FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA

*Inspiring Trust, Assuring Safe & Nutritious Food*

## Training of Trainers Program on Analysis of Veterinary Drug Residues including Antibiotics

# TRAINING MANUAL

**Name:** _____

**Email:** _____

**Organized by:**

FSSAI & GFSP in association with PBTI
at

Punjab Biotechnology Incubator, Mohali, Punjab

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Global Food Safety Partnership

# MANUAL FOR VETERINARY DRUG RESIDUE ANALYSIS INCLUDING ANTIBIOTICS

Food Safety and Standards Authority of India
Ministry of Health and Family Welfare, Government of India

**Manual for veterinary drug residue analysis including antibiotics, 2018.**

**ABOUT THE MANUAL**

The current manual borrows its majority of content and structure from the Training of Trainers (ToT) manual prepared by IFSTL partners USFDA, USDA and JIFSAN and with their consent. This manual is complementary to the workshop laboratory manual and lecture notes. The manual is meant to provide future trainers with an opportunity to make notes on various aspects of logistics associated with giving the training to a group.



## LABORATORY SAFETY

1. Laboratory coats should be worn at all times in the laboratory.
2. Use fume hoods whenever handling volatile or hazardous chemicals.
3. Safety goggles should be worn at all times in the laboratory.
4. Appropriate gloves should be worn as needed.
5. Gloves and lab coats should not be worn outside the laboratory.
6. Appropriate closed-toed shoes should be worn in the laboratory.
7. All samples and transfer containers must be labelled.
8. MSDS are available in a clearly marked binder in the laboratory if you wish to consult them.
9. In the event of an emergency please contact one of the supervising instructors immediately.

# FOREWORD

Laboratories are crucial to national veterinary drug residue monitoring programmes. However, one of the main challenges laboratories encounter is obtaining access to relevant methods of analysis. Thus, in addition to training, providing technical advice and transferring technology, the FSSAI has resolved to develop clear and practical manuals to support laboratories at national level. FSSAI with the support of scientific committee has developed a number of analytical methods as standard operating procedures (SOPs), which are now compiled in various manuals. This manual contains SOPs on chromatographic and spectrometric techniques, as well as veterinary drug residue analysis in different food commodities. Some analytical method validation protocols are also included. The publication is primarily aimed at food safety laboratories involved in testing veterinary drug residues, including under organized national residue monitoring programmes. It is expected to enhance laboratory capacity and competence of various laboratories in India.

Quality Assurance Division
FSSAI

# Contents

## Chapter 1

# 1. INTRODUCTION

## 1.1. BACKGROUND

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. 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.

## 1.2. OBJECTIVE

The purpose of this manual is to aid food testing laboratories primarily in the routine monitoring, determination and control of residues of some veterinary drugs in animal products.

## 1.3. SCOPE

This manual publication consists of analytical techniques, methods in form of SOPs, for testing selected veterinary drug residues in some animal products. It covers a number of chromatographic–spectrometric techniques. The information in the manual is also presented as an informative guide in some respect.

## 1.4. STRUCTURE

The preliminary (and larger) part of the manual consists of chromatographic and spectrometric SOPs for analysis of antimicrobial residues such as chloramphenicol, nitrofuran metabolites and tetracyclines, sulfonamides as well as quinolones. The manual then concludes a reference validation protocol.

## Chapter 2

## 2. Introduction on Veterinary Drugs

***Veterinary drugs*** are defined as any substance applied or administered to any food-producing animal, such as meat or milk producing animals, poultry, fish or bees, whether used for therapeutic, prophylactic, or diagnostic purposes, or for modification of physiological functions or behavior.

***Residues of veterinary drugs***are pharmacologically active substances, whether active principles, excipients or degradation products, and their metabolites which remain in foodstuffs obtained from animals to which the veterinary medicinal product in question has been administered.The definition includes the parent compounds and/or their metabolites in any edible portion of the animal product, and includes residues of associated impurities of the veterinary drug concerned.

There are various functional classes of veterinary drugs. The functional classes as classified by Codex Alimentarius Commission are: Adrenoceptor agonist, Anthelmintic agent, Antibacterial agent, Antifungal and antiprotozoal agent, Antimicrobial agent, Antiparasitic agent, Antiprotozoal agent, Beta-adrenoreceptor blocking agent, Glucocorticosteroid, Growth promoter, Insecticide, Production aid, Tranquilizing agent and Trypanocide.

Veterinary drugs are being increasingly used in farming of livestock, poultry birds and aquaculture. The main purpose of use of these drugs is to prevent outbreak of diseases, prevent mortality during transport, increase freshness or to stimulate growth during farming. As a consequence, veterinary drugs appear in the final product as residues and further continue in the food chain. Consumption of animal products (e.g. fish, shrimp, milk, meat, etc.) containing residues of these compounds for protracted period of time can render undesirable adverse health effects. Major concerns of veterinary drugs residues are development of carcinogenicity, development of resistant bacteria and allergic reaction in hypersensitive individuals.

From an animal and human health perspective, responsible use of antibiotics is of importance and therefore extensive monitoring programs are in place within India. The methods used for analysis of veterinary drugs aim for the detection, quantitation and confirmation of the drug present in products of animal origin.

### 2.1Antibiotics and their veterinary usage

Antibiotics are used to treat infections caused by bacteria and other microorganisms. Traditionally, the term “antibiotics” is used to describe any substance produced by a

micro-organism that is effective against the growth of another micro-organism. Nowadays the term **"antibiotics"** is used interchangeably with the term "antibacterials", and includes synthetic substances like sulfonamides and quinolones as well.

### 2.2 Antibiotic usage and its impact in food chain

Nowadays, the use of antibiotic agents in animal breeding for food production is general practice. Antibiotics are used to treat bacterially infected animals but are also administered as a preventive measure. Furthermore, administration of antibiotics at sub-therapeutic doses has a growth promoting effects, making its use economically advantageous. This is especially of interest since the ban of antimicrobial growth promoting substances in animal feed since 2006. Antibiotic usage in veterinary practice in the India is monitored to obtain insight in the exposure of farm animals to antibiotics.

Antibiotics such as tetracyclines, quinolones, macrolides, sulphonamides and penicillins are frequently used in animal husbandry for the treatment and prevention of diseases. To avoid undesired residues of these substances in food, maximum residue limits (MRLs) have been established within India. Food produced or imported toIndia has to fulfil these residue requirements. Self-monitoring by the food industry, farmers and retailers is an important tool to ensure the consumer safety with regard to antibiotic residues. Nevertheless compliance with this self– monitoring program has to be safeguarded by the competent authorities.

Excessive antibiotic usage in veterinary practice in food producing animals can have adverse effects on human health. Some antibiotics are banned for use in veterinary practice because of their negative effects on health, like bone marrow toxicity, aplastic anemia and carcinogenicity. If these antibiotics are illegally administered, residues might occur in food products of animal origin. The adverse effects of the occurrence of these antibiotics in the food chain do not need any further elaboration.

European Union has classified and fixed limits of pharmacologically active substances in foodstuffs of animal origin (EC regulation 37/2010). The table 1 in the annex provides limits for allowed substances with MRL, whereas Table 2 provides list of prohibited substances for which no MRLs are established.

FSSAI as per Food Safety and Standards (Contaminants, Toxins and Residues) Regulations, 2011 has provided tolerance (Table 1) for antibiotics and also the list of prohibited pharmacologically active substances in fish and fishery products (Table 2).

**Table 1:** Tolerance for Antibiotics in Fish and fishery products as per FSSR (2011)

| Sl.No. | Name of Antibiotic | Tolerance Limit (mg/Kg) |
|---|---|---|
| 1. | Tetracycline | 0.1 |
| 2. | Oxytetracycline | 0.1 |
| 3. | Trimethoprim | 0.05 |
| 4. | Oxolinic acid | 0.3 |

**Table 2:**List of prohibited pharmacologically active substances in fish and fishery products (FSSR, 2011)

1. Nitrofurans including
    i. Furaltadone
    ii. Furazolidone
    iii. Furylfuramide
    iv. Nifuratel
    v. Nifuroxime
    vi. Nifurprazine
    vii. Nitrofurnatoin
    viii. Nitrofurazone
2. Chloramphenicol
3. Neomycin
4. Nalidixic Acid
5. Sulphamethoxazole
6. Aristolochia spp and preparations thereof
7. Chloroform
8. Chloropromazine
9. Colchicine
10. Dapsone
11. Dimetridazole
12. Metronidazole
13. Ronidazole
14. Ipronidazole and other nitromidazoles
15. Clenbuterol
16. Diethylstibestrol (DES)
17. Sulphonamide drugs except sulfadimethoxine, sulfabromomethazine and sulfaethoxypyridazine
18. Fluroquinolones
19. Glycopeptides

USFDA classifies Chloramphenicol, Nitrofurans, Fluoroquinolones and Quinolones, Malachite Green and Steroid Hormones as high enforcement priority aquaculture drugs. Drugs which are prohibited by FDA for extra label use includes Chloramphenicol, Clenbuterol, Diethylstilbestrol (DES), Dimetridazole, Ipronidazole and other Nitroimidazoles, Furazolidone and Nitrofurazone, Fluoroquinolones and Glycopeptides. Only oxytetracycline, florfenicol, sulfadimethoxine and ormetoprim are approved for use for specificfinfish/shellfish species.

*Definition: "Maximum Residue Limit (MRL) ► the maximum concentration of residue resulting from the use of a veterinary medicinal product which may be accepted by the regulatory authority to be legally permitted or recognised as acceptable in or on a food. It is based on the type and amount of residue considered to be without any toxicological hazard for human health as expressed by the acceptable daily intake (ADI), or on the basis of a temporary ADI that utilises an additional safety factor. It also takes into account other relevant public health risks as well as food technology aspects."*

**2.3 Method criteria**

Earlier reference methods were established indicating analytical techniques and methods for detection of specific residues, where screening and confirmatory methods could be distinguished. This concept was superseded by a criteria based approach as laid down in Commission Decision 2002/657/EC. In this document the minimum requirements of analytical methods for both screening and confirmatory methods are described.

*Screening method ►Methods that are used to detect the presence of a substance or class of substances at the level of interest. These methods have the capability for a high sample throughput and are used to sift large numbers of samples for potential non-compliant results. They are specifically designed to avoid false compliant results.*

*Confirmatory method ► Methods that provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest.*

As per USFDA, each analyticalmethod must specify Testing Target Level (TTL)for unapproved animal drug residues. TargetTesting Level (TTL)is basedon the accuracy of the method and lab equipment. Regulatory enforcement action is taken against product with positivefindings at or above TTL.

The Target Testing Levels established by USFDA for various drug-fish species combinations are as follows:

| Species | Residue | TTL |
|---|---|---|
| **Shrimp and crab** | Chloramphenicol (CAP) | 0.3 ppb |
| **Shrimp** | Nitrofurans | 1 ppb |
| **Shrimp** | Fluroquinolones | 5 ppb |
| **Shrimp** | Quinolones-Flumequine, Oxolinic Acid, Nalidixic Acid | 10 ppb |
| **Tilapia** | Methyl testosterone | 0.8 ppb |
| **Tilapia** | Malachite Green | 1 ppb |
| **Tilapia** | Gentian violet | 1 ppb |
| **Tilapia** | Fluroquinolones | 5 ppb |
| **Tilapia** | Sulfonamides | 10 ppb |

**Chapter 3**

## Sample Preparation/Processing for fishery products

The samples used in this laboratory were frozen, and taken out of the freezer ~2 hoursprior to the laboratory session so that they can be processed, but are still very cold. Using of cold matriceshelps reduce the amount of evaporation.Sample size of ~ 350-400gm received in the laboratory is provided to two groups

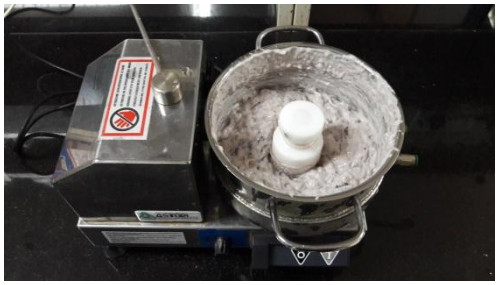
**Procedure for thawing:**
The sample unit is thawed by enclosing it in a film type bag and immersing in water at room temperature (not greater than 35°C). The complete thawing of the product is determined by gently squeezing the bag occasionally so as not to damage the texture of the shrimp, until no hard core or ice crystals are left.

**Processing of samples:**

- A sample of approximately 400gm of shrimp has been assigned to your team.
- Remove the non-edible parts including the shells, cut the samples into small pieces (~2 cm)
- Position the blade and lock the jar onto the food processor base.
- Blend for about 60-120 seconds while turning the blender paddle to gather the ice
- There is no need to clean the blender between the two halves of a same sample if quantity is more than the capacity of blender.
- Wash the blender thoroughly with detergent and water, rinse 5 times and dry**.**
- Clean your food processing, workspace and all glassware.

After grinding, homogenize the material and pack the same in sample pouches, sealed and transferred to deep freezer till analysis (The processing of sample homogenization is given below)






## Chapter 4

## ANALYTICAL TECHNIQUES IN ANTIBIOTIC RESIDUES ANALYSIS

Analytical methods in antibiotic analysis are generally divided in screening and confirmatory methods. Screening methods are usually inexpensive, rapid and suitable for high-throughput analysis, but do not provide unequivocal identification and usually do not result in exact quantitative results. Various bioanalytical screening methods such as microbial inhibition tests (four plate test), immunoassays (lateral flow) and ELISA are available and can be used for screening of antibiotic residues. Confirmatory methods must be instrumental spectrometric techniques and therefore are more expensive and time-consuming, but are supposed to be highly selective in order to provide unequivocal identification. The combination of a bio-based screening method and an instrumental confirmatory method is very strong in residue analysis. With a bio-based screening a fast qualification (compliant or suspect) of samples can be made based on biological activity. Compliant samples can be reported right away and the usually few suspect samples can be subsequently analysed by a more elaborate confirmatory method based on chemical properties of the compound.

Until the last decade of the 20$^{th}$century, the main instrumental techniques used for veterinary drug residue analysis were liquid chromatography (LC) using ultra violet detection (UV), diode array detection (DAD) and fluorescence detection (FLD), and gas chromatography (GC) using flame ionisation detection and electron capture detection. *Chromatography* is a separation technique (Figure 1)based on the different interactions of compounds with two phases, a *mobile phase* and a *stationary phase*, as the compounds travel through a supporting medium; mobile phase: a solvent that flows through the supporting medium; stationary phase: a layer or coating on the supporting medium that interacts with the analytes and supporting medium: a solid surface on which the stationary phase is bound or coat.

The analytes interacting most strongly with the stationary phase will take longer to pass through the system than those with weaker interactions (Figure 1).These interactions are usually chemical in nature, but in some cases physical interactions can also be used.

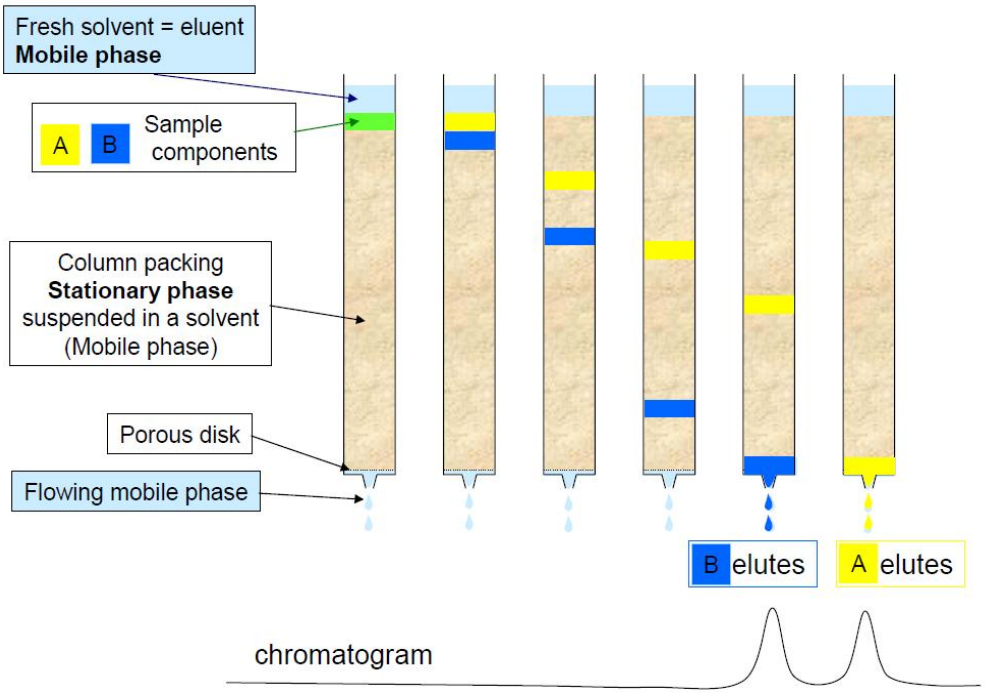



**Figure 1: Chromatographic separation**

Mass spectrometer is an instrument in which ions are analyzed according to their mass-to-charge ratio, and in which the number of ions is determined electrically. For the most part, there are four basic components that are standard in all mass spectrometers (Figure 2). These are i) sample inlet ii) ionization source iii) mass analyzer and iv) ion detector.

High performance liquid chromatography hyphenated with mass spectrometry comprises of a high performance liquid chromatograph (HPLC) attached, via a suitable interface, to a mass spectrometer (MS). The primary advantage HPLC/MS has over GC/MS is 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 HPLC/MS, even proteins may be routinely analysed. Solutions derived from samples of interest are injected onto an HPLC column that comprises a narrow stainless steel tube (usually 150 mm length and 2 mm internal diameter, or smaller) packed with fine, chemically modified silica particles. 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). Components eluting from the chromatographic column are then introduced to the mass spectrometer via a specialised interface. The two most common interfaces used for HPLC/MS are the electrospray ionisation (ESI) and the atmospheric pressure chemical ionisation (APCI)

Although there are many variations of mass spectrometers the process by which all sample molecules are analysed is similar regardless of instrument configuration. Sample

molecules are introduced into the instrument through a sample inlet. Once inside the instrument, the sample molecules are converted to ions in the ionization source, before being electrostatically propelled into the mass analyzer. Ions are then separated according to their m/z within the mass analyzer. The detector converts the ion energy into electrical signals, which are then transmitted to a computer.

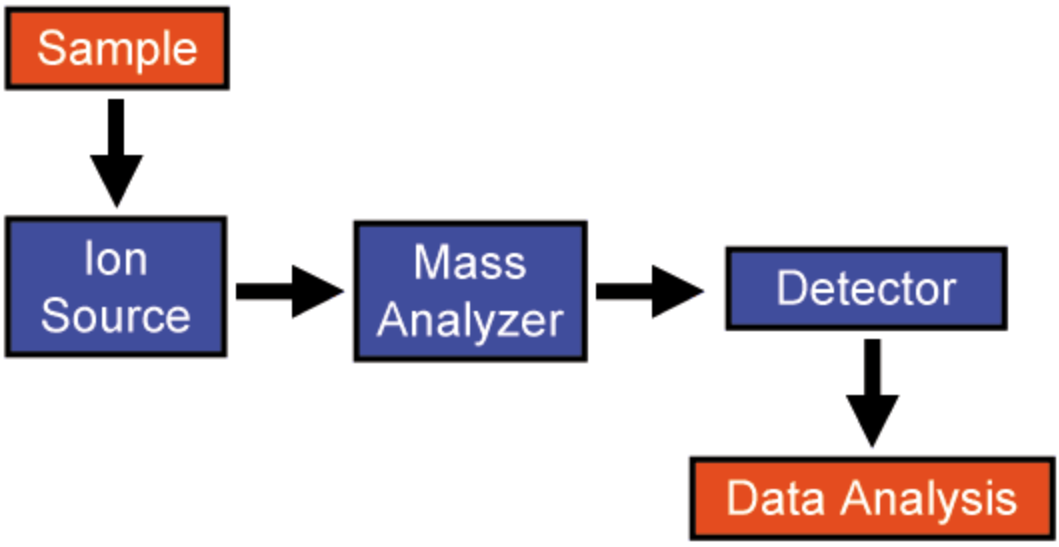



**Figure 2: Basis components of a mass spectrometer**

**Liquid Chromatography to Mass Spectrometry: Interfaces and Ionisation Techniques**

The main obstacle in the development of the hyphenated technique LC-MS was converting the analyte in the mobile phase to gas phase ions in order for them to be analysed by the MS. This resulted in the need for an interface linking the two techniques. This interface works at atmospheric pressureand allows for the liquid to be changed into gas phase and also ionises the analyte. This interface type is known as atmospheric pressure ionisation (API). There are many different designs of this interface but an example of one can be seen in Figure 3.

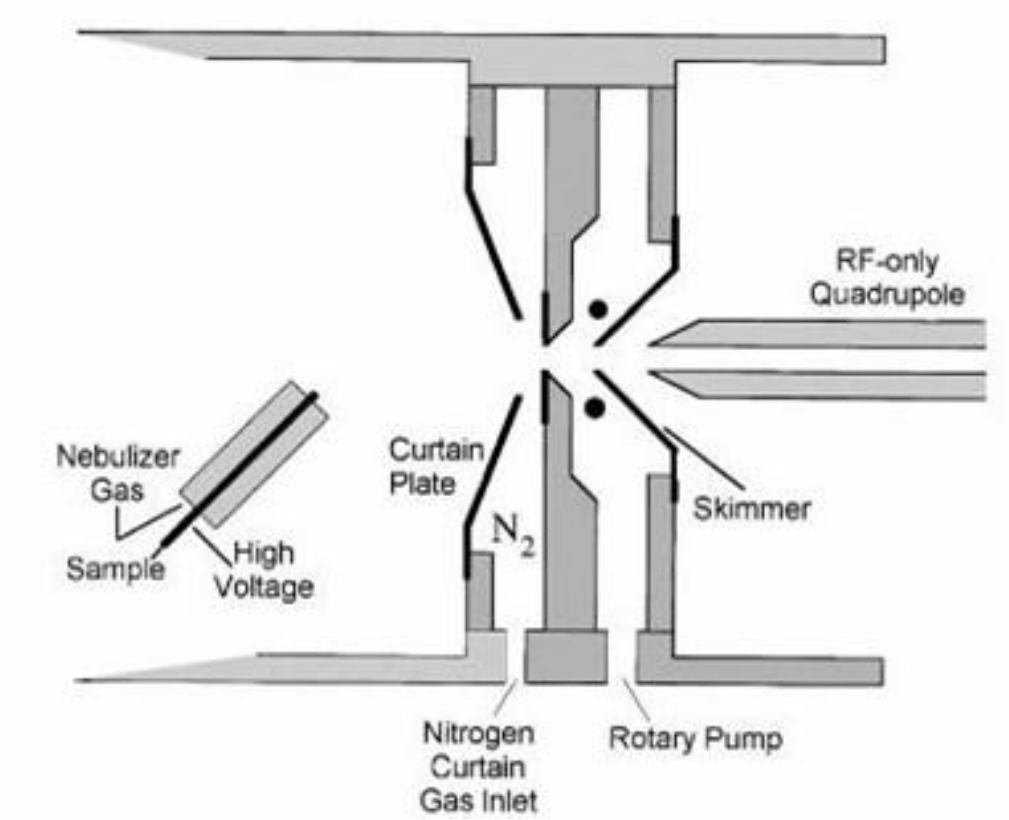



**Figure 3. Diagram of API interface**

Although ionisation is carried out at atmospheric pressure there are numerous different ionisation techniques that may be used with LC-MS. The most common ones used in veterinary residue analysis are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI). From examination of Figure 4 it is clear that ESI works bestover a broader range of different analytes. Compounds with higher polarity and molecular weights can only be analysed by ESI.

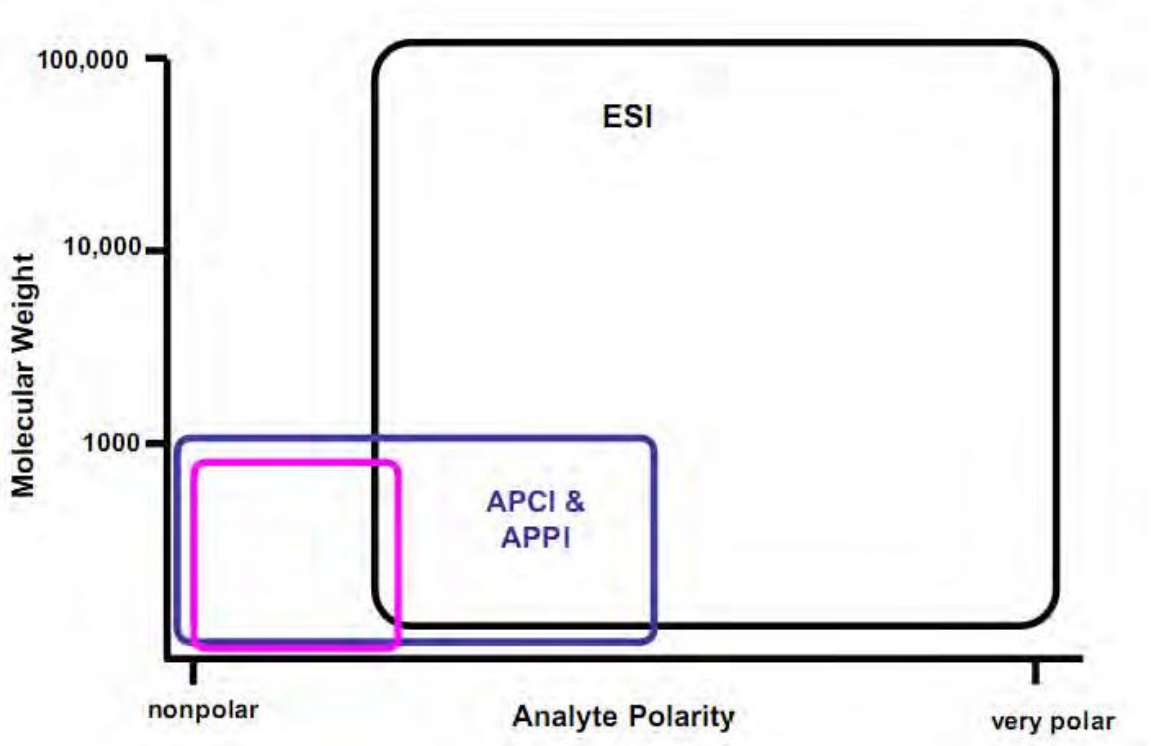
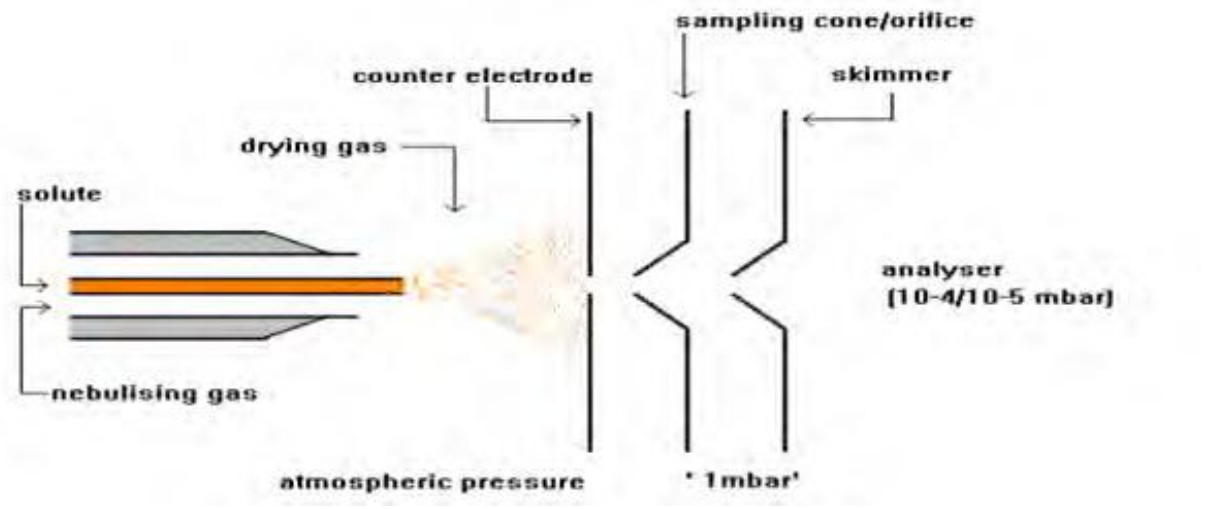



**Figure4. Representation of various ionization techniques such as ESI, APCI, and APPI as afunction of compound polarity and molecular weight.**

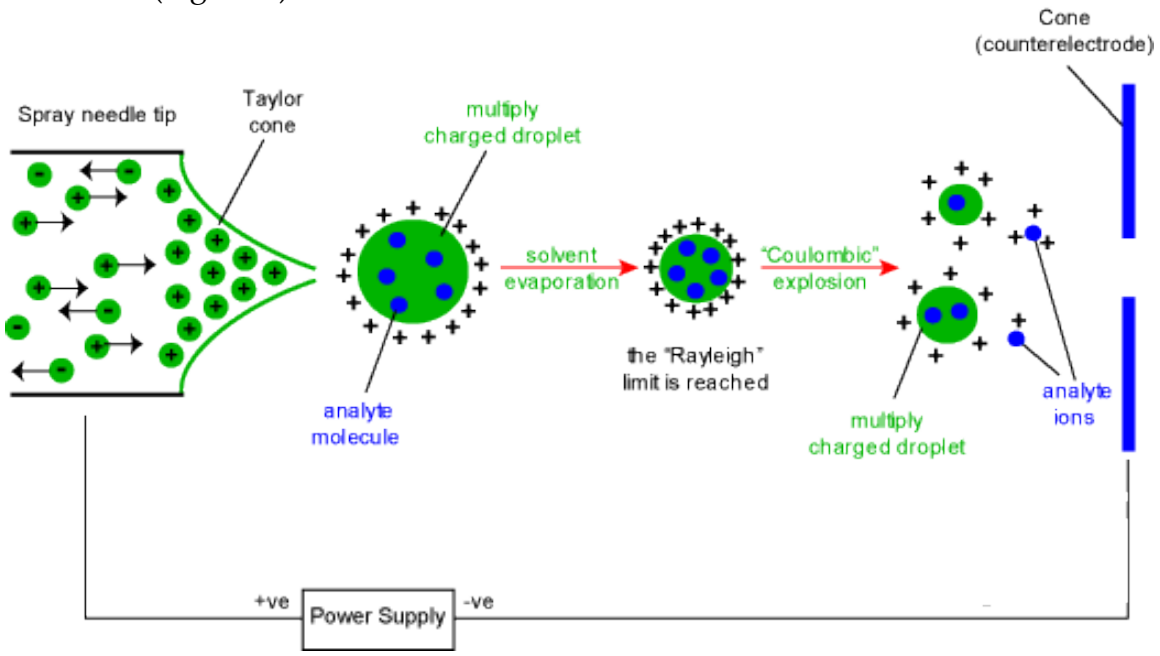
ESI is generally accomplished by forcing the LC mobile phase containing the analyte through a small capillary into an electric field of high positive or negative electrical potential typically of the order of 3-5 kV depending on whether positive ionization (higher voltages) or negative ionization (lower voltages) is required. (Figure5).




(Source http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm)

**Figure 5: Components of an ESI interface**

When the solution reaches the end of the tube the strong electric field forces it to be nebulized into a spray of small highly charged droplets of solution in solvent vapour. Before entering the mass spectrometer the spray passes through a heated chamber, through which a flow of drying gas, typically air or nitrogen, is continually passed at high flow rates evaporating the solvent rapidly. Thus as the charged droplets get smaller, the electrical surface charge density increases until it reaches a point where the repulsive forces between charges of the same polarity at the surface of the droplet are greater than the cohesive forces of surface tension which hold the droplet together. This results in a "Coulombic explosion" (Figure 6), which produces a number of smaller droplets and this continues until charged analyte ions are formed which can be analysed by the mass spectrometer (Figure 7).




Source http://www.bris.ac.uk/nerclsmsf/techniques/hplcms.html)

**Figure 6. A simplified mechanism of ion formation in the electrospray ionization process**

While the use of single mass analyzers is quite common, it is possible for ions to undergo separation by two different mass analysers in the same experiment. This is known as tandem mass spectrometry (Figure 7) and it is a popular technique (golden standard) used in the analysis of veterinary residues in biological and feed matrices. This technique is concerned with the analysis of product ions formed from precursor ions as a result of their fragmentation due to collision induced dissociation. The most commonly used mode in tandem mass spectrometry for this purpose is "selected reaction monitoring"/"multiple reaction monitoring (SRM/MRM) usually carried out on a triple quadrupole instrument.In SRM/MRM mode, the molecular ion of the target compound is isolated in the first mass analyser, it subsequently undergoes fragmentation and only specific product ions are monitored in the second mass analyser (Figure 8). This technique offers many advantages for the analysis of trace levels of substances in complex matrices.

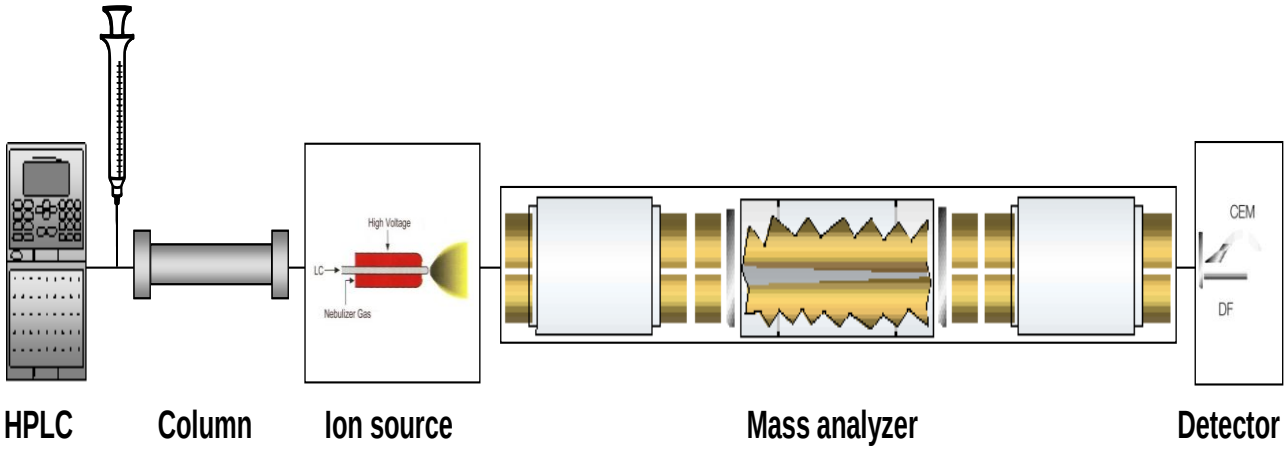



**Figure 7: Ideal HPLC with mass analyzer (Golden standard for quantitation)**

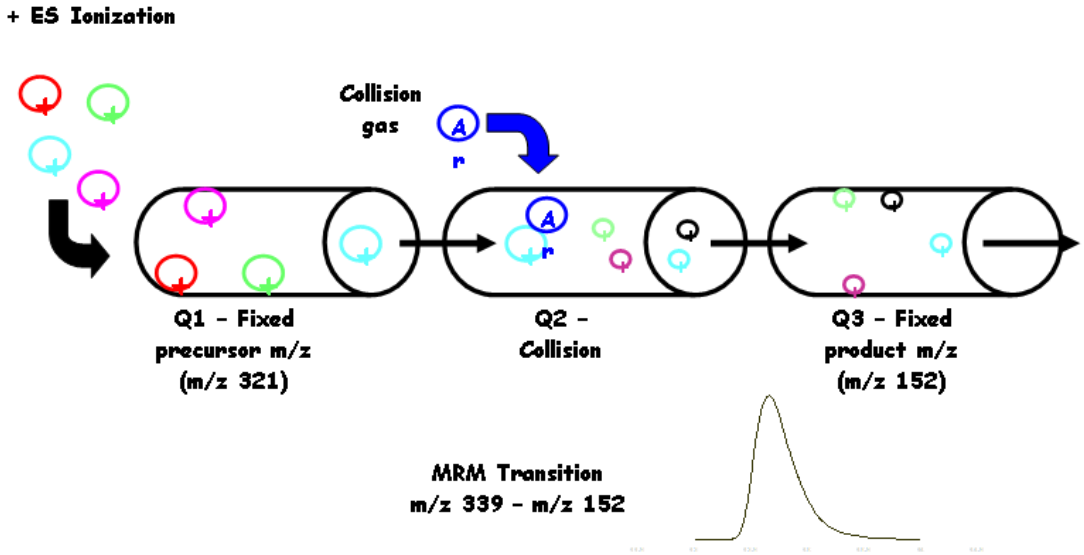



**Figure 8: Fragmentation in SRM/MRM**

## Chapter 5

## DETERMINATION OF CHLORAMPHENICOL – HPLC-MS/MS METHOD

### Introduction

Chloramphenciol (CAP) is a broad-spectrum antibiotic with historical veterinary uses in all major food-producing animals. CAP is biosynthesised by the soil organism *Streptomyces venezuelae*and several other *Actinomycetes*, but is produced for commercial use by chemical synthesis. CAP is a suspected carcinogen and due to its linkages with the development of aplastic anaemia in humans, the drug is banned for use in food-producing animals in the European Union (EU) and in many other countries, including the India, United States of America (USA), Canada, Australia, Japan and China. A minimum required performance limit (MRPL)/Target testing Level (TTL) of 0.3 μg $kg^{-1}$ was assigned by the European Commission and USFDA for the analytical methods testing for CAP in products of animal origin.

### Scope

This procedure is applicable for determination of chloramphenicol in animal origin. Chloramphenicol is extracted from tissue using a simple extraction and sample clean up. The extracted residues are examined using LC-MS-MS using a triple quadrupole mass spectrometer under electrospray ionization ($ESI^-$) conditions. Analytes are identified by comparison against matrix matched standards.

### Applicability

This method is suitable for the screening and confirmation of Chloramphenicol in fish, muscle tissue, egg, liver, kidney, honey etc.

### 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.

Blender, vortex mixer/ rotary shaker, centrifuge tubes (15/50 mL), refrigerated centrifuge, micropipettes, turbovap concentrator under nitrogen, Syringless filter (0.22μ), LC vials.
*Note: Equivalent equipment may be substituted.*

### Reagents/Chemicals

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

**Preparations of standard stock solutions**

Dissolve appropriate amount of Chloramphenicol for a final stock concentration of 1000 mg/L in Methanol (MeOH) which is generally stable for one year if stored in freezer (-18°C approximately) and intermediate standard solutions prepared in methanol is generally stable for 6 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 MeOH: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 MeOH:water) that is prepared from 100 mg/L stock solution.

**Instrumental conditions**

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

*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.*

**Sample processing**

Samples collected fresh must be kept cold before and during shipping to the laboratory. Once received at the laboratory, samples must be frozen (< -10 to -18°C) prior to grinding if they cannot be prepared on the day of receipt. Once frozen, the sample should be allowed to thaw, while keeping it as cold as possible. Dissect away fat and connective tissue. Grind tissue in blender or vertical cutter-mixer until homogeneous. Store samplesfrozen (< -10 to -18°C) prior to analysis.

**Extraction Procedure**

Weigh precisely 1.0gm of defatted milk/ 1.0gm honey/ 2.0gm homogenized edible portion of egg in a centrifuge tube. Add 200 μL of CAP-d5 (20 μg/L) in sample. Add 5 mL ethyl acetate and stir thoroughly for 10 min. on a vortex/ rotary shaker. Centrifuge at about 8000rpm 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 8000rpm @ $4^0$C for 15 min for separation of layers. Transfer upper layer (water) quantitatively into LC vial for injection in HPLC-MS/MS.

**Preparation of calibration curve**

Calibration curve (matrix matched) are prepared in matrix for at least 5 points including blank and treated in the same way like the samples.

**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 (Representative chromatograms and Calibration curves given in figure 9).

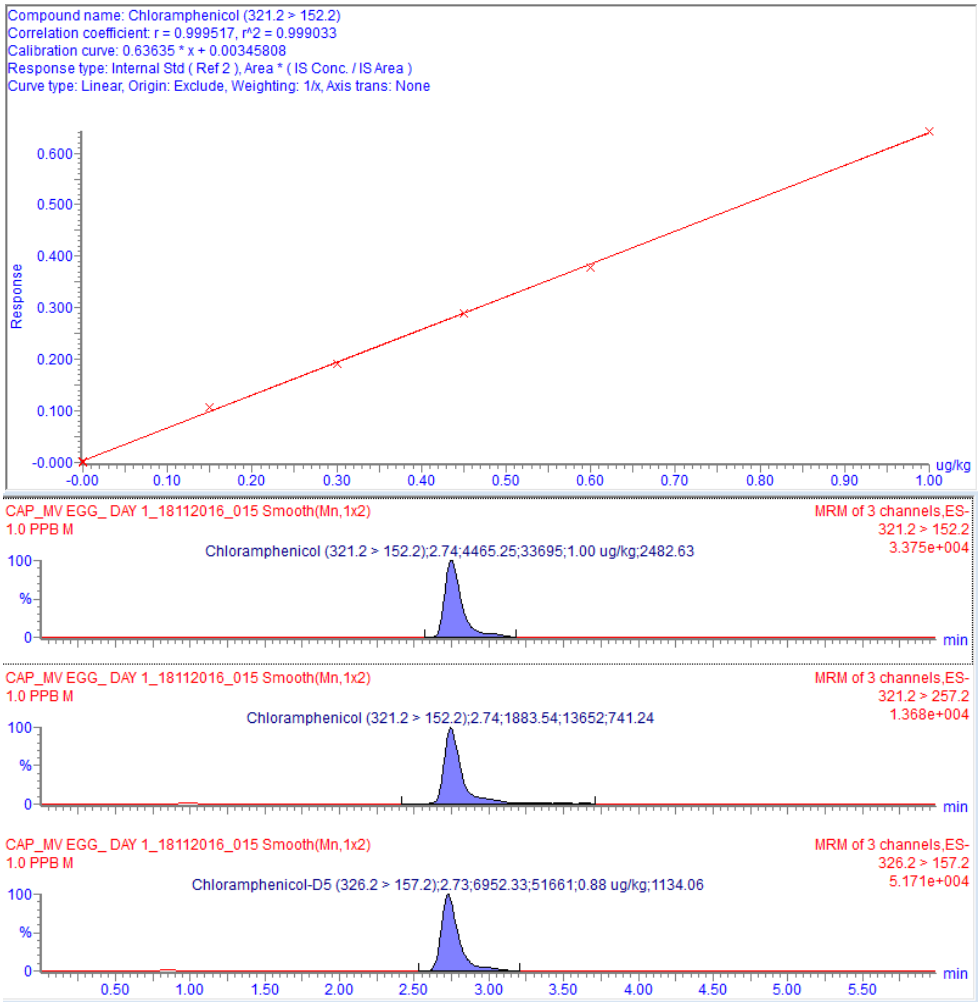



**Figure 9: Representative chromatogram with calibration curve for Chloramphenicol**

The order of Injection shall be as given below:

a. Reagent Blank
b. Compliant control samples
c. Calibration standard (s)
d. Samples to be confirmed/tested
e. Compliant control sample
f. Non-compliant control samples
g. Calibration standards can be injected at the end of the run to verify instrument response.

**Calculations**

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 0.99 (criteria for correlation coefficient given is just a reference and labs can set by themselves)

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. The retention time (or relative retention time) of the analyte in the test portion shall match that of the calibration standard within a specified retention time window. The ratio of the chromatographic retention time of the analyte to that of the internal standard, i.e. the relative retention time of the analyte, shall correspond to that of the calibration solution at a tolerance of ± 2.5 % for LC and retention times shall be identical within a margin of ±5 %.
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

$$\text{Ratio} = \frac{\text{Product Ion \#2}}{\text{Product Ion\#1}}$$

*Note: Ion ratio should be less than 1.*

Ion ratios determined for each analyte shall be within tolerance limits as described in the EU document 2002/657/CDin case of positive samples. Suggested tolerances are based on EU guidelines given in table below.

**Maximum permitted tolerances for relative ion intensities (Ion ratio) using LCMSMS**

| Relative intensity (% of base peak) | Ion Ratio tolerance (%) |
|---|---|
| >50% | ± 20% |
| >20% to 50% | ±25% |
| >10% to 20% | ±30% |
| >10% | ±50% |

*Note: If a sample shows a positive response for a compound which did not meet screening criteria in the associated QC samples, then further testing of that sample is warranted.*

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

**Safety information and precautions**

1. Required Protective Equipment -Safety eyewear, protective gloves, and lab coat.

2. Hazards

| *Procedure Step* | *Hazard* | *Recommended Safe Procedures* |
|---|---|---|
| Antibiotic standards | Some individuals may have allergic reactions to certain β-lactams, sulphonamides, or other drugs. | • Wear appropriate personal protective equipment to avoid dermal contact.<br>• Keep in well-closed containers away from ignition sources. |
| Acetonitrile, Methanol | Flammable | • Avoid contact or prolonged exposure to vapors.<br>• Work in fume hood.<br>• Keep away from flame or heat. |
| Formic acid | Corrosive, Caustic | Wear personal protective equipment, avoid skin contact. |

**References**


a. Chloramphenicol Identification by Liquid Chromatography Tandem MassSpectrometry, 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.

## Chapter- 6

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

**Scope**

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

**Reagents/ Chemicals**

HPLC grade Ethyl acetate, HPLC grade Acetonitrile, HCl 37%, 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 AHD-13C3, SCA-HCl – 13C,15N2, AMOZ-d5 & AOZ-d4 (Brand Sigma or equivalent) and gradient grade water.

**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.

**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% 5mM Ammonium format 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>100

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.*

**Preparations of Solutions Reference standards**:
**Procedure for Preparation of Standards of Nitrofuran Metabolites** Stock solutions of AOZ, AMOZ, AHD, SEM, AHD-13C3, SCA-HCl – 13C,15N2, AOZ-d4 & AMOZ-d5

Weigh 10 mg each of AOZ, AMOZ, AHD, SEM, AHD-13C3, SCA-HCl – 13C,15N2, 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, AHD-13C3, SCA-HCl – 13C,15N2,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, AHD-13C3, SCA-HCl – 13C,15N2, 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 AHD-13C3, SCA-HCl – 13C, 15N2, AOZ-d4 & AMOZ-d5:
1 ml each of AHD-13C3, SCA-HCl – 13C, 15N2, 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 AHD-13C3, SCA-HCl – 13C, 15N2, 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).

**Preparation of Calibration curve**
Calibration curve (matrix matched) are prepared in matrix for at least 5 points including blank and treated in the same way like the samples.

**Sample processing**

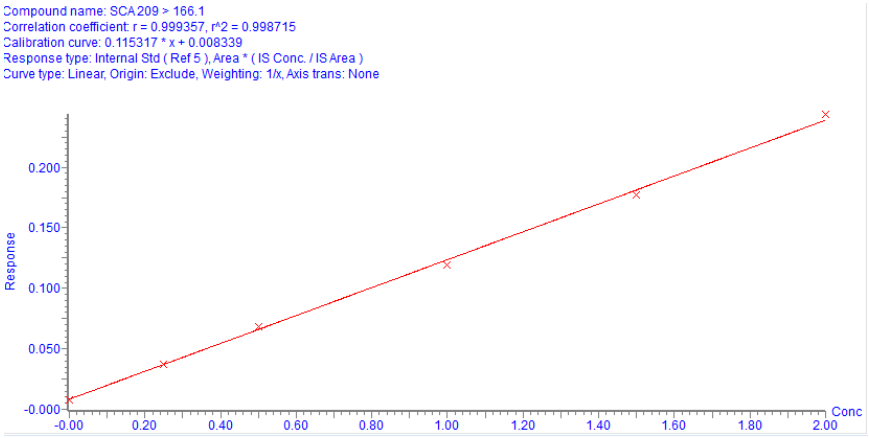
Samples collected fresh must be kept cold before and during shipping to the laboratory. Once received at the laboratory, samples must be frozen (< -10 to -18°C) prior to grinding if they cannot be prepared on the day of receipt. Once frozen, the sample should be allowed to thaw, while keeping it as cold as possible. Dissect away fat and connective tissue. Grind tissue in blender or vertical cutter-mixer until homogeneous. Store samplesfrozen (< -10 to -18°C) prior to analysis.

**Extraction Procedure**

Weigh precisely 4 gm of shrimp sample in a centrifuge tube add 200 µL of 50 ppb IS2 Standard solution each (corresponding to 2.5 ppb in final 1 mL extract volume in LC vial) to this add 10mL of 0.2M HC1 & 300 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 in an Incubator cum Rotary Shaker. Cool the tube to room temperature after incubation Add 300µL 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 8000rpm 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 8000rpm 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. Centrifuge at 8000rpm for 10-20 minutes. Transfer upper layer (water) quantitatively into LC vial for injection in HPLC-MSMS.

**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 (Representative chromatograms and calibration curve given in Figure 10).



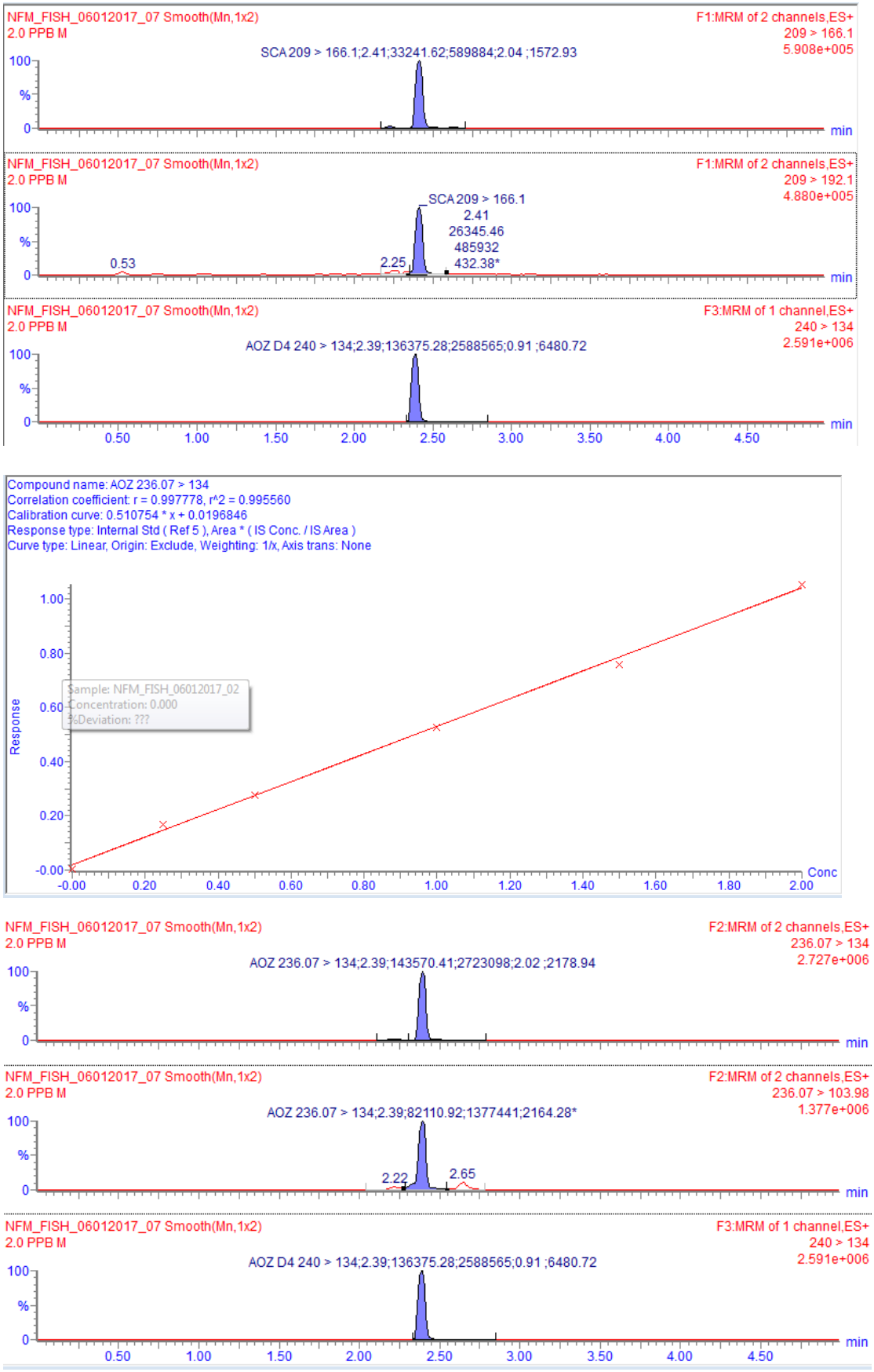



**Figure 10: Representative chromatograms and calibration curve for Nitrofuran metabolites**

The order of Injection shall be as given below:

a. Reagent Blank
b. Compliant control samples
c. Calibration standard (s)
d. Samples to be confirmed/tested
e. Compliant control sample
f. Non-compliant control samples
g. Calibration standards can be injected at the end of the run to verify instrument response.

**Calculations**

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 0.99 (criteria for correlation coefficient given is just a reference and labs can set by themselves)

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. The retention time (or relative retention time) of the analyte in the test portion shall match that of the calibration standard within a specified retention time window. The ratio of the chromatographic retention time of the analyte to that of the internal standard, i.e. the relative retention time of the analyte, shall correspond to that of the calibration solution at a tolerance of ± 2.5 % for LC and retention times shall be identical within a margin of ±5 %.
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

$$\text{Ratio} = \frac{\text{Product Ion \#2}}{\text{Product Ion\#1}}$$

*Note: Ion ratio should be less than 1.*

Ion ratios determined for each analyte shall be within tolerance limits as described in the EU document 2002/657/CD in case of positive samples. Suggested tolerances are based on EU guidelines given in table below.

**Maximum permitted tolerances for relative ion intensities (Ion ratio) using LCMSMS**

| **Relative intensity (% of base peak)** | **Ion Ratio tolerance (%)** |
|---|---|
| >50% | ± 20% |
| >20% to 50% | ±25% |
| >10% to 20% | ±30% |
| >10% | ±50% |

*Note: If a sample shows a positive response for a compound which did not meet screening criteria in the associated QC samples, then further testing of that sample is warranted.*

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

**Safety information and precautions**

1. Required Protective Equipment -Safety eyewear, protective gloves, and lab coat.
2. Hazards

| ***Procedure Step*** | ***Hazard*** | ***Recommended Safe Procedures*** |
|---|---|---|
| Antibiotic standards | Some individuals may have allergic reactions to certain β-lactams, sulphonamides, or other drugs. | • Wear appropriate personal protective equipment to avoid dermal contact.<br>• Keep in well-closed containers away from ignition sources. |
| Acetonitrile, Methanol | Flammable | • Avoid contact or prolonged exposure to vapors.<br>• Work in fume hood.<br>• Keep away from flame or heat. |
| Formic acid | Corrosive, Caustic | Wear personal protective equipment, avoid skin contact. |

**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.

## Chapter- 7

# SIMULTANEOUS DETERMINATION OF MULTI-RESIDUE AND MULTI-CLASS ANTIBIOTIC RESIDUES USING HPLC-MS/MS METHOD

### Scope

Simultaneous detection and quantification of 22 pharmacologically active substances, belonging to three different chemical classes comprising 9 quinolones including fluoroquinolones, 10 sulphonamides and 3 tetracylines by HPLC-MS/MS.

### Reagents/ Chemicals

Reference standards of Sulfanilamide, Sulfadiazine, Sulfapyridine, Sulfamethaxazole, Sulfathiazole, Sulfamerazine, Sulfamethizole, Sulfamethazine, Sulfamethoxypyridazine, Sulfadoxine, Sulfadimethoxine, Oxolinic acid, Ciprofloxacin, Enrofloxacin, Nalidixic acid, Flumequine Tetracycline hydrochloride, Oxytetracycline, Chlortetracycline, 4-epitetracycline, 4-epioxytetracycline, 4-epi chlortetracycline. Stock standard solutions (1000 mg $L^{-1}$) of sulphanomides and tetracylines were prepared individually by dissolving 10mg in methanol, whereas for quinolones, the solvent used was methanol with 2% 2M $NH_4OH$ or NaOH. In all cases, purity percentage and salt correction (wherever applicable) were considered for calculating the concentrations. The individual stock solution were stored in dark coloured glass bottles at -20° C. Working mix-standard solutions of the studied antibiotics (μg $ml^{-1}$, each) were prepared by diluting suitable aliquot of the stock solutions. Acetonitrile, methanol and water (LC-MS grade).Formic acid, n-Hexane (HPLC grade), Sodium hydroxide and Ammonium hydroxide.

### Apparatus

Analytical balances, centrifuge, Nitrogen evaporator, laboratory homogenizer, Centrifuge tubes (15/50 ml)

### Instrument

The chromatographic separation was achieved on an XBridge BEH C18 column [2.5 μm, 2.1x 100 mm , at a flow rate of 0.3-1.0 mL. $min^{-1}$ and the column temperature was maintained at 40°C. The mobile phase consisted of Methanol (solvent B) and 0.1% formic acid in water (solvent A). The gradient is presented in Table 1. The injection volume was 10μl.

| Time (min.) | Mobile phase composition/vol. % | |
|---|---|---|
| | A: 0.1% Formic acid in water | B: Methanol |
| 0.0 | 95 | 5 |
| 2.0 | 80 | 20 |
| 2.5 | 85 | 15 |
| 3.0 | 85 | 15 |
| 5.0 | 70 | 30 |
| 9.0 | 60 | 40 |
| 10.0 | 40 | 60 |
| 11.0 | 95 | 5 |
| 15.0 | 95 | 5 |

**Table 1: UPLC gradient for the elution of target compounds**

*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.*

**Preparations of Solutions Reference standards**:

Stock solutions of Sulfonamides, Tetracyclines and Quinolones:

Stock solutions are prepared independently. Weight the appropriate amount of powder in accordance to the purity and chemical form to obtain stock solutions containing 1000 µg/ml of active substance in a volumetric flask and dissolve in HPLC grade Methanol. Place the volumetric flask in an ultrasonic bath. Store up to 12 months at ≤ -20ºC.

Intermediate standard of 10.0 ppm and working standard solutions of 1.0 ppm were prepared by diluting suitable aliquot of stock standard in Methanol in amber volumetric flasks and stored for one year and Six months respectively at the temperature -20$^0$C.

**Preparation of Calibration curve**
Calibration curve (matrix matched) are prepared in matrix for at least 5 points including blank and treated in the same way like the samples.

**Sample processing**
Samples collected fresh must be kept cold before and during shipping to the laboratory. Once received at the laboratory, samples must be frozen (< -10 to -18°C) prior to grinding if they cannot be prepared on the day of receipt. Once frozen, the sample should be allowed to thaw, while keeping it as cold as possible. Dissect away fat and connective tissue. Grind tissue in blender or vertical cutter-mixer until homogeneous. Store

samplesfrozen (< -10 to -18°C) prior to analysis.The samples were kept for 1 hour after spiking to equilibrate before extraction.

**Extraction Procedure:**

2.0 ± 0.1 g of sample was weighed in a 50-mL polypropylene centrifuge tube (Tarsons, USA); 500µl of 5% formic acid in water and 8.0 mL of acetonitrile (ACN) were added. The mixture was vortex mixed (20s) and centrifuged at 1800 x g for 10 minutes. The acetonitrile layer (supernatant) was transferred into a 15 mL glass test tube and the whole extraction procedure was repeated and the supernatant were combined. The combined supernatant (acetonitrile) was evaporated to dryness under gentle stream of nitrogen at 40°C using a Turbovap. The dried extract was reconstituted with 1m of 95:5 0.1% Formic acid methanol. The mixture was filtered through a 0.20 µm PTFE filter. The collected filtrate was injected into the LC-MS/MS system under the optimized analytical condition.

**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 (Representative chromatograms given in Figure 12).

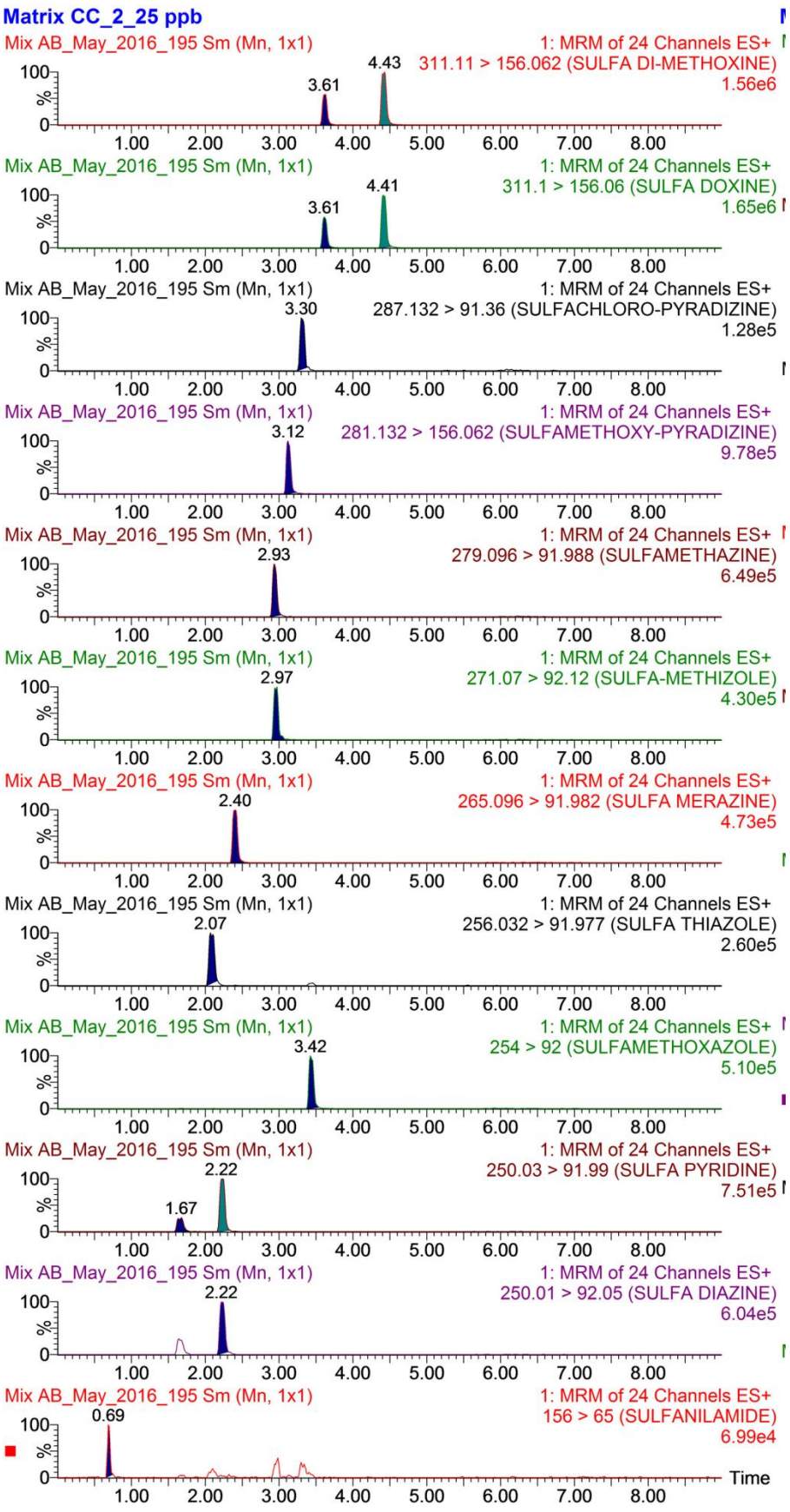



**Figure 12: Representative chromatograms for Multi residues**

The order of Injection shall be as given below:

h. Reagent Blank
i. Compliant control samples
j. Calibration standard (s)
k. Samples to be confirmed/tested
l. Compliant control sample
m. Non-compliant control samples
n. Calibration standards can be injected at the end of the run to verify instrument response.

**Calculations**

For Quantitation of each compound of interest

e. Review the chromatograms to verify that the analyte peaks are within the retention time windows and that the peaks are integrated correctly.
f. Calculate the normalized peak for each component of interest by dividing the component response by the internal standard response:

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

g. Generate a linear curve fit to each analyte in standard curve using normalized response to concentration in tissue (μg/kg or ppb).
h. Standard curve must have a correlation coefficient greater than or equal to 0.99 (criteria for correlation coefficient given is just a reference and labs can set by themselves)

For Confirmation

e. Choose a standard or recovery containing the analyte of interest.
f. 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
g. The retention time (or relative retention time) of the analyte in the test portion shall match that of the calibration standard within a specified retention time window. The ratio of the chromatographic retention time of the analyte to that of the internal standard, i.e. the relative retention time of the analyte, shall correspond to that of the calibration solution at a tolerance of ± 2.5 % for LC and retention times shall be identical within a margin of ±5 %.
h. Calculate the ratio of the response of product ion #2 to product ion #1 in the standard or recovery for the analyte of interest

$$\text{Ratio} = \frac{\text{Product Ion \#2}}{\text{Product Ion\#1}}$$

*Note: Ion ratio should be less than 1.*

Ion ratios determined for each analyte shall be within tolerance limits as described in the EU document 2002/657/CD in case of positive samples. Suggested tolerances are based on EU guidelines given in table below.

**Maximum permitted tolerances for relative ion intensities (Ion ratio) using LCMSMS**

| **Relative intensity (% of base peak)** | **Ion Ratio tolerance (%)** |
|---|---|
| >50% | ± 20% |
| >20% to 50% | ±25% |
| >10% to 20% | ±30% |
| <10% | ±50% |

*Note: If a sample shows a positive response for a compound which did not meet screening criteria in the associated QC samples, then further testing of that sample is warranted.*

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

**Safety information and precautions**

1. Required Protective Equipment -Safety eyewear, protective gloves, and lab coat.

2. Hazards

| ***Procedure Step*** | ***Hazard*** | ***Recommended Safe Procedures*** |
|---|---|---|
| Antibiotic standards | Some individuals may have allergic reactions to certain β-lactams, sulphonamides, or other drugs. | • Wear appropriate personal protective equipment to avoid dermal contact.<br>• Keep in well-closed containers away from ignition sources. |
| Acetonitrile, Methanol | Flammable | • Avoid contact or prolonged exposure to vapors.<br>• Work in fume hood.<br>• Keep away from flame or heat. |
| Formic acid | Corrosive, Caustic | Wear personal protective equipment, avoid skin contact. |

**References**


a. 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
b. Antonia GarridoFrenich, María del Mar Aguilera-Luiz, Jose Luis Martínez Vidal, Roberto Romero-González, Comparison of several extraction techniques for multiclass analysis of veterinary drugs in eggs using ultra-high pressure liquid chromatography–tandem mass spectrometry, Anal. Chim. Acta. 661(2) (2010) 150–160.
c. Heller D.N., Nochetto C.B., Rummel N.G., & Thomas M.H., Development of multiclass methods for drug residues in eggs: hydrophilic solid-phase extraction cleanup and liquid chromatography/tandem mass spectrometry analysis of tetracycline, fluoroquinolone, sulfonamide, and beta-lactam residues, J. Agric. Food Chem. 54(15) (2006) 5267–5278
d. GannaFedorova, Vaclav Nebesky, Tomas Randak, Roman Grabic (2014). Simultaneous determination of 32 antibiotics in aquaculture products using LC-MS/MS, Chemical Papers 68 (1) (2014) 29–36

## Chapter 8

# METHOD VALIDATION PROTOCOL FOR VETERINARY DRUGS INCLUDING ANTIBIOTICS

## I. Purpose and Scope

a. As a result of legislation for the control of residues on food of animal origin, analytical methods for the analysis of the controlled substances needed to be developed. These methods need to be sensitive, selective and fit for the purpose. In order to ensure this the European Commission initiated the construction of a legislation to ensure any method developed met certain quality criteria.

b. This legislation lays down performance criteria for the analytical methods to be used for the analysis of certain substances and residues thereof in live animals and animal products according to the **Council Directive 96/23/EC.** The resulting piece of legislation is **Commission decision 2002/657/EC** and it is concerned with the performance of analytical methods and the interpretation of results.

c. This protocol describes Performance criteria's and validation steps for residues of veterinary drugs (Group A and Group B substances)/Banned substances in various food matrices based on Commission Decision 2002/657/EC

## II. Performance criteria and other requirements for analytical methods (Part 2 of CD)

1. Confirmatory method for Organic Residues:
   a. Confirmatory methods for organic residues shall provide information on chemical structure of analyte.
   b. **Group A substances:** Chromatographic system connected to detector (MS)
   c. **Group B substances:** Chromatographic system connected to detector (DAD, ECD, MS etc.)

2. Common Performance Criteria and requirements:
   a. Confirmatory method used for the residues should be based on chromatography techniques with mass spectrometry identification.
   b. Wherever possible internal standards (IS) to be used. When used in the method IS should be added to the test portion **at the beginning of the extraction procedure.**

c. When no suitable internal standard is available identification of the analyte to be confirmed by co-chromatography. The enhanced peak height/area should be equivalent to the amount of analyte added.
d. With GC/LC, the peak width at half maximum height shall be within 90-110% range of the original width and the retention time shall be identical within a **margin of 5%.**
e. Reference or fortified material containing known amount of analyte at the permitted level or the decision limit as well complaint control material and reagent as blank should be subjected to the entire procedure simultaneously.
f. **The order of injection of extracts is as follows:-**

Reagent blank, compliant control sample, sample to be confirmed, compliant control sample again and finally known non-compliant control sample. Any variation from the above protocol should be justified.

3. Performance Criteria and other requirements for Chromatography:
   a. An Internal standard should be used if a material suitable for this purpose is available. It shall preferably be a related standard with a retention time close to that of the analyte.
   b. For GC MS, capillary columns to be used. For LCMS suitable columns as per the analyte requirement to be used.
   c. The minimum acceptable retention time for the analyte is to be twice the retention time of the void volume of the column.
   d. The ratio of the chromatographic retention time of the analyte to that of the internal standard i.e.**the relative retention time** of the analyte, shall correspond to that of the **calibration solution/ spiked sample** at a tolerance of +**2.5% for LC and +0.5% for GC**.

4. Performance Criteria and other requirements for Mass Spectrometry:
   a. **MS detection** to be carried out either by full scan/selected ion monitoring (SIM)/Multiple Reaction Monitoring (MRM) or other suitable ionization modes.
   b. **Full scan** spectrum should contain all measured diagnostic ions with a relative intensity of more than 10% in the reference spectrum of the calibration standards.
   c. **When SIM modes** are followed the molecular ion shall preferably be one of the selected diagnostic ion and the signal to noise ratio for each ion shall be greater or equal to 3:1.
   d. **Full Scan and SIM:**The relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion or transition, shall correspond to those of the calibration standard, either from calibration

standard solutions or from spiked samples, at comparable concentrations, measured under the same conditions, within the below tolerances—**Criteria for Ion Ratio (Table 1)**

**Table 1: Maximum permitted tolerances for relative ion intensity (Ion ratios) for GCMS/LCMS MS techniques (as per Table-4 of CD/2002/657/EC)**

| Relative intensity [% of base peak] | LC $(MS)^n$ (Relative) |
|---|---|
| >50% | ± 20% |
| >20% to 50% | ± 25% |
| > 10% to 20% | ± 30% |
| ≤ 10% | ± 50% |

e. When MS techniques are carried out by SIM modes for confirmation of substances listed in **GROUP-A**of Council Directive of 2002/657/EC, **minimum of 4 identification points (IP)** is required.
f. For substances listed in **GROUP-B** for confirmation, a minimum of **3 identification points** are required. **Table 2 and Table 3** mentions about the number of IP that each of MS techniques can earn.
g. To qualify the identification points:
   - Minimum one ion ratio to be measured.
   - All relevant measured ion ratios to meet the criteria described above.
   - Maximum of three separate techniques can be combined to achieve minimum number of identification points.

**Table 2. The relationship between a range of classes of mass fragment and identification points earned (Table-5 of CD/2002/657/EC)**

| MS Technique | Identification points earned per ion |
|---|---|
| Low resolution mass spectrometry [LR] | 1.0 |
| LR-MS precursor ion | 1.0 |
| LR-MS transition products | 1.5 |

**Note:**

- ✓ *Each ion to be counted once.*
- ✓ *GC-MS using EI mode is regarded as being a different technique to GCMS using CI.*
- ✓ *Group-A substances, the following techniques earn maximum 1 point per technique. (HPLC-DAD, HPLC with fluorescence detection)*
- ✓ *Transition points include both daughter and granddaughter ions.*

**Table 3. Number of Identification points earned for a range of techniques and combinations thereof (n=an integer) (Table-6 of CD/2002/657/EC)**

| Technique(s) | Number of ions | Identification points |
|---|---|---|
| GC MS MS | 1 precursor and 2 daughters | 4 |
| LC MS MS | 1 precursor and 2 daughters | 4 |
| GC MS MS | 2 precursor ions, each with 1 daughter | 5 |
| LC MS MS | 2 precursor ions, each with 1 daughter | 5 |
| LC MS MSMS | 1 precursor, 1 daughter and 2 granddaughters | 5,5 |

5. Performance criteria for LC/GC technique with different detectors:

***Chromatographic separation:***

- Use of internal standard suitable for the analyte wherever possible with retention time close to that of analyte.
- The retention time of analyte and that of the calibration standard to match under typical same experimental condition.
- Minimum acceptable retention time for the analyte shall be twice the retention time of the void volume of the column.
- The relative retention time [ratio of the retention time of the analyte to that of internal standard] to be within the margin of ±2.5%
  - The nearest peak maximum in the chromatogram shall be separated from the analyte peak by at least 1 peak with width of 10% of the maximum height of the analyte peak.

6. Identification and Confirmation:

- For the confirmation of substances listed in **Group A**of Annex I of Directive 96/23/EC, a minimum of **4 identification points** shall be required.
- For the confirmation of substances listed in **Group B** of Annex I of Directive 96/23/EC, a minimum of **3 identification points**are required.

**STEP 1: Identification of an analyte:**

- RRT of sample to RRT of spike (<2.5%-LC/<0.5%-GC)
- S/N Ratio of sample signal >3
- Presence of 2 MRM transition ions
- Transition Ion ratio tolerance within limits

**STEP 2: Accurate Quantitation:**

5 point calibration with a coefficient correlation R>0.99 (to be fixed by lab)

## III. Validation procedure (Part 3 of CD)

a. Validation procedure adopted is as per section 3.1 of Directive includes "verification of compliance of performance criteria" for the corresponding analytical method used for screening and confirmation and set of common performance characteristics and parameters described under conventional validation approach in Table-10 under section 3.1 of Council Directive.

b. The performance characteristic has been derived taking into consideration 3.1.1, 3.1.2 & 3.1.3 of the Commission Decision 2002/657/EC.

Performance parameters: To ensure that the optimized procedure was suitable (fit for purpose) for the application in routine analysis, the **basic analytical performance parameters** given below were determined and assessed.

a) Specificity
b) Stability
c) Calibration curves
d) Recovery
e) Repeatability
f) Within-laboratory Reproducibility
g) Decision Limit (CC$\alpha$)
h) Detection Capability (CC$\beta$)
i) Ruggedness (minor changes)
j) Uncertainty

a. **Specificity: T***he ability of a method to distinguish between the analyte being measured and other substances. Specificity is mainly a function of the measuring technique described, but can vary accordingly to class of compounds or matrix.*

The power of discrimination between analyte and closely related/other substances.Eg: isomers, metabolites, matrix constituents, etc. is important for which the **following needs to be checked.**

- Analyse appropriate number of representative blank samples [**n≥7**] and check for interference [signal, peak, ion trace] in the region of interest where target analyte is expected.
- Select a range of chemically related compounds like metabolites, derivatives etc., and study the effect of interference.

- Representative blank sample to be fortified at relevant concentration with substances that are likely to interfere with the analyte identification/quantitation.
- The data from the study needs to be checked for false identification, hindrance in identification of target analyte and influence in quantification (notably) of the analyte.

b. **Stability:** ***The degree to what a substance/analyte is subject to degradation under different conditions.***

Insufficient stability of the analyte or matrix constituent in the sample during storage may cause significant deviation in the analysis. Also, **stability of the calibration standard in solution/sample needs to be checked**.

- Stability of analyte in matrix need not be estimated, if justification (reasons) on the basis of published data, information from the Community Referral laboratory etc. are available and documented for the respective residues.
- When there is no sufficient information regarding the stability of analyte in solution/matrix the following procedures needs to be adopted

***Stability of the analyte in solution.***

→ Prepare fresh stock solution of the analyte and dilute as per the test protocol to give aliquots [eg:40] of selected concentration [around MRPL limit or around MRL limit]
→ Measure the analyte content in the freshly prepared solution.
→ Dispense appropriate volumes in to suitable container label and store.
→ The storing time and temperature is selected as per the analyte stability.
→ Analyse the aliquots at appropriate time as per the method and calculate the concentration using the concentration of the freshly prepared as 100%

$$\text{Analyte remaining percentage} = \frac{C' \; x \; 100}{C - fresh}$$

C′ = concentration obtained at different time of storage point.
C-fresh = concentration of fresh solution.

***Stability of the analyte in matrix***

→ Use positive control sample or matrix fortified with analyte.
→ When positive control sample is available, determine the concentration of the analyte when it is fresh. Further aliquots of the material stored under proper condition are processed on different times as per the declared protocol and concentration determined. Incase of non-availability of positive control blank material which has been tested negative for the analyte is homogenized and the material is divided into aliquots. Each aliquot is fortified with the analyte

and stored under appropriate condition. Each aliquot is analysed as per decided time and concentration calculated.

c. **Calibration Curve:***Linearity is the relation between the concentration level and response factor*

Matrix effect needs to be carried out before deciding the type of calibration (i.e**Solvent, Matrix match & Matrix based**) by checking the matrix effect (slope of linearity can be taken). **If the matrix effect is >15%** when comparing the three type **Matrix based calibration** may be considered.

- When any of the above types of calibration curves are used for quantification.
- At least 5 levels including zero should be used for construction of curve.
- The working range of the curve to be described.
- Mathematical formula of the curve and goodness-of-fit of the data to the curve to be described.
- Acceptability range for the parameters of the curve should be described.

d. **Recovery:** ***Recovery means the percentage of true concentration of a substance recovered during the analytical procedure. It is determined during validation, if no certified Reference material (Trueness needs to be carried out) is available.***

Recovery has to be determined using fortified blank matrix.

- 21 aliquots of blank material and fortify 7 aliquots each with 1, 1.5 and 2 times the Minimum Required Performance Level (MRPL) or 0.5, 1.0, 1.5 times Permitted Limit.
- Analyse the samples and calculate the concentration in each sample.
- Calculate the recovery and CV from six results at each level
- % Recovery= 100 x Measured content/fortification level
- The sample should be corrected for recovery.
- The range of recovery should fall within the limits given in Table 4

**Table 4: Minimum recovery limits (as per Table 2 of CD/657/2002/EC)**

| Mass fraction | [EU Guide line] Range |
|---|---|
| ≤ 1 µg/kg | -50% to + 20% |
| > 1 µg/kg to 10 µg/kg | -30% to + 10% |
| ≥ 10 µg/kg | -20% to + 10% |

e. **Repeatability:***Closeness of agreement between independent test results obtained under the same conditions in the same laboratory by the same operator using the same equipment.*

For establishing repeatability the following needs to be carried out:

- Prepare a set of samples of identical matrices, fortified with analyte equivalent to 1.0, 1.5 and 2.0 of MRPL/0.5, 1.0 and 1.5 MRL.
- At each level, analysis to be performed with minimum 6 replicates.
- Analyse the sample and calculate the concentration in each.
- Find the mean, SD and CV%.
- Repeat the steps on at least two different occasions.
- Calculate the overall mean concentration and CVs for the fortified sample.
- **Horwitz equation:** RSDR(%) = $2(1\text{-}0.5^{\log C})$, where C is the mass fraction expressed as a power (exponent) of 10 (e.g. 1 mg/g = $10^{-3}$).
- **Criteria for repeatability: Intra laboratory CV shall be between1/2 to 2/3rd of Table 5.**

*f.* **Within Laboratory Reproducibility:** ***Closeness of agreement between independent test results obtained under different condition. Reproducibility conditions means conditions where independent test results are obtained with the same method on identical test items in the same laboratory by a different operator using the same equipment on different days.***

For establishing reproducibility the following needs to be carried out:

- Prepare a set of samples of identical matrices, fortified with analyte equivalent to 1.0, 1.5 and 2.0 of MRPL/0.5, 1.0 and 1.5 MRL.
- At each level, analysis to be performed with minimum 7 replicates.
- Repeat these steps on two other occasions with different operators, different environmental conditions like different batch of reagents, room temperature, different instruments, etc., if possible.
- Analyse the sample and calculate the concentration in each.
- Find the mean, SD and CV%.
- Calculate the overall mean concentration and CVs for the fortified samples.
- The CVs obtained should within the limits given in Table 5
- **Criteria for within-lab reproducibility: Shall not be greater than Reproducibility (Table 5) at concentration 0.5 x Permitted limit.**

**Table 5: Acceptable reproducibility CVs for range of analyte concentration (as per Table 3 of CD/657/2002/EC)**

| Mass fraction | [EU Guide line] Reproducibility CV (%) |
|---|---|
| 1 μg/kg | (*) |
| 10 μg/kg | (*) |
| 100 μg/kg | 23 |
| 1000 μg/kg (1mg/kg) | 16 |

* For mass fraction lower than 100 μg/kg the application of the Horwitz equation given unacceptably high values. Therefore, the CVs for concentration lower than 100 μg/kg shall be as low as possible or within **22%**.

*g.* **Reproducibility:***Reproducibility can be verified by participating in collaborative studies.*

*h.* **Decision Limit – CC$\alpha$:***Decision limit (CC$\alpha$) means the limit at and above which it can be concluded with a specific error probability of $\alpha$ that a sample is non-compliant.*

*Two ways of establishing CC$\alpha$* can be followed as per commission decision:

***By Calibration curve:***

- This has to be established according to the requirements for identification plus quantification for the analytical methods.
- In the case of substances with no permitted limit (MRPL/Banned substances), CC$\alpha$ can be established by following calibration curve procedure.
- In this case, blank material fortified at and around the MRPL in equidistant steps (i.e: 0.5, 1 and 1.5 times MRPL), samples are analysed and plot the signal against added concentrations. The corresponding concentration at the Y intercept +2.33 (i.e factor)
  times the standard deviation of within the lab reproducibility of the intercept is the decision limit.
- This is applicable to quantitative methods only [$\alpha$ error is equivalent to 1%].
- In the case of substances with established permitted limit [MRL] CC$\alpha$ can be calculated by analysing blank material fortified around the MRL in equidistant steps (i.e0.5, 1.0 and 1.5 times of the MRL level) and plot the signal against the added concentrations. The corresponding concentration at the permitted limit + 1.64 times the standard deviation of within the lab reproducibility of the intercept is the decision limit.
- This is applicable to quantitative methods only [$\alpha$ error is equivalent to 5%].

***By analysing Blank material (This approach should be followed when blank samples are representative)***

- For calculation of CC$\alpha$ (MRPL) 20 blank material is analysed per matrix to be able to calculate the signal to noise ratio at the time window in which the analyte is expected. Three times the signal to noise ratio can be used as decision limit. The can be used both for qualitative and quantitative assays.
- For calculation of CC$\alpha$ (MRL) 20 blank material is fortified with the analyte at Permitted Limit. Analyse the samples and identify the analytes. The concentration at the permitted limit plus 1.64 times the corresponding standard deviation equals the decision limit ($\alpha$ error=5%).

**Note: The CC$\alpha$ established should ideally be 1/3 of MRPL** and CC$\alpha$ should ideally be within 10% of MRL (eg. Within 110µg/kg for compound having 100µg/kg as MRL) in case of MRL compounds when Internal standards (IS) are used. In case IS are not being used the CC$\alpha$ should be ideally be within 20% (eg. within 120µg/kg for compound having 100µg/kg as MRL) of MRL.

*i.* **Detection capability – CCβ:*Detection capability means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β.***

*Two ways of establishing CC*β has been detailed in commission decision:

***By Calibration curve:***

- In this case, blank material fortified at and around the MRPL in equidistant steps (i.e: 0.5, 1 &1.5 times MRPL), samples are analysed and plot the signal against added concentrations. The corresponding concentration at the decision limit +1.64 (i.e safety factor) times the standard deviation of within the lab reproducibility of the mean measured content at the decision limit equals the detection capability.(β=5%)
- In the case of substances with established permitted limit [MRL] CCβ can be calculated by analysing blank material fortified around the MRL in equidistant steps (i.e 0.5, 1.0 and 1.5 times of the MRL level). Analyse the samples and identify the analyte. Calculate the Standard deviation of the mean measured content at the decision limit. The corresponding concentration at the decision limit + 1.64 times the standard deviation of within the lab reproducibility equals detection capability (β=5%).

***By analyzing Blank material:***

For calculation of CCβ (MRPL/MRL) 20 blank material is fortified with the analyte at decision limit. Analyse the samples and identify the analytes. The value of the decision limit plus 1.64 times the corresponding standard deviation equals the decision limit (β=5%).

**NOTE:** CC β should ideally be within **20% of MRL** (eg. Within 120µg/kg for compound having 100µg/kg as MRL) in case of MRL compounds when Internal standards (IS) are used. In case IS are not being used the CC β should be ideally be**within 40%of MRL** (eg. Within 140µg/kg for compound having 100µg/kg as MRL) of MRL. The figures generated for both CC$\alpha$ and CCβ (for MRPL/banned substances) must be verified by blank samples which are fortified with the respective concentration of CC$\alpha$ and CCβ if the figures obtained are **lower than <1/3$^{rd}$ of MRPL** (theoretically/by calculation obtained). At the CCβ this must be performed with at

least 20 replicates for the verification of the β error of <5%, the method should be able to detect/identify the analyte at the CCβ in 95% of the cases. The same procedure has to be followed for CC$\alpha$ however the method should be able to detect/identify in 50% of the cases. **Where the above said percentages obtained as significantly lower it can be concluded that the calculate values of CC$\alpha$ and CCβ are too low, requiring further investigation of these performance characteristics.**

j. **Ruggedness [Minor change]**

- To study the effect of minor reasonable variation on the qualitative and quantitative identification of analyte.
- Factors like change of analyst, change of standard, reagent/solvent batch, rate of heating, pH, etc.
- 8 determinations have to be made with combination various factors and the effect of the minor changes have to be studied.

k. **Measurement Uncertainty (MU)**

The CC$\alpha$ and CCβ once established will take care of the Uncertainty measurements, however as per the requirement of Accreditation bodies (NABL etc.) MU needs to be calculated.

## IV. References


- Council Directive 96/23/EC on measures to monitor certain substances and residues thereof in live animals and the animal products.
- Commission Decision 657/2002/EC Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.
- Guidelines for implementation of Decision 657/2002/EC (SANCO/2004/2726-rev-4-December 2008).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**Annexure-I**

## DETERMINATION OF CHLORAMPHENICOL AND THIAMPHENICOL IN MILK USING LC-MS/MS

### A. Scope

A method is presented for the analysis of Chloramphenicol and Thiamphenicol in milk.

### B. Apparatus/Requirements

- Analytical Balance
- Tube shaker
- Vortex
- Centrifuge
- Nitrogen evaporator apparatus with heating water bath
- 15 mL centrifuge tubes
- 0.2 μm nylon syringe filter
- Variable volume calibrated pipettes
- 5 mL plastic syringe
- Glass autosampler vials - 2 mL
- 10 ml volumetric flasks
- Graduated measuring cylinders 100 ml and 500 ml

### C. Reagents/Chemicals/Solutions

- Reference standards of Chloramphenicol, Chloramphenicol-d5 and thiamphenicol
- Ethyl Acetate, LCMS grade
- Acetonitrile, LCMS grade
- Methanol, LCMS grade
- Formic acid, LCMS grade

### D. Standard preparation

- Stock preparation(1000 PPM):
  Weigh 10 mg standard and make up the volume to 10 ml with methanol in volumetric flask.Purity factor and salt correction (wherever applicable) are to be considered for calculating exact concentration.Stock standard is stored at -20°C.

- Intermediate Standard preparation(10 PPM):

Intermediate standard solutions are prepared by diluting appropriate (0.1 ml) volume from each stock solution to 10 mlin a volumetric flask with methanol.

- Working (Mix) solution:
  Always prepare fresh working solution as per requirement.

**E. Calibration standards**

Matrix matched calibration standards are prepared for at least 5 points including blank and processed in the same way as that of samples.

**F. Extraction procedure**

1) Weigh precisely 1 gm of defatted milk in 15 ml centrifuge tube.
2) Add internal standard chloramphenicol-d5 at a level of 1 ng/gm and keep it aside for 10 minutes.
3) Add 7 ml ethyl acetate and vortex the tube for one minute.
4) Put the sample tubes in rotary shaker for 10 minutes to enable proper mixing of the contents.
5) Centrifuge at 4000 rpm at 4°C for 10 minutes.
6) Decant the upper ethyl acetate layer in another tube.
7) Repeat the extraction by adding 7 ml ethyl acetate in sample.
8) Add the supernatant in previously collected ethyl acetate layer.
9) Evaporate the content to dryness under nitrogen evaporator.
10) Reconstitute the dried sample with 1 ml water containing 10% acetonitrile.
11) Vortex the samples, filter through 0.2 μm syringe filter into a glass vial and inject.

**G. UPLC Conditions**

- Mobile phase A : 0.1% Formic acid in water
- Mobile phase B : 0.1 % Formic acid in acetonitrile
- Column: Acquity UPLC, BEH C18, 2.1 X 50mm
- Column temperature: 40°C
- Injection volume : 10 μl
- Flow rate : 0.4 ml/min
- Run time: 6 min.

## H. UPLC Mobile Phase Gradient

| Time (min) | Mobile Phase A (%) | Mobile Phase B (%) |
|---|---|---|
| Initial | 10 | 90 |
| 0.40 | 10 | 90 |
| 3.00 | 40 | 60 |
| 3.10 | 10 | 90 |
| 6.00 | 10 | 90 |

## I. MS Conditions

| | |
|---|---|
| Polarity | ESI –ve |
| Capillary (kV) | 3 |
| Cone (V) | 50 |
| Source offset (V) | 80 |
| Source temperature ($^0$C) | 150 |
| Desolvation Temperature ($^0$C) | 500 |
| Cone Gas Flow (L/hr) | 150 |
| DesolvationGasflow (mL/min) | 1000 |
| Collision Gas Flow (mL/min) | 0.15 |
| Nebuliser Gas Flow (Bar) | 7.00 |

## J. MRM of analytes

| Sr. No. | Name | Parent | Product ion | Cone Volt | CE |
|---|---|---|---|---|---|
| 1 | Chloramphenicol | 321.04 | 151.84 | 50 | 16 |
| | | 321.04 | 256.93 | 50 | 12 |
| 2 | Chloramphenicol - d5 | 326.08 | 155.95 | 50 | 16 |
| | | 326.08 | 261.17 | 50 | 12 |
| 3 | Thiamphenicol | 354.04 | 184.91 | 10 | 20 |
| | | 354.04 | 289.99 | 10 | 12 |

## K. References

1) Tölgyesi, Á.,Fekete, J., Sharma, V. K., Palffi, É., Bekesi, K., Lukonics, D., &Pleva, G. (2014). A LC-MS/MS confirmatory method for determination of chloramphenicol in real samples screened by competitive immunoassay. ActaAlimentaria, 43(2), 306-314.

2) Penney, L., Smith, A., Coates, B., &Wijewickreme, A. (2005). Determination of chloramphenicol residues in milk, eggs, and tissues by liquid chromatography/mass spectrometry. Journal of AOAC International, 88(2), 645-653.
3) Vora, V. R., &Raikwar, M. K. (2013). Determination of chloramphenicol and thiamphenicol residues in fish, shrimp and milk by ESI-LCMSMS. Int. J. Agr. Food Sci. Tech, 4, 823-828.

**Annexure-II**

## DETERMINATION OF MULTI-RESIDUE AND MULTI-CLASS ANTIBIOTIC RESIDUES IN MILK USING LC-MS/MS

### A. Scope

The method is applicable for simultaneous determination of 18 antibiotic residues in milk including sulphonamides, quinolones and other antibacterial substances.

### B. Apparatus/Requirements

- Analytical Balance
- 50 mL centrifuge tubes
- Vortex
- Tube Shaker
- Centrifuge
- Variable volume calibrated pipettes
- Nitrogen Evaporator Apparatus with Heated Water Bath
- 15 mL glass centrifuge tubes
- 0.2 μm Nylon Syringe Filter
- 5 mL Plastic Syringe
- SPE cartridge (Oasis HLB, 6cc, 200mg)
- Glass Auto sampler Vials & Caps - 2 mL
- Glass Volumetric Flasks - Class A

### C. Reagents/chemicals/solutions

- Reference standards of Sulfanilamide, Sulfadiazine, Sulfathiazole, Sulfamerazine, Sulfamethiazole, Sulfadimidine, Sulfamethazine, Sulfamethoxypyridazine, Sulfadimethoxine, Sulfadoxine, Ciprofloxacin, Enrofloxacin, Erythromycin, Spiramycin, Neospiramycin, Tilmicosin, Tylosin and Trimethoprim.
- Acetonitrile, LCMS grade
- Methanol, LCMS grade
- Citric acid hydrate (0.1M)
- Disodium hydrogen phosphate (0.2M)
- Formic acid, LCMS grade
- Water - Millipore Water

- Trichloroacetic acid
- Buffer :Mixture of 0.1M citric acid hydrate and 0.2 Disodium hydrogen phosphate mix in 60:40 v/v

**D. Standard preparation**

- Stock preparation (1000 PPM):
  Weigh 10 mg standard and make up the volume to 10 ml with methanol in volumetric flask. Purity factor and salt corrections (wherever applicable) are to be considered for calculating exact concentration. Stock standard is stored at -20°C.

- Intermediate Standard preparation (10 PPM):
  Intermediate standard solutions are prepared by diluting appropriate (0.1 ml) volume from each stock solution to 10 ml in a volumetric flask with methanol

- Working (Mix) solution:
  Always prepare fresh working solution as per requirement.

**E. Calibration standards**

Matrix matched calibration standards are prepared for at least 5 points including blank and treated in the same way as that of samples.

**F. Extraction Procedure**

1) Weigh 2.0 grams of milk into a 50 mlpolypropylene tube and spiked with the working standard mixtures.
2) After waiting for 10 minutes, add 100 μL of 20% (w/v) trichloroacetic acid and vortex for 1 minute.
3) Add 10 ml of buffer (pH 4).
4) Vortex for 1 min and put the sample tubes in rotary shaker for 10 minutes to enable proper mixing of the contents.
5) Centrifuge at 4000rpm at 4ºC for 15 min.
6) Collect the supernatant and filter through blotting paper.
7) The filtrate is submitted to a solid-phase extraction (SPE) clean-up procedure.
8) First the SPE cartridge (Oasis HLB) is conditioned with 6ml of methanol and 6ml of water. Then transfer the filtrate of sample to the cartridge.

9) After the washing step with 6ml of a mixture of 5% methanol in water (v/v), the cartridge is dried in air stream.
10) Then the analytes are eluted with 6ml of methanol and elute is evaporated to dryness in a nitrogen stream at 40ºC.
11) The dried sample is reconstituted in 1ml of the water containing 10% acetonitrile.
12) Vortex the samples, filter through 0.2 µn syringe filter into a glass vial and inject.

**G. UPLC Conditions**

- Mobile phase A : 5 mM ammonium formate in water with 0.1% Formic acid
- Mobile phase B : 0.1 % Formic acid in methanol
- Column: Acquity UPLC, BEH C18, 1.8 X 100mm
- Column temperature: 40°C
- Injection volume : 5 µl
- Flow rate : 0.4 ml/min
- Run time: 8 min.

**H. UPLC mobile phase gradient**

| Time (min) | Mobile Phase A (%) | Mobile Phase B (%) |
|---|---|---|
| Initial | 90.0 | 10.0 |
| 0.4 | 90.0 | 10.0 |
| 4.0 | 10.0 | 90.0 |
| 5.0 | 10.0 | 90.0 |
| 5.1 | 90.0 | 10.0 |
| 8.0 | 90.0 | 10.0 |

**I. MS Conditions**

| | |
|---|---|
| Polarity | ESI +ve |
| Capillary (kV) | 3 |
| Cone (V) | 50 |
| Source offset (V) | 80 |
| Source temperature ($^0$C) | 150 |
| Desolvation Temperature ($^0$C) | 500 |
| Cone Gas Flow (L/hr) | 150 |
| DesolvationGasflow (mL/min) | 1000 |

| | |
|---|---|
| Collision Gas Flow (mL/min) | 0.15 |
| Nebuliser Gas Flow (Bar) | 7.00 |

## J. MRM of analytes

| Sr. No. | Name | Parent | Product ion | Cone Volt | CE |
|---|---|---|---|---|---|
| 1 | Sulfanilamide | 173.00 | 92.00 | 25 | 15 |
| | | 173.00 | 156.00 | 25 | 15 |
| 2 | Sulfadiazine | 251.00 | 92.00 | 30 | 27 |
| | | 251.00 | 156.00 | 30 | 15 |
| 3 | Sulfathiazole | 256.00 | 92.00 | 31 | 25 |
| | | 256.00 | 156.00 | 31 | 15 |
| 4 | Sulfamerazine | 265.10 | 92.00 | 35 | 25 |
| | | 265.10 | 156.00 | 35 | 15 |
| 5 | Sulfamethiazole | 271.00 | 92.00 | 30 | 25 |
| | | 271.00 | 156.00 | 30 | 15 |
| 6 | Sulfadimidine | 279.10 | 92.00 | 35 | 30 |
| | | 279.10 | 124.10 | 35 | 20 |
| 7 | Sulfamethazine | 279.10 | 124.10 | 35 | 25 |
| | | 279.10 | 186.00 | 35 | 15 |
| 8 | Sulfamethoxypyridazine | 281.00 | 92.00 | 35 | 30 |
| | | 281.00 | 156.00 | 35 | 15 |
| 9 | Sulfadoxine | 311.00 | 92.00 | 35 | 32 |
| | | 311.00 | 156.00 | 35 | 15 |
| 10 | Sulfadimethoxine | 311.10 | 92.00 | 36 | 32 |
| | | 311.10 | 156.00 | 36 | 20 |
| 11 | Trimethoprim | 291.00 | 230.00 | 24 | 35 |
| | | 291.00 | 261.00 | 24 | 33 |
| 12 | Ciprofloxacin | 332.10 | 288.10 | 32 | 18 |

| | | | | | |
|---|---|---|---|---|---|
| | | 332.10 | 314.10 | 32 | 22 |
| 13 | Enrofloxacin | 360.20 | 245.00 | 32 | 20 |
| | | 360.20 | 316.10 | 32 | 22 |
| 14 | Spiramycin | 422.20 | 101.00 | 22 | 22 |
| | | 422.20 | 174.10 | 22 | 20 |
| 15 | Neospiramycin | 699.00 | 174.00 | 35 | 28 |
| | | 699.00 | 142.00 | 35 | 28 |
| 16 | Erythromycin | 734.50 | 116.03 | 64 | 46 |
| | | 734.50 | 158.00 | 64 | 30 |
| 17 | Tilmicosin | 843.50 | 142.10 | 50 | 40 |
| | | 843.50 | 174.10 | 50 | 40 |
| 18 | Tylosin | 916.50 | 101.10 | 57 | 45 |
| | | 916.50 | 174.10 | 57 | 45 |

## K. References


1) Bohm, D. A., Stachel, C. S., &Gowik, P. (2009). Multi-method for the determination of antibiotics of different substance groups in milk and validation in accordance with Commission Decision 2002/657/EC. Journal of Chromatography A, 1216(46), 8217-8223.
2) Wang, J., & Leung, D. (2009). Determination of spiramycin and neospiramycin antibiotic residues in raw milk using LC/ESI-MS/MS and solid-phase extraction. Journal of separation science, 32(4), 681-688.

**Annexure-III**

## DETERMINATION OF NITROFURAN RESIDUES IN MILK USING LC-MS/MS

### A. Scope

The method involves the release of the bound nitrofuran metabolites by acid hydrolysis and simultaneous derivatization with 2-NBA and detection of derivatives of AOZ. AMOZ, SEM and AHD by LC-MS/MS.

### B. Apparatus/Requirements

- Analytical Balance
- Vortex mixer
- Calibrated micropipettes
- 50 mL centrifuge tubes
- Tube Shaker
- Centrifuge
- Nitrogen Evaporator Apparatus with Heated Water Bath
- 15 ml centrifuge tubes
- 0.2 μm Nylon Syringe Filter
- 5 mL Plastic Syringe
- Glass Auto sampler Vials & Caps - 2 mL
- Glass Volumetric Flasks - Class A
- Graduated cylinders - Class A

### C. Reagents/chemicals/solutions

- Reference standards of nitrofuran metabolites (AMOZ, AOZ, AHD, SEM) and internal standards (Amoz-d5, AOZ d-4, AHD-C13, SEM-IS)
- Ethyl acetate
- Ammonium acetate
- Hydrochloric acid
- 2-Nitrobenzaldehyde
- Anhydrous potassium phosphate dibasic
- Sodium hydroxide
- Dimethyl sulphoxide
- Sodium chloride
- Acetonitrile, LCMS grade
- Methanol, LCMS grade

- Formic acid, LCMS grade
- Water - Millipore Water

**D. Standard preparation**

- Stock preparation (1000 PPM) :
  Weigh 10 mg standard and make up the volume to 10 ml with methanol in volumetric flask. Purity factor and salt correction (wherever applicable) were considered for calculating exact concentration. Stock standard is stored at -20°C.

- Intermediate Standard preparation (10 PPM):
  Intermediate standard solutions are prepared by diluting appropriate (0.1 ml) volume from each stock solution to 10 ml in a volumetric flask with methanol.

- Working (Mix) solution:
  Always prepare fresh working solution as per requirement.

**E. Calibration standards**

Matrix matched calibration standards are prepared for at least 5 points including blank and treated in the same way as that of samples.

**F. Extraction procedure**

1) Weigh precisely 2 gm of milk into a 50 ml polypropylene tube and spike the calibration standards from working standard mixture.
2) Add known concentration of internal standards to all the samples.
3) Add 10 ml of 0.125 M HCL and 400 µl of freshly prepared 2-NBA solution (50 mM in DMSO) to each sample.
4) The samples are then subjected to vortex and incubated at 37°C for 16 h with gentle shaking.
5) After incubation and cooling to room temperature, add 1 ml of 0.1 M K2HPO4 and 1 ml 0.8 M NaOH.
6) Adjust the pH to 7 ± 0.25 by adding 0.8 M aqueous NaOH or 0.125 M HCL.
7) Add 0.5 gm sodium chloride and 15 ml ethyl acetate to the tube and vortex for 1 minute.
8) Centrifuge the samples at 5000 rpm for 5 minutes at 4°C.
9) Transfer ethyl acetate layer into glass tube.
10) Repeat the extraction and combine the ethyl acetate layer with first extract.

11) Evaporate ethyl acetate to dryness under stream of nitrogen at 40°C.
12) Add 2 ml of methanol : water (1:1) and 2 ml hexane to the dried sample.
13) Vortex the contents for 30 seconds and transfer into 15 ml polypropylene tube.
14) Centrifuge at 6000 rpm for 5 minutes.
15) Discard hexane (upper layer), filter the lower layer through 0.2 μm syringe filter into a glass vial and inject.

**G. UPLC Conditions:**

- Mobile phase A : water with 0.1% Formic acid
- Mobile phase B : 20 mM ammonium acetate in methanol
- Column: Acquity UPLC, BEH C18, 1.8 X 100mm
- Column temperature: 40°C
- Injection volume : 5 μl
- Flow rate : 0.4 ml/min
- Run time: 8 min.

**H. UPLC Mobile phase gradient**

| Time (min) | Mobile Phase A (%) | Mobile Phase B (%) |
|---|---|---|
| Initial | 90.0 | 10.0 |
| 0.4 | 90.0 | 10.0 |
| 4.5 | 02.0 | 98.0 |
| 5.0 | 02.0 | 98.0 |
| 5.1 | 90.0 | 10.0 |
| 8.0 | 90.0 | 10.0 |

**I. MS Conditions**

| | |
|---|---|
| Polarity | ESI +ve |
| Capillary (kV) | 1 |
| Cone (V) | 50 |
| Source offset (V) | 80 |
| Source temperature ($^0$C) | 150 |
| Desolvation Temperature ($^0$C) | 500 |
| Cone Gas Flow (L/hr) | 150 |
| DesolvationGasflow (mL/min) | 1000 |
| Collision Gas Flow (mL/min) | 0.15 |
| Nebuliser Gas Flow (Bar) | 7.00 |

## J. MRM of analytes

| Sr. No. | Name | Parent | Product ion | Cone Volt | CE |
|---|---|---|---|---|---|
| 1 | 2NP-AOZ | 236.00 | 104.00 | 50 | 20 |
| | | 236.00 | 134.00 | 50 | 14 |
| 2 | 2NP-AOZ-d4 | 240.00 | 134.00 | 50 | 13 |
| 3 | 2NP-AMOZ | 335.10 | 100.00 | 35 | 26 |
| | | 335.10 | 291.20 | 35 | 12 |
| 4 | 2NP-AMOZ-d5 | 340.10 | 296.20 | 35 | 12 |
| 5 | 2NP-AHD | 249.00 | 104.00 | 50 | 20 |
| | | 249.00 | 134.00 | 50 | 10 |
| 6 | 2NP-AHD-13C3 | 252.00 | 134.00 | 50 | 11 |
| 7 | 2NP-SEM | 209.00 | 166.10 | 30 | 8 |
| | | 209.00 | 192.10 | 30 | 10 |
| 8 | 2NP-AHD-13C3 | 212.00 | 168.00 | 30 | 10 |

## K. References


1) Alkan, F., Kotan, A., &Ozdemir, N. (2016). Development and validation of confirmatory method for analysis of nitrofuran metabolites in milk, honey, poultry meat and fish by liquid chromatography-mass spectrometry. Macedonian Veterinary Review, 39(1), 15-22.
2) Chu, P. S., & Lopez, M. I. (2007). Determination of nitrofuran residues in milk of dairy cows using liquid chromatography− tandem mass spectrometry. Journal of agricultural and food chemistry, 55(6), 2129-2135.
3) Rodziewicz, L. (2008). Determination of nitrofuran metabolites in milk by liquid chromatography–electrospray ionization tandem mass spectrometry. Journal of Chromatography B, 864(1), 156-160.

**Annexure-IV (I-A)**

## DETERMINATION OF SULFONAMIDES IN HONEY USING LC-MSMS BY QUECHERS METHOD

### A. Scope:

Quantitative determination of Sulfonamides residue in Honey

### B. Objective:

This method describes procedure for the quantitative determination of sulfonamides in honey using Acid hydrolysis followed by QuEChERS extraction procedure and HPLC-MSMS analysis

### C. Principle:

The honey sample hydrolyzed by Sonication followed by QuEChERS clean up and Acetonitrile extraction. The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method uses a single step with smaller sample size, acidified Acetonitrile extraction and salting out liquid partitioning from water in the sample with anhydrous sodium sulfate and anhydrous magnesium sulfate. Dispersive clean-up is done to remove organics, sugar, pigments, excess water and other components' with the combination of primary secondary amine, Magnesium sulfate,C18.The extracted sulfonamides dried and reconstituted with water: Acetonitrile before injection to LC-MSMS

### D. Reagent and Chemicals:

- 2M Hcl
- 0.3M Citric Acid
- Ammonia Solution 25%
- Acetonitrile (HPLC)
- Methanol(HPLC)
- Milli Q water
- Anhydrous Magnesium sulfate(GR)
- Anhydrous sodium acetate(GR)
- Acetic acid(GR)
- GCB(Agilent)
- Encapped C18(Agilent)
- Sulfonamides(Sulfanilamide,Sulfadiazine,Sulfathiazole,Sulfamerazine, Sulfamethazine,Sulfamethizole,Sulfamethoxypyridazine,Sulfamethaxozole,Sulfadioxin,Sulfadimethoxin)
- Trimethoprim (Sigma SRM)

## E. Apparatus:

- Blender
- Vortex Mixer
- Centrifuge tubes(15 /50 ml
- Refrigerated Centrifuge
- Micro pipettes
- TurboVap-II Concentrator
- LC Vials
- Weighing balance
- 0.45micronX1cm dia filter

## F. Standard Preparation

- Standard Preparation (1000PPM)
  Weigh 10mg each of sulfonamides,(Sulfanilamide,Sulfadiazine,Sulfathiazole,Sulfamerazine, Sulfamethazine,Sulfamethizole,Sulfamethoxypyridazine,Sulfamethaxozole,Sulfadioxin,Sulfadimethoxin) and Trimethoprim (Sigma SRM) and sulfa pyridine IS diluted to 10ml methanol, further exact weight and purity used for calculating actual concentration.
- Intermediate Mix Standard Preparation (100ppm,10ppm)
  Intermediate standard solutions are prepared by diluting appropriate volume (0.1 ml to 10 ml ) with methanol, Intermediate standard solution of 1ppm(1000ppb) and 0.5ppm were made by serial dilutions with water: Acetonitrile (70:30)
- Working Standards for matrix calibration
  - Blank Sample - 0 ppb
  - 20µL of 1000ppb in 2gm Sample -10ppb
  - 30µL of 1000ppb in 2gm Sample -15ppb
  - 40µL of 1000ppb in 2gm Sample -20ppb
  - 100µL of 1000ppb in 2gm Sample -50ppb
  - Internal Standards 40µL of 1000ppb in 2 gm all Sample and matrix standards-20ppb

## G. Extraction Procedure:

- Weigh 2g sample (±0.05g) in 50ml polypropylene Tube
- Spike Standards and IS (if necessary),Vortex for 30 Sec and keep it for 30 min
- Add 6.0ml of 2M Hcl and Sonicate for 45 Min at 35 deg

- Add 10.0ml of 0.3M Citric Acid and adjust PH with 25% Ammonia(~1.7ml) solution.
- Add 10.0ml of 1%Acetic Acid in Acetonitrile, Vortex for 30 Sec
- Add 2.0g of Sodium Acetate and 4gm of Magnesium Sulfate vortex for 1 min.
- Chill to 4 deg and centrifuge at 4000rpm for 5 min/4deg
- Transfer 6ml of extract to 15ml PP tube containing 400mg PSA+600mgMgSo4+5mgGCB,Vortex for 1min and chill to 4deg
- Centrifuge at 4000rpm for 5min/4deg
- Transfer 5ml of extract to test tube and dry under nitrogen flow at 50 deg
- Reconstitute with 1ml of water :Acetonitrile (70:30)
- Transfer to LC-MSMS Vial and inject 20µL

## H. HPLC Condition:

Mobile Phase A: 0.1% Formic Acid in Water
Mobile Phase B: 0.1% Formic Acid in Acetonitrile:Water (50;50)
Column:Poreshell 120EC-C18(100mmX4.6mmX2.7µm)
Column Temperature:40ºC
Injection Volume:20µL
Run Time:20 min
Post run :4 min

## I. HPLC Mobile Phase Gradient:

| Time | A% | B% | Flow |
|---|---|---|---|
| 0 | 90 | 10 | 0.5ml |
| 6 | 77 | 23 | 0.6ml |
| 15 | 40 | 60 | 0.6ml |
| 17 | 40 | 60 | 0.6ml |
| 20 | 90 | 10 | 0.5ml |

## J. MS Condition:

| MS Conditions | ESI+ive |
|---|---|
| Gas Temperature | 350 deg |
| Gas Flow | 10 L/Min |
| Nebulizer Pressure | 40Psi |
| Capillary Voltage | 4000V |

| | |
|---|---|
| MS1 Heater | 100 deg |
| MS2 Heater | 100 deg |

## K. MRM of analytes:

| S.No | Name of the Compound | Parent Ion | Fragment Voltage | Product Ion-1/CE | Product Ion-2/CE | Retention Time |
|---|---|---|---|---|---|---|
| 1 | Sulfanilamide | 173 | 70 | 156/2 | 108/13 | 3.2 |
| 2 | Sulfadiazine | 251.1 | 100 | 156/12 | 108/13 | 6.8 |
| 3 | Sulfa pyridine(IS) | 250 | 120 | 156/13 | 184/16 | 7.2 |
| 4 | Sulfamethazine | 279 | 100 | 186/14 | 124/21 | 8.8 |
| 5 | Sulfamethiazole | 256 | 100 | 156/12 | 108/23 | 7.0 |
| 6 | Sulfamerazine | 265 | 110 | 156/13 | 172/14 | 7.6 |
| 7 | Sulfamethizole | 271 | 110 | 156/13 | 108/23 | 9.1 |
| 8 | Sulfamethoxy pyridine | 281 | 130 | 156/15 | 126/18 | 9.9 |
| 9 | Sulfamethoxazole | 254 | 110 | 156/13 | 108/24 | 11.42 |
| 10 | Sulfadoxin | 311.1 | 100 | 156/15 | 108/24 | 11.39 |
| 11 | Sulfadimethoxin | 311 | 120 | 156/20 | 108/24 | 14.39 |
| 12 | Trimethoprim | 291 | 140 | 230/23 | 123/26 | 7.66 |

## L. References:


- Verzegassi,L,Savy-perroud,MC,and Stadler,R.H,. Application of liquid chromatography-electrospary ionization tandem mass spectrometry to detection of 10 sulfonamides in Honey" Nestle research centre,Journal of chromatography A,977(2002)77-87
- AOAC Official method 2007.01 Pesticide Residue in Foods by Acetonitrile extraction and partitioning with magnesium sulphate,J.AOAC Intl.90:485(2007)
- AnatasiosEconomou, olympiapetraki,DespinaTsipi,Elenibotitsi. Determination of liquid chromatography tandem mass spectrometric method for the determination of sulfonamides,trimethoprim and dapsone in honey and validation according to commission Decesion2002/657/EC for banned compounds,Talanta 97(2012)32-41

**Annexure IV (I - B)**

## DETERMINATION OF SULFONAMIDES IN HONEY USING LC-MSMS BY LIQUID-LIQUID EXTRACTION (LLE) METHOD

**A. Scope:**

Quantitative determination of sulfonamides residue in Honey.

**B. Objective:**

The method describes the procedure for the Quantitative determination of Sulfonamides in the Honey sample using liquid-liquid extraction followed by LCMS/MS Analysis.

**C. Principle:**

Sulfonamides are extracted from Honey sample by liquid-liquid partitioning with Acetonitrile and after salting out, the organic layer is evaporated on Nitrogen concentrator and finally reconstituted in water acidified with formic acid and injected on LCMSMS.

**D. Reagent and Chemicals:**

- Methanol (HPLC)
- Acetonitrile (HPLC)
- Formic acid
- Milli-Q-water
- NaCl
- Sulfonamides (Sulfanilamide, Sulfathiazole, Sulfamerazine, Sulfamethizole, Sulfamethazine, Sulfamethoxypyridazine, Sulfadimethoxine, Sulfadoxine, Sulfapyridine, Sulfamethoxazole).

**E. Apparatus:**

- Calibrated Micro pipettes 20 to 200µl and 100-1000µl capacity ranges.
- Nitrogen concentrator
- Refrigerated Centrifuge
- Vortex mixer
- Rotospin
- LC Vials
- Weighing Balance
- Centrifuge Tubes(15/50 mL)
- Syringe with 0.45µ syringe filter (Hydrophilic).

**F. Standard Preparation**:

- Standard Stock Preparation (1000 ppm)

Weigh 10 mg each Sulfonamide standard and transfer it to 10 mL volumetric flask. Add Acetonitrile and make up the volume up to the mark with it and calculate the actual concentration.

- Intermediate Mix Standard Preparation(10 ppm)

  Intermediate Standard solutions are prepared by diluting appropriate volume (0.1 mL to 10mL) with Acetonitrile in a 10 mL volumetric Flask.

- Spiking Solution for matrix Calibration :

  Spiking Solution for matrix matched calibration is prepared by serial dilution of intermediate solution.

  **Working Standards for matrix Calibration**

| | |
|---|---|
| Blank Sample | : 0 ppb |
| 25µL of 1000ppb in 5g Sample | : 5 ppb |
| 50µL of 1000ppb in 5g sample | : 10 ppb |
| 75µL of 1000ppb in 5g sample | : 15ppb |
| 100µL of 1000ppb in 5g sample | : 20ppb |
| 250µL of 1000ppb in 5g sample | : 50ppb |

**G. Extraction Procedure:**

- Weigh 5.0 ±0.1gm honey in 50ml centrifuge tubes.
- Spike Standard and Internal Standard (if necessary), vortex for 2 minutes and keep it for 10 min at room temperature.
- Add 5ml water, vortex for 1 minutes and rotospin for 10 minutes.
- Add 10 ml Acetonitrile vortex for 2 minutes and rotospin for 10 minutes.
- Add 1gm of NaCl in it and rotospin for 5 minutes.
- Centrifuge the tubes at 4000 rpm for 5 minutes at 4 °C.
- Collect the upper organic layer and dry it under the gentle stream of Nitrogen in Nitrogen concentrator at 50°C.
- Reconstitute the analyte in 1 ml (Water with 0.2% Formic Acid).

- Filter the reconstitute with 0.2µm syringe filter into the vial and inject on LC/MS/MS.

**H. LC Conditions:**

Mobile Phase : A- 0.2%Formic acid in 1L Water

Mobile Phase : B- 0.2%Formic acid in 1L Methanol

Column : Eclipse plus C18 (100mm x 4.6mm x 3.5µm)

Column Oven Temp. : 40°C

Injection Volume : 20µL

Run Time : 15 min.

Flow : 0.4 mL/min

Gradient

| Time | Mobile Phase A (%) | Mobile Phase B (%) | Flow (mL/min) |
|---|---|---|---|
| 0.01 | 90 | 10 | 0.4 |
| 6.0 | 75 | 25 | 0.4 |
| 12.0 | 40 | 60 | 0.4 |
| 12.2 | 90 | 10 | 0.4 |
| 15 | 90 | Stop | 0.4 |

**I. MS Conditions:**

Curtain Gas : 25 psi

IS Voltage : 5500v

Temperature : 500°C

GS-1 : 50 psi

GS-2 : 50 psi

CAD : Medium

## J. MRM of Analytes:

| Analyte | Q1 | Q3 | DP | EP | CE | CXP | DWELL | Retention Time (Min) |
|---|---|---|---|---|---|---|---|---|
| **Sulfanilamide** | 173.1 | 93.2 | 51 | 10 | 31 | 6 | 80 | 3.73 |
| | 173.1 | 156.0 | 51 | 10 | 9 | 12 | 80 | |
| | 173.1 | 108.1 | 51 | 10 | 21 | 8 | 80 | |
| **Sulfadiazine** | 251.1 | 156.2 | 66 | 10 | 21 | 12 | 80 | 6.99 |
| | 251.1 | 92.1 | 66 | 10 | 35 | 6 | 80 | |
| **Sulfathiazole** | 263.3 | 156.1 | 65 | 10 | 21.4 | 12 | 80 | 7.54 |
| | 263.3 | 92.2 | 65 | 10 | 35.5 | 7 | 80 | |
| **Sulfamerazine** | 265.3 | 92.0 | 65 | 10 | 37.1 | 5 | 80 | 8.91 |
| | 265.3 | 156.1 | 65 | 10 | 33.3 | 11 | 80 | |
| | 265.3 | 107.9 | 65 | 10 | 35 | 6 | 80 | |
| **Sulfamethizole** | 271.2 | 156.1 | 65 | 10 | 20.4 | 10.3 | 80 | 10.16 |
| | 271.2 | 92.0 | 65 | 10 | 39 | 5 | 80 | |
| **Sulfamethazine** | 279.4 | 186.2 | 72 | 10 | 24.5 | 15 | 80 | 10.51 |
| | 279.4 | 124.0 | 72 | 10 | 34.5 | 9.5 | 80 | |
| **Sulfamethoxypyridazine** | 281.2 | 156.0 | 66 | 10 | 25.1 | 11.5 | 80 | 10.66 |
| | 281.2 | 92.1 | 66 | 10 | 39 | 5 | 80 | |
| **Sulfadimethoxine** | 311.1 | 156.0 | 76 | 10 | 29 | 10 | 80 | 13.59 |
| | 311.1 | 92.1 | 76 | 10 | 45 | 6 | 80 | |
| **Sulfadoxine** | 311.1 | 156.0 | 61 | 10 | 25 | 12 | 80 | 12.17 |
| | 311.0 | 92.0 | 61 | 10 | 43 | 6 | 80 | |
| **Sulfapyridine (IS)** | 250.0 | 156 | 61 | 10 | 23 | 12 | 80 | 8.18 |
| | 250.0 | 92.1 | 61 | 10 | 37 | 6 | 80 | |
| **Metronidazole** | 172.2 | 128.1 | 56 | 10 | 19 | 10 | 80 | 6.28 |
| | 172.2 | 82.1 | 56 | 10 | 35 | 4 | 80 | |

## K. References :


- André Schreiber.2014. Simultaneous Analysis of Chloramphenicol and Tetracycline Antibiotics in Food Samples Using the AB SCIEX Triple Quad™ 3500 System AB SCIEX Concord, Ontario (Canada).

- 2002/657/EC (Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results).

**Annexure-IV (II)**

## DETERMINATION OF NITROFURAN METABOLITES (AMOZ,AOZ,AHD,SEM) IN HONEY USING LC-MSMS

**A. Scope**

Determination and conformation of Nitro furan Metabolites residue in Honey

**B. Objective**

Determination and conformation of Nitro furan Metabolites residue in Honey By HPLC-MSMS

**C. Reagent and Chemicals**

- Ethyl Acetate
- Acetonitrile(HPLC grade)
- Carbon tetra Chloride
- N-Hexane
- Nitrofuran metabolites reference Standard
- Internal Standard

**D. Apparatus**

- Blender
- Vortex Mixer
- Centrifuge tubes(15 /50 ml
- Refrigerated Centrifuge
- Micro pipettes
- TurboVap-II Concentrator
- LC Vials
- Weighing balance
- 0.45micronX1cm dia filter

**E. Standard Preparation**

Standard stock and intermediate solution prepared in methanol are stable for one year and six months respectively at 4ºC and working standards are prepared in methanol- water every month

**F. Extraction Procedure**

- Weigh 1.0±0.05g of honey in 15ml centrifuge tube.
- Add 1.0ml of demonized water and place all the tubes in oven at 50ºC for 30 min to soften

- Fortify the sample with 100µL of internal standard which corresponds to 5µg/kg
- A sample then thoroughly vortex until the honey fully dissolved in water
- Add 5ml of ethyl acetate(HPLC grade) and vortex for 30sec.
- Add 1gm of Sodium chloride shake for 30 sec then centrifuge at 5000rpm for 10min at 4ºC
- Transfer the organic layer in to turbovap concentrator tube,repeat the extraction with another 5ml ethyl acetate and collect in the same turbovap concentrator tube.
- Evaporate contents to dryness under nitrogen pressure at 50-60ºC
- Dissolve the residue in 1ml of CCL4:n Hexane(1:1) by vortex for 30 seconds
- Add 1ml of mobile phase,mix and then centrifuge at 5000rpm for 10min at 4ºC
- Transfer the upper mobile phase layer in to LC Vials and inject in to HPLC

**G. HPLC Condition**

Mobile Phase A: 20mM Ammonium Formate with 0.01% Formic acid
Mobile Phase B: Acetonitrile
Column: Analytical C18 columnX4.6mmX5µm
Column Temperature:NA
Injection Volume:10µL
Run Time:15 min
Flow Rate :0.3 ml/Min

**H. HPLC Mobile Phase Gradient:**

| Time | A% | B% | Curve |
|---|---|---|---|
| 0 | 90 | 10 | 1 |
| 2 | 90 | 10 | 6 |
| 4 | 50 | 50 | 6 |
| 8 | 50 | 50 | 6 |
| 10 | 10 | 90 | 6 |
| 10.5 | 90 | 10 | 6 |
| 15 | 90 | 10 | 6 |

## I. MS Condition:

| MS Conditions | ESI+ive |
|---|---|
| Capillary Voltage | 3500V |
| Cone Voltage | 25V |

## J. MRM of Analytes:

| S.No | Name of the Compound | Parent Ion | Product Ion-1/CE | Product Ion-2/CE |
|---|---|---|---|---|
| 1 | NP AMOZ | 335 | 291/14 | 262/14 |
| 2 | NP AMOZ d5 | 340 | 296/13 | |
| 3 | NP AOZ | 236 | 134/12 | 104/12 |
| 4 | NP AOZ d4 | 240 | 134/11 | |
| 5 | NP AHD | 249 | 134/13 | 178/13 |
| 6 | NP SEM | 209 | 166/10 | 192/10 |

## K. References:


- Determination and conformation of Nitrofuran Residues in honey using LC-MSMS.J.Agric.Food Chem.2007,Vol55,No 4 1103-1108
- Detection and identification of metabolites of furazolidone(AOZ),Furaltadone(AMOZ),Nitrofurantoin(AHD) and Nitrofurazone(SEM) by LC-MSMS confirmatory analysis by state institute for quality control of agricultural products(RIKILT) Netherlands 2003.

**Annexure- IV (III)**

## DETERMINATION OF CHLORAMPHENICOL IN HONEY USING LC-MSMS

**A. Scope**

Quantitative determination of Chloramphenicol residues in Honey

**B. Objective**

Detection and Identification of Chloramphenicol residue in Honey HPLC-MSMS

**C. Principle**

HPLC MSMS with software, Analytical column Lichro CART 125-4 purosphar star RP-18e(5µm) or similar phase column, vortex mixer, centrifuge tubes(15ml),Refrigerator Centrifuge, micro pipette, Turbovap concentrator, LC vial

**D. Reagent and Chemicals**

- CAP reference standard
- CAP-d5(internal standard)
- Ethyl Acetate
- Carbon Tetra Chloride
- Water(LC-Milli Q Water)
- Methanol (HPLC grade)
- Acetonitrile(HPLC grade)

**E. Apparatus**

- Blender
- Vortex Mixer
- Centrifuge tubes(15 /50 ml
- Refrigerated Centrifuge
- Micro pipettes
- TurboVap-II Concentrator
- LC Vials
- Weighing balance
- 0.45micronX1cm dia filter

**F. Standard Preparation**

Chloramphenicol 1000ppm stock solution and 100ppm&10ppm intermediate solution in acetonitrile are prepared once in a six months. Other dilutions of 100ppb,10ppb and 1ppb in water are prepared once in 3 months or on exhaustion.0.3-5.0 ppb working standard are prepared in water on the day of analysis.1.0ppm stock standard on chloramphenicol d5 is prepared in acetonitrile water (50:50) once in three months

***Note***: Preparation of CAP standard dilution please refer PTH/CHEM/SOP/001 page3of 3

**G. Extraction Procedure**

- Weigh 1.0±0.05g of honey in 15ml centrifuge tube.
- Add 1.0ml of demonized water and mix well.
- Add 200µL of CAP-d5 (20ppb)in sample.
- Add 5ml of ethyl acetate and stir thoroughly for 10 min on a vortex.
- Centrifuge at 8500rpm at 4deg for 15 min.
- Transfer the upper layer in a clean turbovap concentrator tube, repeat extraction with another 5ml of ethyl acetate and collect ethyl acetate in the same turbovap and dry under nitrogen pressure in turbo II concentrator at 40-60deg.
- Dissolve the dry residue in 1ml CCL4 and hexane (1:1 V/V) by vortexing using vortex mixer. Add 1ml of deionised water and mix well by vortexing.
- Transfer the content in 15ml centrifuge tube and centrifuge at 8500rpm at 4 deg for 15 min.
- Transfer the upper phase(water) in quantitatively in to a LC vial20µL

**H. HPLC Condition**

Mobile Phase A: Water
Mobile Phase B: Acetonitrile
Column: Lichro CART 125-4 purosphar star RP-18e(5µm) or similar phase column
Column Temperature: NA
Injection Volume: 10µL
Run Time: 10 min
Flow Rate: 1.0 ml/Min

## I. HPLC Mobile Phase Gradient:

| Time | A% | B% | Curve |
|---|---|---|---|
| 0 | 80 | 20 | 1 |
| 2 | 80 | 20 | 1 |
| 3 | 2 | 98 | 6 |
| 4 | 2 | 98 | 6 |
| 4.1 | 80 | 20 | 6 |
| 6 | 80 | 20 | 1 |
| 10 | 80 | 20 | 1 |

## J. MS Condition:

| MS Conditions | ESI-ive |
|---|---|
| Capillary Voltage | 3000V |
| Cone Voltage | 3V |
| Collision Energy | 18 |

## K. MRM of analytes:

| S. No | Name of the Compound | Parent Ion | Product Ion-1/CE | Product Ion-2/CE |
|---|---|---|---|---|
| 1 | Chloramphenicol | 321 | 152 | 257 |

## L. References:


- Chloramphenicol identification by liquid chromatography Tandem mass spectrometry by Benard DELEPINE-AFFSSA-FOUGERES, Laboratory for studies and researches on Veterinarymedicine and disinfectant
- HPLC analysis of chloramphenicol in milk. Veterinary drug residues in milk producing animals and their products. Reference material and methods 2nd edition a book on behalf on European communities
- Detection,Confirmation and quantification in honey,shrimps and chicken using LC-MS triple Quadrapole,2007

**Annexure- V**

## DETERMINATION OF CHLORAMPHENICOL IN SHRIMP USING LC-MSMS

### A. Scope

Qualitative confirmation and quantitative detection of Chloramphenicol (CAP) in shrimp at sub-ppb level by LC-MS/MS.

### B. Reagents

a. Ethyl Acetate: High Purity
b. N-Hexane: Chrom AR HPLC Grade
c. Methanol: HPLC Grade
d. Acetonitrile: HPLC Grade
e. Water: Generated from Milli-Q Ultra Pure Water System
f. Glacial Acetic Acid
g. Ammonium Acetate
h. Sodium Chloride
i. Sodium Sulfate
j. Chloramphenicol: Reference Standard -Sigma (meets USP testing specifications)
k. Diluent: 1:1 Methanol: Ultrapure Water

### C. Apparatus

Analytical balances, Centrifuge, Vortex/rotary shaker, Separating funnel, Nitrogen evaporator, Laboratory homogenizer, Centrifuge tubes

### D. Instrument

The chromatographic separation is achieved on a Kinetin C18 column (2.6 μm, 2.1* 100 mm, at a flow rate of 200 μL/min and the column temperature is maintained at 40 °C. The mobile phase consists of 0.1% acetic acid and 10 mM ammonium acetate in water (solvent A) and 0.1% acetic acid and 10 mM ammonium acetate in 95:5 CH3CN: Water (solvent B). the gradient is presented in Table 1. The injection volume is 10 μl.

| Time (min.) | Mobile phase composition/vol. % | |
|---|---|---|
| | A | B |
| 0 | 100 | 0 |
| 15 | 20 | 80 |
| 15.5 | 100 | 0 |
| 20.5 | 100 | 0 |

Table 1: HPLC gradient for the elution of target compounds

E. Autosampler Conditions

Syringe Flush Volume and Wash Volume- 6 ml

Sample Tray Temperature- 10 °C

F. Mass Spectrometer Conditions

Mode: Negative ion Electrospray-with metal needle option. The metal needle is connected with a zero dead volume union to the fused silica capillary delivering the mobile phase to the ESI source; this eliminated the frequent need to reposition the capillary.

*Note that the parameters need to be optimized for each instrument. The following conditions were found to maximize response on our instrument.*

Precursor Ion (m/z) : 321

Product Ions (m/z) : 257,194,176,152

Spray Voltage : 1.5 kV

Collision Voltage : 26 V

Source offset Voltage : 5 V

Electron Multiplier Voltage: 1.27 kV

Capillary Temperature : 350 °C

N2 Sheath Gas : 80 Arbitrary Units

N2 Auxiliary Gas : 35 psi

Collision Gas : Ar

**G. Preparation of Standards**

Calibration and spike standards were prepared in 50:50 Methanol: Ultra Pure Water (Diluent) from CAP reference standard. Preparation of a typical set of standards is given below.

-37.1 mg CAP dissolved in 50.0 ml methanol to yield 742000 ng/ml (#1)

(note: the following standards were diluted with Diluent)

-3.00 mL #1 to 100.0 ml to yield 22,300ng/ml (#2)

-3.00 mL #2 to 50.0 ml to yield 1,340 ng/ml (#3)

-3.00 mL #3 to 100.0 ml to yield 40 ng/ml

-The 40 ng/ml standard is used directly and diluted to yield six standards 1.0 through 40 ng/ml. Spike recoveries are performed at CAP concentration of

0.15,0.3,0.45 and 0.6 ng/ml in the shrimp along with reagent blank. These were used for the calibration curves.

**H. Sample processing**

Freshly collected samples must be kept cold before and during shipping to the laboratory. Once received at the laboratory, samples must be frozen (<-10 to -18°C) prior to grinding, if they cannot be prepared on the day of receipt. Once frozen, the sample should be allowed to thaw, while keeping it as cold as possible. Grind the tissue in a blender until homogenous.

**I. Extraction procedure**

Weigh precisely 10 g of homogenized shrimp in a centrifuge tube. Add 200 ml of CAP-d5 (20 µg/L) in sample. Add 20ml ethyl acetate. Keep it at room temperature for 15 minutes. Stir thoroughly for 10 minutes on a vortex / rotary shaker. Centrifuge at 3000 rpm for 5 minutes. Transfer the upper ethyl acetate layer in a clean turbovap tube concentrate under N2. Repeat the extraction with another 10 ml ethyl acetate and collect all ethyl acetate layer in the same turbovap tube and dry under N2 at 45 °C. Add 2 ml methanol to sample tube and vortex for 30 seconds. Make up to 25 ml with 4% NaCl solution and shake for 5 seconds. Transfer the contents to a 125ml separating funnel. Add 20ml hexane to sample tube and shake for 5 seconds and transfer the same to separating funnel and shake for 30 seconds. Collect the bottom aqueous portion to sample tube and discard hexane layer. Again add 20 ml hexane into sample tube and separate out the hexane layer. Repeat the above procedure with 15 ml ethyl acetate twice. Decant the ethyl acetate layer though Na2SO4 to a sample tube. Evaporate the contents at 45 ºC in a nitrogen evaporator. Wash down the sides of tube with 2 ml ethyl acetate and evaporate. Add 0.5 ml methanol: water (v/v :50/50) and vortex for 30 seconds. Filter through 0.2 µm syringe filter. The collected filtrate (corresponded to 10 g shrimp/ml) was injected into the LC-MS/MS system under optimized analytical condition.

**J. LC-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 from calibration curve into LC system and obtain the MS chromatogram

**References**

FDA/ORA/DFS No. 4290- LC/MS/MS Analysis of Chloramphenicol in Shrimp by Barbara K. Neuhaus, Jeffrey A. Hurlbut and Walter Hammack