MANUAL OF METHODS

OF

ANALYSIS OF FOODS

ANTIBIOTICS AND HORMONE RESIDUES

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA

MINISTRY OF HEALTH AND FAMILY WELFARE

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MANUAL FOR ANALYSIS OF ANTIBIOTICS AND HORMONE RESIDUES

TABLE OF CONTENTS

Sr. No.	Title/METHOD	Page No.
PART A		
1.0	Introduction	2
1.1	Safety Requirements for handling	2
1.2	Determination of Chloramphenicol – HPLC-MSMS Method	3
1.3	Determination of Nitrofuran metabolites – HPLC-MSMS Method	8
1.4	Determination of Tetracyclines – HPLC-UV/ MSMS Method	14
1.5	Determination of Sulphonamides– HPLC- MSMS Method	20
1.6	Determination of Quinolones – HPLC- MSMS Method	27
1.7	Determination of Nitroimidazoles– HPLC- MSMS Method	32
PART B		
1.0	Introduction	44
1.1	RP-HPLC Method for the determination of oxytocin in milk	44
1.2	Quantitative analysis of oxytocin by LC-MS/MSn	47
1.3	HPLC Method For The Determination Of Oxytocin In Pharmaceutical Dosage Form And Comparison With Biological Method	48
	Annexure 1	50

Note: The test methods given in the manuals are validated/ standardized test methods. However, it would be the responsibility of the respective testing laboratory to confirm that the above methods are validated in its laboratory and gives proper result in their laboratory.

MANUAL FOR ANALYSIS OF ANTIBIOTICS AND HORMONE RESIDUES

PART A

1.0 INTRODUCTION

The presence of residues of banned substances/ substances permitted but exceeding the prescribed limits by the regulatory authorities in case of veterinary drugs, pharmaceutical products and pharmaceutically active substances in products of animal origin (like muscle, liver, kidney, fish-flesh, egg, milk, honey etc) and from various species (like bovine, ovine, porcine, caprine, poultry, rabbit, farmed fish etc) is a matter of concern for public health. The presence of these substances may lead to allergies, suspected to be carcinogens, mutagens or may lead to emergence of resistant microbes. As a consequence, national food safety authorities and regulatory authorities have banned the use/ strictly regulated its use in veterinary practice or established legal guidance to ensure proper use of these veterinary drugs, pharmaceutical products and pharmaceutically active substances.

The successful implementation of national regulation and surveillance monitoring depends on availability of reliable analytical techniques. Various techniques are available, employed and are in practice like Immunoassay for screening and liquid chromatography with Ultra-Violet/ Fluorescence detection/ Mass spectrometry to determine and identify the commercially available veterinary drugs, pharmaceutical products and pharmaceutically active substances in products of animal origin.

1.1 SAFETY REQUIREMENTS FOR HANDLING

1. Required Protective Equipment — Protective clothing, eyewear, and gloves, where applicable.

2. Hazards:

Reagents	Hazard	Recommended Safe procedures
Reference Standards	Standards can be carcinogenic, mutagen or allergen	Wear protective clothing and gloves when handling standards.

Reagents	Hazard	Recommended Safe procedures
Acetonitrile , Methanol, Hexane, Dichloromethane, Carbon tetrachloride, Ethyl acetate	Flammable. Explosive hazard. Vapors will explode if ignited. Irritating to skin and mucous membranes.	Keep container tightly closed and away from fire. Use under a fume hood. Avoid breathing vapors.
Concentrated Acids: HCl, Acetic, HFBA, TCA, Formic, and solutions.	Corrosive substances. Danger of chemical burns. Potential for inhalation of corrosive fumes.	Prepare solutions in a fume hood. Wear protective equipment and avoid contact with skin.
NaOH and solutions made from same.	Corrosive substances Danger of chemical burns.	Wear gloves when preparing solutions, and take care to avoid splashes or spills.

1.2 DETERMINATION OF CHLORAMPHENICOL – HPLC-MS/MS METHOD

1.2.1 Scope:

Determination of Chloramphenicol in foods of animal origin

1.2.2 Reagents/Chemicals:

Ethyl acetate (HPLC grade), Acetonitrile/ ACN (HPLC or Gradient grade), Carbon tetrachloride (AR/HPLC), Hexane (AR), Certified reference standard of chloramphenicol base (CAP), Internal standard deuterated Chloramphenicol-d5 (CAP-d5) and gradient grade water.

1.2.3 Apparatus:

Blender, vortex mixer/ rotary shaker, centrifuge tubes (15/50 mL), refrigerated centrifuge, micropipettes, turbovap concentrator under nitrogen, LC vials.

1.2.4 Instrument:

Triple quadrupole HPLC-MS/MS and Analytical Column RP-18 end-capped, 250/150/100x4.6/3.0/2.1mm, 3-5 µm particle size or its equivalent.

HPLC Conditions:

a) Mobil phase: Water & Acetonitrile gradient,
Flow rate: 0.3-1.0 mL per min. Depending on column ID & length

b) Run time: 6-12 min.
MSMS Conditions: ES Negative
MRM of 321 >152 (for quantitation) & 321>257 (for confirmation)
MRM of Internal Standard (CAP-d5) 326>156

Note: The instrument parameters listed here are examples that worked with the equipment listed in the method. The analyst should optimize parameters for the instrument used.

1.2.5 Preparations of standard stock solutions:

Dissolve appropriate amount of Chloramphenicol for a final stock concentration of 1000 mg/L in acetonitrile (ACN) which is stable for one year if stored in freezer (-18°C approximately) and intermediate standard solutions prepared in acetonitrile is stable for 3 month if stored in the refrigerator (1-5°C approximately). The working standard solutions of µg/L levels for calibration curve are prepared by dilution in water on the day of analysis.

Prepare a 20 µg/L concentration of Internal Standard of CAP-d5 in water (stable for three months) from intermediate solution (of 1 mg/L prepared in 50:50 v/v ACN: water) that is prepared from 100 mg/L stock solution.

1.2.6 Laboratory Sample: Division into subsamples:

The Laboratory sample has to be divided into subsamples of at least 30 gm. The subsamples should be stored in freezer at approximately at -18°C.

1.2.7 Extraction Procedure:

Weigh precisely 5gm of defatted milk/ 2.5gm honey/ 2.5gm homogenized edible portion in case of tissue sample in a centrifuge tube. Add equal amount of water i.e. 2.5 mL in case of honey and tissue samples. Add 200 µL of CAP-d5 (20 µg/L) in sample. Add 10 mL ethyl acetate and stir thoroughly for 10 min. on a vortex/ rotary shaker. Centrifuge at about 5,000 gm for 15 minutes. Transfer the upper ethyl acetate layer in a clean Turbovap tube concentrator under nitrogen, repeat extraction with another 5 ml ethyl acetate & collect all ethyl acetate layers in the same Turbovap tube and dry under nitrogen at about 45°C. Dissolve the dried residue in 1mL Hexane: Carbon tetrachloride (1:1, v/v) by vortexing using a vortex mixer. Add 1mL water and mix properly by vortexing. Centrifuge at about 5,000gm gravity for 15 min for separation of layers. Transfer upper layer (water) quantitatively into LC vial for injection in HPLC-MS/MS.

1.2.8 HPLC-MS/MS Analysis: Inject appropriate and equal volumes (based on response/ Signal to noise ratio and sensitivity of the instrument) of final extract of sample as well as calibration dilutions of standards for calibration curve into LC system and obtain the MS chromatogram.

1.2.9 Injection Sequence:

a. Inject Solvent Blank
b. Inject calibration standard(s)
c. Inject the recovery sample
d. Inject the blank sample and verify the absence of analytes above 5% of the recovery or sample concentration(s).
e. Inject sample extract(s).

f. Re-inject the calibration standard at the appropriate level at least after every 20 injections and at the end of the run to verify instrument response.

Note: If significant carryover is detected, inject wash solution as needed until it is reduced to an acceptable level.

1.2.10 Calculations:

1. For Quantitation of each compound of interest:

a. Review the chromatograms to verify that the analyte peaks are within the retention time windows and that the peaks are integrated correctly.

b. Calculate the normalized peak for each component of interest by dividing the component response by the internal standard response:

$$\text{Normalized Response Component 1} = \frac{\text{Response of Component 1}}{\text{Response of Internal Standard}}$$

c. Generate a linear curve fit to each analyte in standard curve using normalized response to concentration in tissue (µg/kg or ppb).

d. Standard curve must have a correlation coefficient greater than or equal to 0.995.

e. Blank must exhibit a response of less than 5% of the recovery used contemporaneously in the set.

2. For Confirmation:

a. Choose a standard or recovery containing the analyte of interest.

b. Identify 2 product ion peaks in the sample and verify that their peaks are present with a signal to noise ratio ≥ 3. Auxiliary ions may be used if necessary.

c. Identify the retention time of the two product ion peaks in the standard or recovery and in the sample of interest. The sample peak retention times must be within ± 5% of the standard or recovery retention times.

d. Calculate the ratio of the response of product ion #2 to product ion #1 in the standard or recovery for the analyte of interest:

   Ratio = Product ion#2/ Product Ion #1 Note: Ion ratio should be less than 1. If not, then invert the ratio.

e. Ion ratios determined for each analyte shall be within tolerance limits as described in the EU document 2002/657/EC incase of positive samples. Suggested tolerances are based on EU guidelines and range from ± 20% for peaks greater than 50% of the base peak and to ± 50% for those less than or equal to 10% of the base peak.

Software provided in the instrument can be used for auto Quantitation by using linear regression (y=mx+b), where y=peak area/ height, x= Chloramphenicol concentration in ppb/ µg/kg, m=slope of curve, & b= intercept of y) for samples taking in to account dilution factor, if any.

1.2.11 References:

a) Chloramphenicol Identification by Liquid Chromatography Tandem Mass-Spectrometry, by AFSSA (now ANSES), Laboratoire de Fougères, la haute marche, Javene, 35133 Fougères, France

b) Commission Decision 2002/657/EC of 12 August 2002, implementing Council directive 96/23/EC concerning the performance of analytical methods and interpretation of results.

c) Joint FAO/WHO Food Standard Programme. Codex Alimentarius Commission. Report of the thirty fifth session of the Codex Committee on Pesticide Residues, Rotterdam, The Netherlands. 31stMarch - 5th April 2003. pp. 46-55.

1.3 DETERMINATION OF NITROFURAN METABOLITES – HPLC-MS/MS METHOD

1.3.1 Scope:

Determination of nitrofuran metabolites (SEM, AHD, AOZ & AMOZ) in foods of animal origin.

1.3.2 Reagents/ Chemicals:

HPLC grade Ethyl acetate, HPLC grade Acetonitrile, HCl 32%, 2- Nitrobenzaldehyde (AR/GR), Carbon tetrachloride, Tri-sodium-phosphate-do-deca-hydrate, Sodium hydroxide pellets, Ammonium acetate, Semicarbazide (SEM) as metabolite of Nitrofurazone, 3-amino-2-oxazolidinone (AOZ) as metabolite of Furazolidone, 1-aminohydantoin (AHD) as metabolite of Nitrofurantoin, 3-amino- 5-morpho linomethyl-2-oxazolidinone (AMOZ) as metabolite of Furaltadone and Internal Standards namely AMOZ-d5 & AOZ-d4 (Brand Sigma or equivalent) and gradient grade water.

1.3.3 Apparatus:

Blender, Vortex mixer/ rotary shaker, Centrifuge tubes (15/50 mL), Refrigerated centrifuge, Micropipettes, Turbovap concentrator under Nitrogen, LC vials, Analytical balance, Incubator cum rotary stirrer, pH meter.

1.3.4 Instrument:

Triple quadrupole HPLC-MS/MS and Analytical Column RP-18 end-capped, 250/150/100x4.6/3.0/2.1mm, 3-5µm particle size or its equivalent

HPLC Conditions:

a) Gradient Mobile Phase:

Mobile Phase A=0.1% Formic acid in water, B = ACN

Flow rate: 0.3-1mL/min. depending upon column length and ID.

b) Run time: 10-15 min based on column length & ID

MSMS Conditions: ES +ve mode

a) MRM of NPAMOZ: 335>291, 335>262

b) MRM of NPAMOZd5 – 340>296

c) MRM of NPAOZ - 236>134, 236>104

d) MRM of NPAOZd4 - 240>134

e) MRM of NPAHD - 249> 134, 249> 178

f) MRM of NPSEM: - 209>166, 209>192

Note: The instrument parameters listed here are examples that worked with the equipment listed in the method. The analyst should optimize parameters for the instrument used.

1.3.5 Preparations of Solutions Reference standards:

1.3.5.1 Procedure for Preparation of Standards of Nitrofuran Metabolites

Stock solutions of AOZ, AMOZ, AHD, SEM, AOZ-d4 & AMOZ-d5

- Weigh 10 mg each of AOZ, AMOZ, AHD, SEM, AOZ-d4 & AMOZ-d5 pure standards separately & diluted to 50 mL in Methanol to get 200 µg/mL (200ppm) individual standard solutions of AOZ, AMOZ, AHD, SEM, AOZ-d4 & AMOZ-d5.

- 2.5 mL of each above 200 ppm individual standard solutions are separately diluted & volume made up to 10 mL with methanol to get 50ppm (Stock solution) individual standard solutions of AOZ, AMOZ, AHD, SEM, AOZ-d4 & AMOZ-d5.

Intermediate mix metabolite standard Solution of AOZ, AMOZ, AHD & SEM

- 1 mL each of AOZ, AMOZ, AHD & SEM Individual Stock solutions diluted & volume made up to 50 mL with methanol to get1 ppm Intermediate mix metabolite standard solution (MM1).

Intermediate mix metabolite internal standard solution of AOZ-d4 & AMOZ-d5

- 1 ml each of AOZ-d4 & AMOZ-d5 Individual Stock solutions diluted & volume made up to 50 ml with Methanol to get1 ppm Intermediate mix metabolite internal standard solution (IS1)

Working mix metabolite Standard solution of AOZ, AMOZ, AHD & SEM

- 500 µL of MM1 diluted and volume made up to 10 mL IN Methanol: Water (50:50) to get 50 ppb working mix metabolite standard solution (MM2).

Working mix metabolite standard solution of AOZ-d4 & AMOZ-d5

- 500 µL of IS1 diluted & volume made up to 10 mL in Methanol:Water (50:50) to get 50ppb working mix metabolite standard solution (IS2).

1.3.6 Preparation of Calibration curve:

The mixed calibration standards should be prepared afresh for sample analysis: - Transfer 0 µL, 40 µL, 80 µL, 160 µL, 240 µL, 320 µL and 400 µL of 50 ppb MM2 in separate centrifuge tubes of 50 mL already having blank matrix 4 gm. To all the tubes add 40 µL of 50 ppb IS2 Standard solution each (corresponding to 40 ppb in final 1 mL extract volume in LC vial) to this add 10 mL of 0.2 M HCl & 250 µL of 100 mM, 2-NBA solution in methanol, screw cap the tube & vortex for about a minute. Incubate overnight (at least 16 hrs.) at 37°C ± 2°C, alternatively 50°C ± 2°C for four hours in an Incubator cum Rotary Shaker. Cool the tube to room temperature after incubation. Add 250 µL of 0.3 M Tri-sodium-phosphate solution and adjust the pH to neutral using 2M NaOH solutions if required. Add 10 mL ethyl acetate and hand mix/ vortex for 5 minutes each tube, ensuring no emulsion formation. Centrifuge at 4000gm for 10 minutes. Transfer the ethyl acetate layer in a clean concentrator tube and repeat extraction with 10 mL ethyl acetate by vortexing for 5 minutes and centrifuge at 5000 gm for 10 minutes. Collect this ethyl acetate also to the same concentrator tube & dry under nitrogen in Turbovap II concentrator at about 45°C. A wash with Hexane: Carbon tetrachloride (50:50 v/v), may be required if coloration or fat content is observed. Use 1 mL

of this mix & vortex for a minute & then add 1 mL water in the same tube and vortex properly, then Centrifuge at about 5000gm for 10-20 minutes. Transfer the clean upper water layer in to a LC vial for injection into HPLC-MS/MS. Each of these vials has concentration equivalent to 0.0, 0.5, 1.0, 2.0, 3.0, 4.0 & 5.0 µg/kg of derivatized mix AMOZ, AOZ, AHD & SEM standards. These are used for preparation of calibration curve for sample analysis. The calibration curve shall have at least 5 points including 0.0µg/kg.

Prepared Nitrofuran metabolites & their Internal Standards stock solution in Methanol are stable for one year when stored at 4°C in dark and Mixture of Standards is stable for six month when stored at 4°C. The working standard solutions are stable for one week.

1.3.7 Laboratory Sample: Division into subsamples

The Laboratory sample has to be divided into subsamples of at least 30gm. The subsamples should be stored in freezer at approximately at -18°C.

1.3.8 Extraction Procedure:

Weigh precisely 4 gm of defatted milk/ honey/ homogenized edible portion in case of tissue sample in a centrifuge tube add 40 µL of 50 ppb IS2 Standard solution each (corresponding to 4 ppb in final 1 mL extract volume in LC vial) to this add 10 mL of 0.2M HC1 & 250 µL of 100mM, 2-NBA solution in methanol, screw cap the tube & vortex for about a minute. Incubate overnight (at least 16 hrs.) at 37°C ± 2°C, alternatively 50°C ± 2°C for four hours in an Incubator cum Rotary Shaker. Cool the tube to room temperature after incubation Add 250µL of 0.3 M Tri-sodium-phosphate solution and adjust the pH to neutral using 2M NaOH solutions if required. Add 10 mL ethyl acetate and hand mix/ vortex for 5 minutes each tube, ensuring no emulsion formation. Centrifuge at about 4000 gm for 10 minutes. Transfer the ethyl acetate layer in a clean concentrator tube and repeat extraction with 5ml ethyl acetate by vortexing for 5 minutes and centrifuge at 5000 gm for 10 minutes, collect this ethyl acetate also to the same concentrator tube & dry under nitrogen in Turbovap concentrator at about 45°C. A wash with Hexane: Carbon Tetrachloride (v/v:

50/50), may be required if coloration or fat content is observed use 1 mL of this mix & vortex for a minute & then add 1mL Water in the same tube and vortex properly, then Centrifuge at 5000 gm for 10-20 minutes. Transfer upper layer (water) quantitatively into LC vial for injection in HPLC-MSMS.

1.3.9 HPLC-MS/MS Analysis: Inject appropriate and equal volumes (based on response/ Signal to noise ratio and sensitivity of the instrument) of final extract of sample as well as calibration dilutions of standards for calibration curve into LC-MS/MS system and obtain the MS Chromatogram.

Injection Sequence

a. Inject Solvent Blank
b. Inject calibration standard(s)
c. Inject the recovery sample
d. Inject the blank sample and verify the absence of analytes above 5% of the recovery or sample concentration(s).
e. Inject sample extract(s).
f. Re-inject the calibration standard at the appropriate level at least after every 20 injections and at the end of the run to verify instrument response.

Note: If significant carryover is detected, inject wash solution as needed until it is reduced to an acceptable level.

1.3.10 Calculations:

1. For Quantitation of each compound of interest:

a. Review the chromatograms to verify that the analyte peaks are within the retention time windows and that the peaks are integrated correctly.

b. Calculate the normalized peak for each component of interest by dividing the component response by the respective internal standard response:

$$\text{Normalized Response Component 1} = \frac{\text{Response of Component 1}}{\text{Response of Internal Standard}}$$

c. Generate a linear curve fit to each analyte in standard curve using normalized response to concentration in tissue (µg/kg or ppb).

d. Standard curve must have a correlation coefficient greater than or equal to 0.995.

e. Blank must exhibit a response of less than 5% of the recovery used contemporaneously in the set.

2. For Confirmation:

a. Choose a standard or recovery containing the analyte of interest.

b. Identify 2 product ion peaks in the sample and verify that their peaks are present with a signal to noise ratio ≥ 3. Auxiliary ions may be used if necessary.

c. Identify the retention time of the two product ion peaks in the standard or recovery and in the sample of interest. The sample peak retention times must be within ± 5% of the standard or recovery retention times.

d. Calculate the ratio of the response of product ion #2 to product ion #1 in the standard or recovery for the analyte of interest:

Ratio = Product ion#2/ Product Ion #1 Note: Ion ratio should be less than 1. If not, then invert the ratio.

e. Ion ratios determined for each analyte shall be within tolerance limits as described in the EU document 2002/657/EC incase of positive samples. Suggested tolerances are based on EU guidelines and range from ± 20% for peaks greater than 50% of the base peak and to ± 50% for those less than or equal to 10% of the base peak.

Software provided in the instrument can be used for auto Quantitation by using linear regression (y=mx+b), where y=peak area/ height, x= nitrofuran metabolite concentration in

ppb/ µg/kg, m=slope of curve, & b= intercept of y) for samples taking in to account dilution factor, if any.

1.3.11 References:

a) Detection and Identification of Metabolites of Furazolidone (AOZ), Furaltadone (AMOZ), Nitrofurantoin (AHD) AND Nitrofurazone (SEM) by LCMS-MS confirmatory analysis by State Institute for Quality Control of Agricultural products (RIKILT) Netherlands.

b) A method for the determination Nitrofuran veterinary drug residues by LCMS-MS by P. Hancock, A. Newton, G. Kearney, Thorsten Bernsmann, Peter Furst and Hans (j) A. van Rhijn; Waters Corporation, Manchester UK CVUA Munster, 48151 Munster, Germany.

c) Commission Decision 2002/657/EC of 12 August 2002, implementing Council directive 96/23/EC concerning the performance of analytical methods and interpretation of results

d) Joint FAO/WHO Food Standard Programme. Codex Alimentarius Commission. Report of the thirty fifth session of the Codex Committee on Pesticide Residues, Rotterdam, The Netherlands. 31stMarch - 5th April 2003. pp. 46-55.

1.4 DETERMINATION OF TETRACYCLINES – HPLC-UV/DAD / LC-MS/MS METHOD

1.4.1 Scope:

Determination of Tetracyclines in foods of animal origin

1.4.2 Instruments & Apparatus:

Mortar- pestle or blender, polypropylene centrifuge tubes of 50mL, separating funnels, conical flasks, R.B. flasks, rotary vacuum evaporator, syringe filter (0.2 µm), vials, cartridge C_{18} , Solid Phase Extractor & Centrifuge, Liquid Chromatograph –UV/ Dual

Absorbance / MSMS Detector, HPLC Column –reverse phased deactivated silica packing C_8 / C_{18}, 5 μm, 250 x 4.6 mm id, Turbovap concentrator under Nitrogen.

1.4.3 Chemical/reagents:

Citric acid monohydrate AR, Disodium hydrogen phosphate AR, Phosphoric Acid AR, Oxalic acid AR, Acetonitrile LC, Methanol (LC grade), Water (LC grade).

1.4.4 Standards:

USP reference standard of Tetracycline hydrochloride (TC), Oxytetracycline hydrochloride (OTC) & Chlortetracycline hydrochloride (CTC) and their epimers, (Sigma – Aldrich)

Weigh 108±0.1mg each of the Tetracycline hydrochloride in weighing dishes and transfer with methanol into separate 100 mL standard volumetric flask. Make up to volume with Methanol to get 1000-ppm stock solution. Dilute 1 mL of stock to 100 mL with methanol to obtain 10-ppm intermediate solution (I). Again dilute 10 mL of intermediate (I) solution to 100 mL to obtain 1-ppm intermediate solution (II). Pipette out 1 mL, 2 mL, 3 mL, 4 mL & 5 mL of intermediate solution (II) to 10 mL flask and dilute to volume with methanol to obtain appropriate calibration standards.

1.4.5 Preparation of reagent:

1. McIlvaine buffer pH 4.0
    a. 0.1M Citric acid monohydrate – 21.01 gm/L (If anhydrous – 19.213 gm/L) in water
    b. 0.2 M Na_2HPO_4 – 28.4 gm/L

    Mix 61.45 mL of solution (a) and 38.55 mL of solution (b). Adjust pH to 4.00 with dilute H_3PO_4

2. 0.01 M methanolic oxalic acid (1.26 gm Oxalic acid /L methanol)

HPLC Operating Conditions: UV- Detection at 350 nm

1. Mobile Phase – Methanol: Acetonitrile: 0.01 M Oxalic acid in water (73:17:10)
2. Flow rate: 1 mL/minute

HPLC conditions: Mass spectrometry

LC Conditions :

Column Reverse phase, C8, 2.1 × 150 mm, 5 μm; Flow rate 0.3 mL/min

Mobile phase A: water/0.1% formic acid: B: methanol

Gradient 0–10 min, B from 5% to 30%, 10–12 min, B from 30% to 40%, 12.5–18 min, B 65%, 18.5–25 min, B 95%, 25.5 min, B 5.0%, Total run 28 min; Post time 5 min

Temperature 30 °C, Injection 5 μL

MS Source Settings

Source ESI: Ion polarity Positive

MRM Settings

Name of the Fragment	Precursor ion	Product ions
4-Epitetracycline	445	410,427
4-Epioxytetracycline	461	426,444
Tetracycline	445	410,427
Oxytetracycline	461	426,443
4-Epichlortetracycline	479	444, 462
Chlortetracycline	479	444,462

Note: The instrument parameters listed here are examples that worked with the equipment listed in the method. The analyst should optimize parameters for the instrument used.

1.4.6 Test Procedure (Tissue Extraction):

Accurately weigh 5.0 ±0.05 gm of well ground homogenized edible portion of muscle tissue into a 50 mL polypropylene centrifuge tube. Macerate/blend the tissue with 20 mL, 20 mL, 10 mL of McIlvaine buffer repeatedly each for at least 30sec, collecting the extract after each addition. Centrifuge each of the extract at 2500 gm for 10 minutes. Filter the supernatant through GF/B filter paper in a Buchner funnel and moisten with McIlvaine buffer- EDTA solution. Collect the filtrate in a 125mL sidearm flask, applying gentle vacuum to side arm.

1.4.6.1 Extraction from Milk

1. Weigh a 5 gm milk sample (accurate to 0.01 gm) into a 50-mL colorimetric tube.
2. Dissolve with 0.1 mol/L Na_2EDTA-Mcllvaine buffer solution, and adjust the volume to 50 mL.
3. Vortex mix for 1 min.
4. Ultrasonically extract in an ice water bath for 10 min, then transfer to a 50-mL polypropylene centrifugal tube.
5. Cool down to 0~4°C.
6. Centrifuge at a rotate speed of 5000g for 10 min (below 15°C) and filter with fast filter paper.

1.4.6.2 Column Chromatographic separation (SPE)

1. Fit the C_{18} cartridge tube on to the SPE extractor.
2. Condition the tube with 20 mL of methanol followed by 20 mL water and discard the eluate.
3. Add the sample filtrate to the tube (maintain a flow rate not exceeding 4 mL/minute)
4. Wash with 20 mL distilled water and let the cartridge dry when the water rinse is complete and continue to draw air through the cartridge for $\geq$2min.
5. Elute with two 5 mL portions of methanolic oxalic acid
6. Collect the elute and evaporate to less than 1 mL under Nitrogen using Water bath at about 40°C

7. Make up to 1 mL with methanol and filter through 0.2 μm filter into an HPLC vial and inject.

1.4.6.3 LC Analysis

Inject appropriate volumes (based on response/ Signal to noise ratio and sensitivity of the instrument) of filtered sample extract as well as calibration dilutions of standards for calibration curve into LC system operated isocratically at a mobile phase flow rate of 1.0 mL/minute; with UV detector set at 350 nm/ MS detector and obtain the Chromatogram.

Injection Sequence

a. Inject Solvent Blank
b. Inject calibration standard(s)
c. Inject the recovery sample
d. Inject the blank sample and verify the absence of analytes
e. Inject sample extract(s).
f. Re-inject the calibration standard at the appropriate level at least after every 20 injections and at the end of the run to verify instrument response.

Note: If significant carryover is detected, inject wash solution as needed until it is reduced to an acceptable level.

1.4.7 Calculations:

1. For Quantitation of each compound of interest:

a. Review the chromatograms to verify that the analyte peaks are within the retention time windows and that the peaks are integrated correctly.

b. Calculate the normalized peak for each component of interest by dividing the component response by the internal standard response (in case available and used as stated below):

$$\text{Normalized Response Component 1} = \frac{\text{Response of Component 1}}{\text{Response of Internal Standard}}$$

c. Generate a linear curve fit to each analyte in standard curve using normalized response to concentration in tissue (µg/kg or ppb).

d. Standard curve must have a correlation coefficient greater than or equal to 0.995.

e. Blank must exhibit a response of less than 5% of the recovery used contemporaneously in the set.

2. For Confirmation:

a. Choose a standard or recovery containing the analyte of interest.

b. Identify 2 product ion peaks in the sample and verify that their peaks are present with a signal to noise ratio ≥ 3. Auxiliary ions may be used if necessary.

c. Identify the retention time of the two product ion peaks in the standard or recovery and in the sample of interest. The sample peak retention times must be within ± 5% of the standard or recovery retention times.

d. Calculate the ratio of the response of product ion #2 to product ion #1 in the standard or recovery for the analyte of interest:

Ratio = Product ion#2/ Product Ion #1 Note: Ion ratio should be less than 1. If not, then invert the ratio.

e. Ion ratios determined for each analyte shall be within tolerance limits as described in the EU document 2002/657/EC incase of positive samples. Suggested tolerances are based on EU guidelines and range from ± 20% for peaks greater than 50% of the base peak and to ± 50% for those less than or equal to 10% of the base peak.

Software provided in the instrument can be used for auto Quantitation by using linear regression (y=mx+b), where y=peak area/ height, x= Tetracycline concentration in ppb/ µg/kg, m=slope of curve, & b= intercept of y) for samples taking in to account dilution factor, if any.

1.4.8 Reference:

2. AOAC Official Methods of Analysis, 2005, 995.09, Ch. 23.1.17, p-22 to 26
3. Agilent LC/MS Applications for Drug Residues in Foods, Solution Guide, April 9, 2009.
4. Commission Decision 2002/657/EC of 12 August 2002, implementing Council directive 96/23/EC concerning the performance of analytical methods and interpretation of results

1.5 DETERMINATION OF SULPHONAMIDES – HPLC-MS/MS METHOD

1.5.1 Scope:

Determination of sulphonamides in foods of animal origin

1.5.2 Instruments & Apparatus:

Mortar cum pestle or blender, separating funnels, conical flasks, syringe filter (0.2 μm), vials, cartridge C_{18} , Solid Phase Extractor & Centrifuge, shaker, HPLC-MS/MS (Triple quadrupole) Detector, HPLC Column - Cat. No. Eclipse XDB-C18 RRHT 1.8 μm, 2.1 x 50 mm, Zorbax or equivalent

1.5.3 Chemical/reagents:

Sodium di-hydrogen orthophosphate (AR), Sodium chloride, Sodium hydroxide (AR), Chloroform (AR), Water (LC), Methanol (LC grade)

1.5.4 Reference Standards:

Sulfapyridine (SPY) (internal standard)
Sulfathiazole (STZ)
Sulfadiazine (SDZ)
Sulfaquinoxaline (SQX)
Sulfaethoxypyridazine (SEP)
Sulfadimethoxine (SDM)

Sulfachloropyridazine (SCP)
Sulfamethazine (SMZ)
Sulfamethoxazole (SMX)
Sulfamethoxypyridazine (SMP)
Sulfadoxine (SDX)
Sulfamerazine (SMRZ)
Sulfisoxazole (SSXZ)
Sulfamethizole (SMZL)
(All of Sigma – Aldrich)

1.5.5 Preparation of reference standard Stock solutions (1 mg/mL):

Weigh 100 ± 0.1 mg of the sulfonamide of interest including the internal standard (Sulfapyridine) into separate 100 mL volumetric flasks. Dissolve and bring to volume with acetone.

Note**:** If needed, a smaller amount of a sulfonamide stock solution may be made. If using a sodium salt of the sulfonamide then the weight must be corrected as needed. Dissolve the sodium salt of the sulfonamide with a few drops of distilled water and then bring to volume with acetone.

Recommended working standards (used for fortification):

i. Mixed standard solution (5.0 µg/mL):
   Pipette 0.5 mL of each stock sulfonamide solution (Except the internal standard, SPY) into a 100 mL volumetric flask. Bring to volume with phosphate buffer.
ii. Internal Standard Solution (IS) (2.50µg/mL):
   Pipette 0.5 mL of the 1 mg/mL Stock Solution (SPY) into a 200 mL volumetric flask. Bring to volume with phosphate buffer.

Note: When quantitating large potential positives at levels above the routine curve, it may be necessary to make a more concentrated mixed standard solution (e.g 50 µg/mL) to bracket the expected concentration (s) of analyte in the sample (s).

1.5.6 Standard Solution Stability:

Place all working standards in polyethylene or polypropylene bottles and store refrigerated (2 - 8°C). Shelf life is 3 months.

Place all stock solutions prepared in acetone in polyethylene or polypropylene bottles and store at < -10°C. Shelf life is 6 months.

1.5.7 Sample Preparation:

Samples of liver and muscle are processed until homogeneous. All samples are stored refrigerated or frozen until analyzed.

1.5.8 Analytical Procedure:

1. Preparation of standard curve and recoveries.
   a. Weigh a 2.5 ± 0.1gm portion of a blank control matrix for the blank.
   b. Weigh four (4) additional 2.5 ± 0.1gm portions of a blank control matrix in 50 mL polypropylene centrifuge tubes.

      Fortify each tube as follows:

Level of Fortification (ppm of tissue)	Fortification Solution (µg/mL)	Amount spiked (µL)
0.05	5.0	25
0.10	5.0	50
0.20	5.0	100
Recovery (0.10 ppm)	5.0	50

   c. Add 100 µL of Internal Standard Solution (2.5 µg/mL) to all standards for calibration curve, recoveries, blanks and internal checks.

1.5.9 Preparation of samples to be analyzed:

Weigh 2.5 ± 0.1gm of each thawed sample into a 50 mL polypropylene centrifuge tube. Add 100 µL of Internal Standard Solution (2.5 µg/mL) to all samples for a 0.1 ppm level fortification.

Note: Sample amounts less than 2.5gm may be used when confirming suspected high positive values. Also, a higher concentration of the internal standard may be used to allow for sample extract dilution.

1.5.10 Extraction:

a. Add 6.0 mL ethyl acetate to standards and samples and vortex for 2 minutes. Let samples stand for at least 10 minutes.

   Manual – Vortex or shake by hand to break up sample. Place samples on horizontal shaker for approximately 10 minutes on high or vortex for 2 minutes. Let stand at least 10 minutes or centrifuge for 5 minutes at 2500 rpm.

b. Filter ethyl acetate through a fast flow filter column and collect filtrate into a clean 15 mL centrifuge tube.

c. Add 1.0 mL 3.2 M HCl.

d. Vortex for 30 seconds and let stand for at least 5 minutes

   Manual – Shake approximately 5 minutes on high or vortex for 30 seconds. Let stand at least 5 minutes or centrifuge for 5 minutes at 2500 rpm.

e. Aspirate ethyl acetate to waste.

f. Add 5.0 mL hexane and vortex for 30 sec., let stand for 5 minutes.

   Manual – Shake approximately 5 minutes on low or vortex for 30 sec., and let stand for at least 5 minutes or centrifuge for 5 minutes at 2500 rpm.

g. Aspirate hexane to waste.

h. Add 2.0 mL 3.5M sodium acetate.

i. Add 3.0 mL ethyl acetate, vortex for 30 sec., and let stand for at least 5 minutes.

   Manual – Shake 5 minutes on low or vortex for 30 sec. and let stand for at least 5 minutes or centrifuge for 5 minutes at 2500 rpm.

j. Transfer ethyl acetate to a clean centrifuge tube.

k. Evaporate final extract to dryness under nitrogen with a water bath temperature set to 40 ± 5°C.

l. Add 100 µL of methanol to the residue. Vortex on high speed to dissolve. Dilute the samples and standards with 400 µL of mobile phase A. The volume of mobile phase A added must be consistent across all samples and standards. Vortex again on high speed to mix.

m. Transfer to centrifugal filter tubes (Cat. No. 82031-356, VWR).

n. Centrifuge at approximately 3000 rpm until sufficient volume of filtrate has been collected for HPLC analysis (approximately 5 to 10 minutes).

o. Transfer to LC auto sampler vials.

Note: Extract stability for SSXZ & STZ is 24 hours for liver and muscle for quantitation when refrigerated. All other analytes are stable for LC Instrument Settings:

Note: The instrument parameters listed here are examples that worked with the equipment listed in the method. The analyst should optimize parameters for the instrument used.

1.5.11 LC analysis:

Inject appropriate volumes (based on response/ Signal to noise ratio and sensitivity of the instrument) of filtered sample extract as well as calibration dilutions of standards for calibration curve into LC system and obtain the Mass Chromatogram.

Column Temperature: 70°C

Injection Volume: 4 µL

Initial Flow Rate: 0.65 mL/min

HPLC Mobile Phase:

A (0.1% Formic Acid): Add 2 mL Formic Acid to approximately 500 mL of water in 2 L volumetric flask. Bring to volume with water.

B (20% Isopropanol in 0.1% Formic Acid): Add 800 mL HPLC Mobile Phase A (0.1% Formic Acid) to a 1 L HPLC mobile phase container. Add 200 mL Isopropanol to the same container. Mix thoroughly.

Gradient Program:

Time (min)	Flow Rate (mL/min)	A (%)	B (%)
0	0.65	95	5
2	0.65	95	5
5	0.65	0	100
5.1	0.65	95	5
10	0.65	95	5

Mass Spec/ LC interface Settings

Note: The analyst should optimize parameters for the instrument used.

Polarity ES+

Analyte	**Parent Ion**	**Product Ions**	**Analyte**	**Parent Ion**	**Product Ions**
SDZ	251	156, 108	SMX	254	156, 108
STZ	256	156, 108	SSXZ	268	156, 113
SPY	250	156, 108	SDX	31	156, 108
SMRZ	265	156, 92	SEP	295	156, 92
SMZL	271	156, 92	SDM	311	156, 108
SMP	281	126, 188	SQX	301	156, 108
SMZ	279	186, 124	SCP	285	156, 108

(1) **Very Important** - If the parent and daughter ions for two analytes are not completely resolved by mass, then the analytes must be completely resolved chromatographically (baseline resolution). For example, SDM and SDX must be completely resolved chromatographically; so must SDZ and SPY.

(2) Fragmentation Energies - The fragmentation energies are instrument specific and should be optimized on each instrument for each parent ion.

(3) Product Ions – The first product ion listed is the recommended Quantitation ion, though the other ions may be used in case of unusual interferences or changes in instrument conditions. The auxiliary ions are at much lower abundances but can be used to help identify a compound if an unusual interference is present

(4) Collision Energies (CE) - The CE settings are instrument specific and should be optimized on each instrument for each product ion.

Injection Sequence:

a. Inject Solvent Blank
b. Inject calibration standard(s)
c. Inject the recovery sample
d. Inject the blank sample and verify the absence of analytes above 5% of the recovery or sample concentration(s).
e. Inject sample extract(s).
f. Re-inject the calibration standard at the appropriate level at least after every 20 injections and at the end of the run to verify instrument response.

Note: If significant carryover is detected, inject wash solution as needed until it is reduced to an acceptable level.

1.5.12 Calculations:

1. For Quantitation of each compound of interest:

a. Review the chromatograms to verify that the analyte peaks are within the retention time windows and that the peaks are integrated correctly.

b. Calculate the normalized peak for each component of interest by dividing the component response by the internal standard response:

$$\text{Normalized Response Component 1} = \frac{\text{Response of Component 1}}{\text{Response of Internal Standard}}$$

c. Generate a linear curve fit to each analyte in standard curve using normalized response to concentration in tissue (µg/kg or ppb).

d. Standard curve must have a correlation coefficient greater than or equal to 0.995.

e. Blank must exhibit a response of less than 5% of the recovery used contemporaneously in the set.

2. For Confirmation:

a. Choose a standard or recovery containing the analyte of interest.

b. Identify 2 product ion peaks in the sample and verify that their peaks are present with a signal to noise ratio ≥ 3. Auxiliary ions may be used if necessary.

c. Identify the retention time of the two product ion peaks in the standard or recovery and in the sample of interest. The sample peak retention times must be within ± 5% of the standard or recovery retention times.

d. Calculate the ratio of the response of product ion #2 to product ion #1 in the standard or recovery for the analyte of interest:

Ratio = Product ion#2/ Product Ion #1 Note: Ion ratio should be less than 1. If not, then invert the ratio.

e. Ion ratios determined for each analyte shall be within tolerance limits as described in the EU document 2002/657/EC incase of positive samples. Suggested tolerances are based on EU guidelines and range from ± 20% for peaks greater than 50% of the base peak and to ± 50% for those less than or equal to 10% of the base peak.

1.5.13 References:

1. Quantitation and Confirmation of Sulfonamides by Liquid Chromatography - Tandem Mass Spectrometry (LC-MS-MS), CLG-SUL4.01, USFDA method of 05/11/2011
2. Agilent LC/MS Applications for Drug Residues in Foods, Solution Guide, April 9, 2009.
3. Commission Decision 2002/657/EC of 12 August 2002, implementing Council directive 96/23/EC concerning the performance of analytical methods and interpretation of results

1.6 DETERMINATION OF QUINOLONES – HPLC/MSMS METHOD

1.6.1 Scope: Determination of Quinolones in foods of animal origin

1.6.2 Materials and Methods

1.6.2.1 Chemicals:

Flumequine (FLU), oxolinic acid (OXO), nalidixic acid (NAL), cinoxacin (CIN), piromidic acid (PIR) and pipemidic acid (PIP), marbofloxacin (MAR), norfloxacin (NOR), ciprofloxacin (CIP), lomefloxacin (LOM), danofloxacin(DAN), enrofloxacin (ENR), sarfloxacin (SAR), difloxacin (DIF), ofloxacin (OFL), enoxacin (ENO), orbifloxacin (ORB). The major metabolites of ENR are CIP, and desethylene ciprofloxacin (des-CIP). Sodium hydroxide and ultra pure sodium sulfate anhydrate, Formic acid and acetonitrile of HPLC grade. All reagents used should be of analytical grade.

1.6.3 Instrumentation and Conditions

1.6.3.1 Instrumentation:

The instruments for sample preparation consisted of a shaker, a centrifuge and a 4.6mm, 5 μm) analytical column. The triple-quadrupole MSMS coupled to HPLC using an electro-spray ionization interface in positive ionization mode (ESI+). Data acquisition by software

1.6.3.2 LC and MS/MS Conditions:

The injection volume is 20 μL and the analysis carried out with gradient elution using A eluent (20mM ammonium formate in 0.1% formic acid) and B eluent (acetonitrile) as the mobile phase at a flow rate 0.7 mL/min. The program of gradient elution is listed below table. In order to establish the optimized multiple reactions monitoring (MRM) conditions for individual compounds, the mass spectrometric conditions are to be optimized using infusion with a syringe pump/ direct injection of each QNs individually to select the most suitable ion transitions. Due to the presence of the amino group in most QNs that is easily protonated in acidic medium; the ion spray source is set in positive mode. The ESI/MS/MS and other chromatographic conditions may require optimization.

Table: Timetable of gradient elution program

Time (min)	A (%)*	B (%)
0	85	15
7	30	70

8	5	95
9	5	95
10	85	15
12	85	15

*A: 20mM ammonium formate in 0.1% formic acid, B: acetonitrile.

HPLC column: Suitable HPLC column. Please see reference.

Table: Optimize MS/MS for MRM transitions selected for quantification and identification of the Quinolones as follows;

Quinolone	Retention time (min)	Precursor ion (*m/z*)	Quantification	Identification
NAL	6.83	233	233 ➔215	233 ➔187
FLU	7.27	262	262 ➔244	262 ➔202
OXO	6.13	262	262 ➔244	262 ➔216
CIN	5.60	263	263 ➔217	263 ➔245
PIR	7.38	289	289 ➔271	289 ➔243
PIP	3.57	304	304 ➔286	304 ➔217
des-CIP	4.25	306	306 ➔288	306 ➔268
NOR	4.35	320	320 ➔302	320 ➔276
ENO	4.11	321	321 ➔303	321 ➔234
CIP	4.50	332	332 ➔314	332 ➔288
LOM	4.58	352	352 ➔308	352 ➔265
DAN	4.52	358	358 ➔340	358 ➔283
ENR	4.76	360	360 ➔342	360 ➔316
OFL	4.35	362	362 ➔318	362 ➔261
MAR	4.23	363	363 ➔345	363 ➔320
SAR	5.24	386	386 ➔368	368 ➔342
ORB	4.93	396	396 ➔352	396 ➔295
DIF	5.42	400	400 ➔382	400 ➔356

LC Instrument Settings:

Note: The instrument parameters listed here are examples that worked with the equipment listed in the method. The analyst should optimize parameters for the instrument used.

1.6.4 Preparation of Standard Solutions:

For the QNs with good solubility in alkaline solution (15), a 200 μg/mL stock standard solution is prepared for each of 18 QNs by dissolving the appropriate amount of standard in 50% acetonitrile containing 2% of 0.1 N NaOH. Working standard solutions are prepared by serial dilution of standard solutions with 10% acetonitrile. Stock and working standard solutions are stored at 4°C in brown volumetric flasks for at least 3 months without any degradation. Five working standard solutions ranging from 0.5 to 10 ng/mL are prepared for external standard calibration. The working standard solutions are spiked into blank matrix samples to the desired concentrations (0.25, 0.5, 1.0, 2.5 and 5.0 ng/g). Then, the fortified sample is allowed to stand for 15 min at room temperature and samples are extracted as detailed in sample preparation below.

1.6.5 Sample Preparation:

Thawed grounded tissue sample (fish, shrimp, pork and Chicken)/ milk/ honey and weigh 2.0 g/mL of sample and place in a 50mL polypropylene centrifuge tube. Twenty milliliters acetonitrile containing 1% formic acid is added to the sample, which then is reversely shaken for 5 min at high setting. Two grams of sodium sulfate anhydrate is added to each tube, which then is reversely shaken again for another 5 min, followed by centrifugation for 10 min (6000 rpm, 4°C). The acetonitrile extract is evaporated in a rotary evaporator at 40°C. The residue is resuspended in 2 mL of 10% Acetonitrile containing 0.1% formic acid, poured into a 16 × 12mm glass tube and defatted by extraction with 4 mL hexane while mixing on a Vortex mixer in 15 sec twice.

The mixture is centrifuged for 5 min (4000 rpm, 4°C) and the aqueous supernatant is transferred and filtered through 0.22 μm Nylon membrane before injection into LC/ESI/MS/MS system.

Injection Sequence:

a. Inject Solvent Blank

b. Inject calibration standard(s)
c. Inject the recovery sample
d. Inject the blank sample and verify the absence of analytes above 5% of the recovery or sample concentration(s).
e. Inject sample extract(s).
f. Re-inject the calibration standard at the appropriate level at least after every 20 injections and at the end of the run to verify instrument response.

Note: If significant carryover is detected, inject wash solution as needed until it is reduced to an acceptable level.

1.6.6 Calculations:

1. For Quantitation of each compound of interest:

a. Review the chromatograms to verify that the analyte peaks are within the retention time windows and that the peaks are integrated correctly.

b. Calculate the normalized peak for each component of interest by dividing the component response by the internal standard response:

$$\text{Normalized Response Component 1} = \frac{\text{Response of Component 1}}{\text{Response of Internal Standard}}$$

c. Generate a linear curve fit to each analyte in standard curve using normalized response to concentration in tissue (µg/kg or ppb).

d. Standard curve must have a correlation coefficient greater than or equal to 0.995.

e. Blank must exhibit a response of less than 5% of the recovery used contemporaneously in the set.

2. For Confirmation:

a. Choose a standard or recovery containing the analyte of interest.

b. Identify 2 product ion peaks in the sample and verify that their peaks are present with a signal to noise ratio ≥ 3. Auxiliary ions may be used if necessary.

c. Identify the retention time of the two product ion peaks in the standard or recovery and in the sample of interest. The sample peak retention times must be within ± 5% of the standard or recovery retention times.

d. Calculate the ratio of the response of product ion #2 to product ion #1 in the standard or recovery for the analyte of interest:

Ratio = Product ion#2/ Product Ion #1 Note: Ion ratio should be less than 1. If not, then invert the ratio.

e. Ion ratios determined for each analyte shall be within tolerance limits as described in the EU document 2002/657/EC incase of positive samples. Suggested tolerances are based on EU guidelines and range from ± 20% for peaks greater than 50% of the base peak and to ± 50% for those less than or equal to 10% of the base peak.

1.6.7 Reference:

1. Simultaneous Determination of 18 Quinolones Residues in Marine and Livestock Products by Liquid Chromatography/ Tandem Mass Spectrometry Journal of Food and Drug Analysis, Vol. 18, No. 2, 2010, Pages 87-97
2. Preparation & LCMSMS Analysis of Fluoroquinolones in Honey, Florida Dept of Agriculture & customer services, Method CR 405, Sept 2006
3. Quantitation and Confirmation of Sulfonamides by Liquid Chromatography - Tandem Mass Spectrometry (LC-MS-MS), CLG-SUL4.01, USFDA method of 05/11/2011
4. Commission Decision 2002/657/EC of 12 August 2002, implementing Council directive 96/23/EC concerning the performance of analytical methods and interpretation of results

1.7 DETERMINATION OF NITROIMIDAZOLES – HPLC-MSMS METHOD

1.7.1 Scope: Determination of Nitroimidazoles in foods of animal origin

1.7.2 Materials and Methods

1.7.2.1 Reagents and solutions:

Note: Equivalent reagents may be substituted if necessary.

1.7.2.1.1 Reagents:

a. Water - 18 megaohm Millipore grade, filtered before use.

b. Acetonitrile - LC grade.

c. Formic acid - Fluka Chemika.

1.7.2.1.2 Solutions:

a. 0.1% Formic acid in water: Add 1 mL of formic acid to water. QS with water to 1liter and filter through a 0.45 µm filter before use.

b. 0.1% Formic acid in acetonitrile: Add 1 mL of formic acid to acetonitrile. Adjust volume to 1 L with acetonitrile.

1.7.2.2 Reference standards:

Metronidazole (MNZ), Dimetridazole (DMZ), Ronidazole (RNZ), Ipronidazole (IPZ) and their hydroxyl metabolites MNZ-OH, HMMNI and IPZ-OH, Carnidazole (CRZ), Ornidazole (ONZ), Ternidazole (TRZ) & Tinidazole (TNZ), internal Standard DMZ-d3, RNZ-d3, IPZ-d3, IPZ-OH-d3 of Sigma/ any other.

1.7.2.3 Preparation of Standards:

a. To prepare 0.5 mg/mL of standards: Weigh each 25 mg (to nearest 0.1 mg) into a 50mL volumetric flask and dilute to volume with ethyl acetate. Stable for at least one year

b. Fortification Solution, 5 µg/mL: Pipette 1.00 mL of each of nitroimidazole stock solutions into a 100 mL volumetric flask, and dilute to volume with ethyl acetate. Stable for at least one year

c. Working Standards: for spiking and Calibration curve should be prepared afresh on the day of analysis.

Storage Conditions: All standards should be kept tightly closed and refrigerated at -20˚C when not in use.

1.7.3 Equipment:

Note: An equivalent may be substituted for any equipment listed below.

1. Instrumentation:

a. HPLC -MS/MS – Triple quodrupole tandem mass spectrometer.

b. ESI mode +ve

2. Apparatus:

a. Balance - capable of weighing 50gm within 0.1gm,

b. Centrifuge tube - 250 mL polypropylene

c. Centrifuge - Capable of holding 250 mL centrifuge tubes,

d. Round bottomed flask - 500 mL with 24/40 ground glass joint

e. Rotary evaporator

f. Separating funnel -125 mL, with stopper,

g. Scintillation vial - 20 mL,

h. pH meter or pH indicator paper

i. Pasteur pipet - Short,

j. C-18 SPE column - High capacity (1000 mg),

k. Vacuum Manifold.

l. Glass culture tube - 13 x 100 mm,

m. Graduated centrifuge tubes - 10 mL,

n. N-Evap - Cat. No. 111, Organomation Inc.

o. Acrodisc LC-13 PVDF filter - 0.45 μm,

p. Microfilterfuge filter - 0.2 μm nylon filter,

q. Analytical column - RP-18, 150 x 4.6mm column, 3μ particle size

r. Guard column - Guard cartridge C18, 4mm x 3.0mm

s. Meat Grinder

1.7.4 Sample Preparation:

1. **Muscle Tissue:** Pass tissue through a meat grinder (5/32" plate) and mix, or blend in a food processor until homogeneous. Store frozen until the sample is ready for extraction.
2. **Milk and Honey Samples** Milk and honey can as such be stored at -20°C until analysis. Portions of these samples

1.7.5 Analytical Procedure:

1.7.5.1 Extraction in muscle tissue

a. Add 10gm of K_2HPO_4, 10gm NaCl, and 50 ± 1gm of thawed tissue to a 250 mL plastic centrifuge tube and is fortified with mixed internal standard which corresponds to 6 ng/mL. Note: Prepare a negative and positive control at this time. Weigh two blank tissues (tissue shown to contain no analyte or interfering chromatographic peaks). Fortify one at 1 ppb level by adding 10 μL of Fortification Solution.

b. Add 100 mL ethyl acetate and shake by hand or mechanical shaker for 1 minute.

c. Centrifuge at 1500 rpm for 5 minutes. Decant supernatant into a 500 mL round bottom flask being careful to not decant the salts.

d. Repeat steps b and c, combining the extracts.

e. Evaporate the ethyl acetate to an oily residue using a rotary evaporator with a water bath temperature of 50-55°C.

f. Add 2 mL of 1M HCI and 2.5 mL of ethyl acetate to each flask and swirl contents.

g. Transfer extract to a 125 mL separating funnel containing 15 mL of hexane.

h. Repeat step f and add the second HCI/ethyl acetate rinse to the hexane.

i. Gently swirl or rotate separating funnel for 1 minute, to partition analytes across interface, taking care to avoid forming large emulsions. Allow layers to separate. After this period, attempt to disrupt any emulsions that remain by carefully swirling the separating funnel.

j. Collect bottom aqueous layer in a scintillation vial or small beaker. Leave behind any emulsion.

Note: If a sizeable emulsion layer remains at this point, transfer to a 50 mL centrifuge tube and centrifuge at 2500 rpm for 10 minutes. Add the bottom aqueous layer from the centrifuge tube to the previously collected aqueous solution.

k. Carefully adjust to pH 4.8 - 5.2 by the dropwise addition of 5N NaOH or 1N HCI.

Caution: Increased volumes of extract applied to the SPE column may result in decreased DMZOH recoveries. Analyst should take care not to overshoot the pH endpoint.

1.7.5.1.1 Solid Phase Extraction:

a. Attach a Baker high capacity C18 SPE column to vacuum chamber.

b. Using a mild vacuum to assist drainage, pass the following through the column:

i. 10 mL methanol

ii. 10 mL chloroform

iii. 10 mL methanol

iv. 20 mL distilled water

Do not let the column fully drain at any time during this step.

c. When water meniscus is within 1/8 inch of C18 bed add sample extract and allow to flow through column by gravity or gentle vacuum until fully drained. Apply vacuum for several seconds to remove residual water from the column.

d. Elute analytes from column with 3 mL of methanol using mild vacuum. Collect eluate in a 13 X 100mm glass culture tube using an N-Evap maintained at 50 ± 5°C, and evaporate samples to dryness using a gentle stream of N_2.

e. Add 1.0 mL water and mix thoroughly.

f. Filter solution through 0.22 µm syringe filter into a glass HPLC vial for analysis

1.7.5.2 Extraction in Milk:

Milk (1mL) is pipetted into polypropylene centrifuge tubes (15mL) and fortified with mixed internal standard (30 µL) which corresponds to 6 ng/mL and Acetonitrile (2mL) is added and vortexed. NaCl (0.5gm) is added to this slurry which is shaken (30 sec) and then centrifuged (4350 x gm for 10min). The top organic layer from each sample is then transferred to amber vials (5mL) and evaporated (50°C) to dryness under a stream of nitrogen. The extracts is reconstituted in Water: Acetonitrile (95:5, 200µL) and filtered through 0.2µm PVDF syringe filters. An aliquot of appropriate amount is injected onto the LC column.

1.7.5.3 Extraction in Honey:

Honey (3 gm) is weighed into polypropylene centrifuge tubes (50 mL). These are then placed in an oven at 50°C for 30 min to soften. The samples are then fortified with mixed internal standard which correspond to2 µg/kg. Water (5mL) is then added to each sample and these are then placed back in the oven for a further 10 min. The samples are then thoroughly vortexed until the honey is fully dissolved in the water. To this acetonitrile (10mL) is added and the tubes are vortexed (20sec). NaCl (2gm) is added to this slurry which is then shaken (30sec) and centrifuged (4350 x gm for 10min). The top organic layers are then transferred to polypropylene tubes (15 mL) and evaporated (50°C) to 6mL under nitrogen. Hexane (5 mL) is added and this is vortexed (30sec). The hexane layer is then discarded and the extracts are evaporated to dryness at 50ºC under a nitrogen stream. They are then reconstituted in Water: ACN (200 µL of 95:5) and filtered through 0.2 µm PVDF syringe filters. An aliquot of appropriate amount is injected onto the LC column.

1.7.6 LC and MS/MS Conditions

1.7.6.1 HPLC Analysis:

a. **Recommended Instrumental Settings and Conditions.** Note: Actual settings may be adjusted, if necessary to optimize performance.

Set up and tune LC/MS system.

Note: The following instrument conditions reflect optimal conditions for the specific instruments used to develop this method. It may be necessary to modify these parameters to optimize performance of any given instrument.

a. HPLC conditions:

Flow rate 0.4 mL/min Flow ramp 2.00, Column Temperature 25°C
Mobile Phase:
0.1% Formic acid in water: Add 1 mL of formic acid to water. QS with water to 1 liter and filter through a 0.45 µm filter before use (A).
0.1% Formic acid in acetonitrile: Add 1 mL of formic acid to acetonitrile.
b. Adjust volume to 1 L with acetonitrile (B).
Ratio: 40/60, (A)/ (B), Run Time: 10 minutes
c. Analytical column - Phenomenex RP-18, 150 x 4.6mm column, 3µ particle size
d. Guard column - Phenomenex SecurityGuard cartridge C18, 4mm x 3.0mm ID.

b. MS Tuning Parameters:

ESI polarity positive Capillary Voltage 3 kV Cone Voltage 18 V Extractor Voltage 2.00 V RF Lens 0.1 Source Temperature 140 °C Desolvation Temperature 450 °C Desolvation Gas Flow 650 L/hr Cone Gas Flow 150 L/hr MS1 Low Mass Resolution 15.0 MS1 High Mass Resolution 15.0 Ion Energy 10.5 Entrance Lens -5 Collision Gas Flow 18 Exit Lens 3 MS^2 Low Mass Resolution 14.5 MS^2 High Mass Resolution 14.5 Ion Energy 20 Multiplier Voltage 650 V

c. MRM:

Analyte	**Parent Ion**	**Product Ions**	**Analyte**	**Parent Ion**	**Product Ions**
MNZ-OH	188	123,126	TNZ	248	121,82
HMMNI	158	110,140	IPZ-OH	186	168,122
MNZ	172	82,128	ORZ	220	128,82
RNZ	201	140,55	CRZ	245	118,75
DMZ	142	96,81	IPZ	170	124,109
TRZ	186	128,82	DMZ-d3	145	99
RNZ-d3	204	143	IPZ-d3	189	171
IPZ-OH-d3	189	171			

Injection Sequence:

a. Inject Solvent Blank
b. Inject calibration standard(s)
c. Inject the recovery sample
d. Inject the blank sample and verify the absence of analytes above 5% of the recovery or sample concentration(s).
e. Inject sample extract(s).
f. Re-inject the calibration standard at the appropriate level at least after every 20 injections and at the end of the run to verify instrument response.

Note: If significant carryover is detected, inject wash solution as needed until it is reduced to an acceptable level.

1.7.7 Calculations:

1. For Quantitation of each compound of interest:

a. Review the chromatograms to verify that the analyte peaks are within the retention time windows and that the peaks are integrated correctly.

b. Calculate the normalized peak for each component of interest by dividing the component response by the internal standard response:

$$\text{Normalized Response Component 1} = \frac{\text{Response of Component 1}}{\text{Response of Internal Standard}}$$

c. Generate a linear curve fit to each analyte in standard curve using normalized response to concentration in tissue (µg/gm or ppm).

d. Standard curve must have a correlation coefficient greater than or equal to 0.995.

e. Blank must exhibit a response of less than 5% of the recovery used contemporaneously in the set.

2. For Confirmation:

a. Choose a standard or recovery containing the analyte of interest.

b. Identify 2 product ion peaks in the sample and verify that their peaks are present with a signal to noise ratio ≥ 3. Auxiliary ions may be used if necessary.

c. Identify the retention time of the two product ion peaks in the standard or recovery and in the sample of interest. The sample peak retention times must be within ± 5% of the standard or recovery retention times.

d. Calculate the ratio of the response of product ion #2 to product ion #1 in the standard or recovery for the analyte of interest:

   Ratio = Product ion#2/ Product Ion #1 Note: Ion ratio should be less than 1. If not, then invert the ratio.

e. Ion ratios determined for each analyte shall be within tolerance limits as described in the EU document 2002/657/EC incase of positive samples. Suggested tolerances are based on EU guidelines and range from ± 20% for peaks greater than 50% of the base peak and to ± 50% for those less than or equal to 10% of the base peak.

1.7.8 Reference:

1. Rapid multi-class multi-residue method for the confirmation of Chloramphenicol and eleven Nitroimidazoles in milk and honey by liquid chromatography tandem mass Spectrometry, [Article published in Food Additives and Contaminants, xxx (2010) xxx–xxx, Author Name Mark Cronly, Patrice Behan, Barry Foley, Sheila Martin, Michael Doyle, Edward Malone and Liam Regan
2. Screen for Nitroimidazoles by HPLC, SOP No: CLG-NIMZ1.01, United States Department of Agriculture Food Safety and Inspection Service, Office of Public Health Science
3. Confirmation of Nitroimidazoles by ESI – LC/MS/MS, SOP No: CLG-NIMZ2.00 United States Department of Agriculture Food Safety and Inspection Service, Office of Public Health Science
4. Commission Decision 2002/657/EC of 12 August 2002, implementing Council directive 96/23/EC concerning the performance of analytical methods and interpretation of results

1.7.9 Validation of above Methods:

Guidelines given under European commission decision 2002/657/EC have been published for validation of analytical procedures for the purpose. The principles described in this section are considered practical and suitable for validation of veterinary drug residue analytical methods. The guidance is not normative. The analyst should decide on the degree of validation required to demonstrate that the method is fit for the intended purpose, and should produce the necessary validation data accordingly. For instance, the requirements for testing for compliance with MRPL/ MRLs or providing data for intake estimation may be quite different.

An analytical method is the series of procedures from receipt of a sample to the production of the final result. Validation is the process of verifying that a method is fit for the intended purpose. The method may be developed in-house, taken from the literature or otherwise obtained from a third party. The method may then be adapted or modified to match the requirements and capabilities of the laboratory and/or the purpose for which the method will be used. Typically, validation follows completion of the development of a method and it is assumed that requirements such as calibration, system suitability, analyte stability, etc. have been established satisfactorily. When validating and using a method of analysis, measurements must be made within the calibrated range of the detection system used. In general, validation will precede practical application of the method to the analysis of samples but subsequent performance verifications an important continuing aspect of the process. Requirements for performance verification data are a sub-set of those required for method validation.

Proficiency testing (or other inter-laboratory testing procedures), where practicable, provides an important means for verifying the general accuracy of results generated by a method, and provides information on the between laboratory variability of the results. However, proficiency testing generally does not address analyte stability or homogeneity and extractability of analyst in the processed sample.

Where uncertainty data are required, this information should incorporate performance verification data and not rely solely on method validation data. Whenever, a laboratory undertakes method development and/or method modification, the effect of analytical variables should be established, e.g. by using ruggedness tests, prior to validation. Rigorous controls must be exercised with respect to all aspects of the method that may influence the results, such as: sample size; partition volumes, variations in the performance of the clean-up systems used; the stability of reagents or of the derivatives prepared; the effects of light, temperature, solvent and storage on analytes in extracts; the effects of solvent, injector, separation column, mobile

1.7.10 Confirmatory Tests:

When analyses are performed for monitoring or enforcement purposes, it is especially important that confirmatory data are generated before reporting on samples containing residues of veterinary drugs etc that are not normally associated with that commodity, or where MRPL/ MRLs appear to have been exceeded. Samples may contain interfering chemicals that may be misidentified for which appropriate care shall be taken.

Confirmatory tests may be quantitative and/or qualitative but, in most cases, both types of information will be required. Particular problems occur when residues must be confirmed at or about the limit of determination but, although it is difficult to quantify at this level, it is essential to provide adequate confirmation of both level and identity.

1.7.11 Expression of results:

For regulatory purposes, only confirmed data should be reported, expressed as defined by the MRPL/MRL. Null values should be reported as being less than lowest calibrated level, rather than less than a level calculated by extrapolation. Results shall be corrected for recovery. If results are reported corrected for recovery, then both measured and corrected values should be given. The basis for correction should also be reported. Where positive results obtained by replicate determinations (e.g. on different LC columns, with different detectors or based on different ions of mass spectra) of a single test portion

(sub-sample), the lowest valued value obtained should be reported. Where positive results derive from analysis of multiple test portions, the arithmetic mean of the lowest valid values obtained from each test portion should be reported. Taking into account, in general, a 20-30% relative precision, the results should be expressed only with 2 significant figures (e.g. 0.11, 1.1, 11 and 1.1 x 102). Since at lower concentrations the precision may be in the range of 50%, the residue values below 0.1 should be expressed with one significant figure only (e.g. 0.01, 0.001).

PART B

METHOD FOR ANALYSIS OF OXYTOCIN

1.0 INTRODUCTION

Oxytocin is a cyclic octapeptide hormone released by the posterior pituitary and showing uterotonic and galactogenic activity. It is chemically L-Hemi-cystinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-L-hemi-cystinyl-L-propyl-L-leucyl glycinamide. Its 20 membered ring consists of five amino acids- cysteine, tyrosine, isoleucine, glutamine, and asparagines, further three amino acids, proline, leucine and glycinamide. Oxytocin is involved in the contraction of uterus and milk ejection in receptive mammals. In the brain oxytocin is classically viewed as primarily involved in the milk let down reflex and in the stimulation of uterine smooth muscle during parturition. When oxytocin is injected into cows, there is a result over production of milk and traces of oxytocin can be found in the same. When excess of oxytocin is found to be present in milk it may cause headache, nausea, abdominal pain, drowsiness etc.to the user.

Radioimmunoassay (RIA) and enzyme immunoassay (EIA) give the required sensitivity, but there are concerns that cross-reactivity may affect the results. An LC-MS/MS method would resolve this issue by providing greater specificity. In addition, recoveries and response linearity can be verified by spiking samples with stable isotope labeled analogues

Since there is no uniform harmonized method for analysis of oxytocin, literature review was done and the following three best suited methods were taken:

1. RP- HPLC
2. LC-MAS
3. HPLC

1.1 RP-HPLC METHOD FOR THE DETERMINATION OF OXYTOCIN IN MILK

1.1.1 Scope: Determination of oxytocin in milk

1.1.2 Instruments & Apparatus:

A reverse phase HPLC system (Schimadzu Prominence 20 AT) consisting of LC-20 AT pump, M- 20A PDA (Photo Diode Array) detector, Phenyl hexyl column (250mm x 4.6mm id, 5μm), 20μl Rheodyne injection syringe and LC Solution software. All the chemicals used should be of HPLC grade and AR grade

Optimized chromatographic conditions are listed in Table.1. Standard stock solution is prepared in 10 mL volumetric flask by dissolving accurately weighed quantities of oxytocin (10 mg) in HPLC grade water followed by dilution up to the mark with HPLC grade water(1000 μg/mL). From this further dilution is prepared to get the concentration of 0.5μg/mL, 1 μg/mL,1.5μg/mL, 2μg/mL, and 2.5μg/mL. These concentrations are taken for studying Linearity range of oxytocin.

Table 1: Optimized Chromatographic Conditions

S.No	Parameters	Optimized conditions
1.	HPLC Model	Schimadzu Prominence LC 20 AT
2.	Column	Phenyl hexyl column (250mm x 4.6 mm id , 5μm)
3.	Mobile phase	Acetonitrile: 0.03M Phosphate buffer(21:79), pH 3.5(Dil.Orthophosphoric acid)
4.	Flow rate	1 mL/min.
5.	Detection Wavelength	At 197 nm
6.	Injection volume	20μL
7.	Retention time	4.78
8.	Temperature	Room Temperature

1.1.3 Preparation of the Sample:

Sample concentration of milk is prepared by taking 1 ml of sample solution with 1 mL of ice cold solution of acetone. This solution is mixed and centrifuged at 3500 rpm in the centrifugal apparatus. After centrifugation process, the acetone layer is taken and mixed with 1 mL of petroleum ether. This solution is mixed and kept for 5 minutes. After 5 minutes,

the ether layer is discarded and the lower layer is evaporated to dryness, to this 0.2 mL buffer solution is added and filtered. The filtered solution is injected.

1.1.4 Method Validation:

The method is validated in terms of linearity, accuracy, Intra-day and inter-day, reproducibility and specificity. The limit of detection (LOD) and limit of quantification (LOQ) is also determined. The accuracy of the method is evaluated by carrying out recovery studies. For that, known concentration of the standard solution is added to the sample solution and recovery is calculated. The intra-day precision is determined by analyzing standard solutions in the linearity range of the calibration curve in triplicate on the same day. While inter- day precision is calculated by analyzing corresponding standard solutions daily, for a period of one week.

1.1.5 References:

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1.2 QUANTITATIVE ANALYSIS OF OXYTOCIN BY LC-MS/MSN

1.2.1 Scope: Detection of oxytocin at endogenous plasma concentrations by LC-MS/MS.

1.2.2 Sample Preparation:

Immunoprecipitaton (IP) was performed with the Seize Primary IP kit from Pierce, following the protocol supplied by the manufacturer. Oxytocin antibodies can be purchased from Millipore Corporation. Synthetic oxytocin are obtained from Bachem, Torrance, CA. Recovery is performed using phosphate buffered saline (PBS) spiked with oxytocin. The incubation time is 6 hours at room temperature. The initial sample volume is 200uL and each elution volume is 100uL at pH 2.8. The elution volumes are analyzed without further modification.

1.2.3 Sample Analysis:

Samples to generate the calibration curve are analyzed on an suitable HPLC and MS system. The column is a suitable 2.1x30mm 3.5um cartridge, with a flow rate of 400uL/min. Initial conditions are 98% solvent A (0.1% formic acid in water)/2% solvent B (0.1% formic acid in acetonitrile) ramped to 25:75 in 2 minutes, returned to initial conditions at 3 minutes, and equilibrated for 2 minutes, for a total run time of 5 minutes. The injection volume is 20uL. The most intense transition is chosen for monitoring during sample analysis. Angiotensin is spiked at 1 pg/µL before chromatographic analysis as an internal standard. Samples to test the IP assay are analyzed with the same HPLC method on an LCQ Classic ion trap mass spectrometer. The spiking level comes out to be 100pg/µL.

1.2.4 References:

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1.3 HPLC METHOD FOR THE DETERMINATION OF OXYTOCIN IN PHARMACEUTICAL DOSAGE FORM AND COMPARISON WITH BIOLOGICAL METHOD

1.3.1 Scope: Determination of oxytocin in pharmaceutical dosage form.

1.3.2 Materials:

Suitable acetonitrile are used, each HPLC-grade, phosphoric acid and chlorobutanol (Sigma), citric acid, sodium citrate and acetic acid (BDH) is used, each analytical reagent grade. An oxytocin solution prepared from oxytocin for Bioassay, 4th International Standard; 12.5 IU/amp, is used as standard. Biological activity and content of oxytocin is determined in an oxytocin synth. Preparation, ampoules 5 IU/mL.

1.3.3 Method:

HPLC analysis of oxytocin is performed by the method of Krummen et al. (5) with own modifications. Another column is chosen and the chromatographic conditions are optimized. A Liquid Chromatograph is used, operated in conjunction with an integrator, UV-VIS detector and auto-injector. An 5 μ column is used; the mobile phase is (18:82 v/v) acetonitrile-water adjusted with orthophosphoric acid to pH of 2.1. Detection is carried out at a wavelength λ = 220 nm. Flow rate is 1 mL/min; sample volume is 20 μL.

1.3.4 Preparation of oxytocin standard solution:

An international standard oxytocin (12.5 IU/ampoule) is dissolved in a placebo solution containing 0.5 gm chlorobutanol, 0.11 gm sodium acetate trihydrate and 0.25 gm concentrated acetic acid per 100 mL water. A series of solutions are prepared covering a concentration range from 1 to 10 IU/mL. These solutions are used to study whether or not the relationship between the peak area and oxytocin concentrations is linear. Repeatability and intermediate precision is studied on Oxytocin synth preparation, 5 IU/mL, with reference to an international standard of oxytocin at the same concentration. Samples of the standard and of the preparation examined are applied onto the column directly from ampoules.

1.3.5 References:

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Annexure-1

Maximum residue limits of antibiotics and other pharmacologically active substances in different countries

	EU μg/kg	**Codex μg/kg**	**Canada ppm**	**Japan ppm**	**FSSR ppm**
Tetracycline	Muscle- 100 Liver-300 Kidney-600 Milk-100 Eggs-200	Liver-600 Kidney-1200 Milk-100 muscle -200	Liver-0.6 Kidney-1.2 Milk-0.1 Muscle-0.2	Live 0.6 Kidney- 1.2 Milk- 0.1 Muscle-0.2	Seafood – 0.1
Chloramph-enicol	MRL cannot be established	-	-	-	Prohibited
Nitrofuran metabolites	MRL cannot be established	-	-	-	Prohibited
Sulphon-amides	Muscle- 100 Fat- 100 Liver- 100 Kidney-100 Milk-100	-	-	-	Prohibited

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