was composed of 0.1% v/v orthophosphoric acid and acetonitrile at a ratio of 500:500 volumes and delivered at a flow rate of one ml per minute. The sensitivity of the assay system to detect 5,7-dichloroquinolin-8-ol was 20 $ng.g^{-1}$ tissue. There was a significant ($p \leq 0.05$) increase in concentration of 5,7-dichloroquinolin-8-ol in liver and kidney between each successive sampling days of 5, 10, 20, 30 and 40 in T_1, T_2 and T_3 groups of experimental birds exposed to halquinol. However, 5,7-dichloroquinolin-8-ol in breast muscle samples belonging to T_1 and T_2 group were below the detection limit of assay, while its concentration was significantly ($p \leq 0.05$) increased in experimental birds belonging to T_3 (240 ppm) group between each successive sampling days *viz*: 5, 10, 20, 30 and 40 of halquinol exposure. On termination of halquinol exposure @ 60 ppm (T_1),

the concentration of 5,7-dichloroquinolin-8-ol in experimental birds which received halquinol @ 60 ppm were high in liver and kidney tissues.

Consequent to withdrawal of halquinol exposure in all the treatment groups (T_1, T_2 and T_3) there was significant decrease ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol in liver and kidney tissues on each successive sampling days and reached to non-detectable levels on day '4'. The depletion of 5,7-dichloroquinolin-8-ol from breast muscles of experimental birds exposed to halquinol @ 240 ppm was relatively rapid and the residues were non-detectable on day '3' following its withdrawal from diet.

However the depletion of the major metabolite *viz* 5-chloro-quinolin-8-ol glucuronide was slow when compared to halquinol which supports the fact that the metabolites of drug takes a day or two longer than the parent compound.In the present study the concentration of 5-chloro-quinolin-8-ol glucuronide was not detectable by day 5, a day longer than what required for halquinol.Hence the withdrawal period for halquinol and its metabolites in poultry was established as 5 days

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VII. BIBLIOGRAPHY

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ABSTRACT

VIII. ABSTRACT

Halquinol is a quinoline derivative, a mixture of 5,7-dichloroquinolin-8-ol, 5-chloroquinolin-8-ol and 7-chloroquinolin-8-ol. The present study was carried out in broiler chickens to determine the distribution of halquinol and its metabolites into edible tissues and to establish pre-slaughter withdrawal period for meat following dietary inclusion of halquinol. Experimental birds consisted of 400, day-old broiler chicks (Vencobb-400). They were divided into four groups of 100 chicks each. Group I birds served as control (C) and received poultry diet without any antimicrobial substances in it, while experimental birds in group II (T_1), III (T_2), IV (T_3) received poultry diet containing halquinol @ 60, 120 and 240 ppm respectively for a period of 40 days. Edible tissues samples *viz:* liver, kidney and breast muscles were collected by sacrificing experimental birds (n=6) on day 5, 10, 20, 30 and 40 of the halquinol exposure through diet and at 2 h and on day 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 following withdrawal of halquinol.

The tissue clean-up and analytical procedures were standardized to quantify 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol by employing Liquid Chromatography Mass Spectroscopy (LCMS) with permissible limits of repeatability, precision and sensitivity. The detection limit of the assay system for both the constituent molecules was 20 $ng.g^{-1}$tissue. There was significant ($p \leq 0.05$) increase in concentration of 5,7-dichloroquinolin-8-ol and its metabolite *viz* 5-chloro-quinolin-8-ol glucuronide in liver and kidney between each successive sampling days.

There was a significant decrease ($p \leq 0.05$) in concentration of 5,7-dichloroquinolin-8-ol and 5-chloro-quinolin-8-ol glucuronide, in liver and kidney tissues on each successive sampling days following withdrawal of halquinol and reached to non-detectable levels on day '4' and 5 respectively, following withdrawal of halquinol exposure in all the treatment groups (T_1, T_2 and T_3).

Therefore it is suggested to adopt four days pre-slaughter withdrawal period in broiler chickens when halquinol being used as growth promoter.