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on

Analysis of Formaldehyde, Antibiotics and Pesticides in Fish and Fish Products

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OVERVIEW OF ANTIBIOTICS AND VETERINARY DRUGS, PESTICIDES, FORMALDEHYDE- CLASSIFICATION, USES, SAFETY ISSUES

Introduction

"Antibiotic" is a chemical substance produced by a living organism, generally a microorganism, that is detrimental to other microorganisms. Antibiotics commonly are produced by soil microorganisms and probably represent a means by which organisms in a complex environment, such as soil, control the growth of competing microorganisms. Microorganisms that produce antibiotics useful in preventing or treating disease include the bacteria and the fungi. Antibiotics came into worldwide prominence with the introduction of penicillin in 1941. Since then they have revolutionized the treatment of bacterial infections in humans and other animals. In 1928 Scottish bacteriologist Alexander Fleming noticed that colonies of bacteria growing on a culture plate had been unfavourably affected by a mold, *Penicillium notatum*, which had contaminated the culture. A decade later British biochemist Ernst Chain, Australian pathologist Howard Florey, and others isolated the ingredient responsible, penicillin, and showed that it was highly effective against many serious bacterial infections. Toward the end of the 1950s scientists experimented with the addition of various chemical groups to the core of the penicillin molecule to generate semisynthetic versions. A range of penicillins thus became available to treat diseases caused by different types of bacteria, including staphylococci, streptococci, pneumococci, gonococci, and the spirochaetes of syphilis.

Conspicuously unaffected by penicillin was the tubercle *bacillus* (*Mycobacterium tuberculosis*). This organism, however, turned out to be highly sensitive to streptomycin, an antibiotic that was isolated from *Streptomyces griseus* in 1943. As well as being dramatically effective against tuberculosis, streptomycin demonstrated activity against many other kinds of bacteria, including the typhoid fever bacillus. Two other early discoveries were gramicidin and tyrocidin, which are produced by bacteria of the genus *Bacillus*. Discovered in 1939 by French-born American microbiologist René Dubos, they were valuable in treating superficial infections but were too toxic for internal use. In the 1950s researchers discovered the cephalosporins, which are related to penicillins but are produced by the mold *Cephalosporium acremonium*. The following decade scientists discovered a class of antibiotics known as quinolones. Quinolones interrupt the replication of DNA-a crucial step in bacterial reproduction and have proven useful in treating urinary tract infections,

infectious diarrhea, and various other infections involving elements such as bones and white blood cells.

Use and administration of antibiotics

The principle governing the use of antibiotics is to ensure that the patient receives one to which the target bacterium is sensitive, at a high enough concentration to be effective but not cause side effects, and for a sufficient length of time to ensure that the infection is totally eradicated. Antibiotics vary in their range of action. Some are highly specific. Others, such as the tetracyclines, act against a broad spectrum of different bacteria. These are particularly useful in combating mixed infections and in treating infections when there is no time to conduct sensitivity tests. While some antibiotics, such as the semisynthetic penicillins and the quinolones, can be taken orally, others must be given by intramuscular or intravenous injection.

Categories of antibiotics

Antibiotics can be categorized by their spectrum of activity- namely, narrow-, broad-, or extended-spectrum agents. Narrow-spectrum agents (e.g., penicillin G) affect primarily gram-positive bacteria. Broad-spectrum antibiotics, such as tetracyclines and chloramphenicol, affect both gram-positive and some gram-negative bacteria. An extended-spectrum antibiotic is one that, as a result of chemical modification, affects additional types of bacteria, usually those that are gram-negative. (The terms *Gram-positive* and *Gram-negative* are used to distinguish between bacteria that have cell walls consisting of a thick meshwork of peptidoglycan [a peptide-sugar polymer] and bacteria that have cell walls with only a thin peptidoglycan layer, respectively.)

Veterinary drugs

Veterinary drugs are used for therapeutic and prophylactic purposes in animals to regulate infections of bacterial and prevent outbreaks of animal diseases. Veterinary drugs residues are historically associated to aquaculture products, as its worldwide growth has been accompanied by an increase in their use, mainly for the treatment or prevention of parasitic and microbial diseases. Considering fish as an aquatic animal, several drugs are used in aquaculture. These drugs are administered as medicated via feed, and some via immersion. However, fishes do not metabolize antibiotics effectively, and the majority of the administered dose is excreted. Ultimately, veterinary drugs in food animal production have become a primary global concern. Veterinary drugs residues were also found in wild fish, especially if caught close to aquaculture plants. Antibiotic residues in fishery products, which are influenced by the administration through feed in water and by environmental chemical and physical variables (e.g. sediment characteristics, water currents, temperature, light and pH) can also contribute to the

development of antibiotic resistance, a major concern for human and animal health worldwide. Misuse of veterinary drugs can result in high residue levels in fishery products.

Common antibiotics

Some common antibiotics are listed in the table.

Antibiotic	
Drug class	**Common uses**
Gentamicin, Tobramycin	infections of the respiratory and urinary tracts, blood, abdominal cavity; pelvic inflammatory disease
Cephalosporins (inhibit cell wall synthesis)	
cefaclor	infections of the respiratory and urinary tracts and skin; otitis media
cefamandole	infections of the respiratory and urinary tracts, skin, bone and joints, and blood; peritonitis
cefazolin	infections of the respiratory and genitourinary tracts, skin, bone and joints, and blood; endocarditis
ceftriaxone	infections of the respiratory and urinary tracts, skin, blood, abdominal cavity, and bone and joints; pelvic inflammatory disease; gonorrhea; meningitis
cefuroxime	infections of the respiratory and urinary tracts, skin, bone and joints, and blood
cephalexin	infections of the respiratory and urinary tracts, skin, and bone; otitis media
Chloramphenicols (inhibit protein synthesis)	
chloramphenicol	infections of the eyes, ears, and skin; cystic fibrosis; prevention of infection in minor wounds
Fluoroquinolones (interfere with DNA synthesis)	
ciprofloxacin	infections of the respiratory and urinary tracts, skin, eyes, abdominal cavity, and bone and joints; diarrhea; gonorrhea; sinusitis; pneumonia; prostatitis; anthrax
norfloxacin	urinary tract infections, STDs caused by Neisseria gonorrhoeae, eye infections, prostatitis
Lincosamides (inhibit protein synthesis)	

clindamycin	infections of the respiratory tract, skin, and abdominal cavity; acne; pelvic inflammatory disease
Macrolides (inhibit protein synthesis)	
azithromycin	infections of the respiratory tract and skin; STDs; otitis media; chronic obstructive pulmonary disease; pneumonia
clarithromycin	infections of the respiratory tract and skin; otitis media
erythromycin	infections of the respiratory tract, skin, and eyes; STDs; pertussis; diphtheria; intestinal amebiasis; otitis media; acne; Legionnaire disease; prevention of infection in minor wounds
Nitrofurans (inactivate essential cell components)	
nitrofurantoin	urinary tract infections
Penicillins (inhibit cell wall synthesis)	
amoxicillin	various streptococcal and staphylococcal infections
ampicillin	infections of the respiratory and urinary tract and blood; meningitis; gonococcal infections; endocarditis
penicillin G	streptococcal and staphylococcal infections
piperacillin	infections of the respiratory and genitourinary tracts, skin, abdominal cavity, bone and joints, and blood
ticarcillin	infections of the respiratory and gastrointestinal tracts; streptococcal and pseudomonas infections; gonorrhea; tonsillitis; Lyme disease; impetigo; otitis media; meningitis
Tetracyclines (inhibit protein synthesis)	
tetracycline	rickettsia, pneumonia, chlamydia, intestinal amebiasis, acne, prevention of infection in minor wounds
Miscellaneous antibiotics	
aztreonam	infections of the respiratory and genitourinary tracts, skin, abdominal cavity, and blood
imipenem-cilastatin	infections of the respiratory and genitourinary tracts, skin, abdominal cavity, bone and joints, and blood; endocarditis
isoniazid	tuberculosis
metronidazole	infections of the vagina and gastrointestinal tract

rifampin	tuberculosis
trimethoprim-sulfamethoxazole	urinary tract infections, shigellosis, otitis media, bronchitis, traveler's diarrhea
vancomycin	infections resistant to penicillins and cephalosporins

Mechanisms of action

Antibiotics produce their effects through a variety of mechanisms of action. A large number work by **inhibiting bacterial cell wall synthesis**; these agents are referred to generally as β-lactam antibiotics. Production of the bacterial cell wall involves the partial assembly of wall components inside the cell, transport of these structures through the cell membrane to the growing wall, assembly into the wall, and finally cross-linking of the strands of wall material. Antibiotics that inhibit the synthesis of the cell wall have a specific effect on one or another phase. The result is an alteration in the cell wall and shape of the organism and eventually the death of the bacterium.

Other antibiotics, such as the aminoglycosides, chloramphenicol, erythromycin, and clindamycin, **inhibit protein synthesis** in bacteria. The basic process by which bacteria and animal cells synthesize proteins is similar, but the proteins involved are different. Those antibiotics that are selectively toxic utilize these differences to bind to or inhibit the function of the proteins of the bacterium, thereby preventing the synthesis of new proteins and new bacterial cells.

Antibiotics such as polymyxin B and polymyxin E (colistin) **bind to phospholipids** in the cell membrane of the bacterium and interfere with its function as a selective barrier; this allows essential macromolecules in the cell to leak out, resulting in the death of the cell. Because other cells, including human cells, have similar or identical phospholipids, these antibiotics are somewhat toxic.

Some antibiotics, such as the sulfonamides, are competitive **inhibitors of the synthesis of folic acid (folate)**, which is an essential preliminary step in the synthesis of nucleic acids. Sulfonamides are able to inhibit folic acid synthesis because they are similar to an intermediate compound (para-aminobenzoic acid) that is converted by an enzyme to folic acid. The similarity in structure between these compounds results in competition between para-aminobenzoic acid and the sulfonamide for the enzyme responsible for converting the intermediate to folic acid. This reaction is reversible by removing the chemical, which results in the inhibition but not the death of the microorganisms. One antibiotic, rifampin, interferes

with ribonucleic acid (RNA) synthesis in bacteria by binding to a subunit on the bacterial enzyme responsible for duplication of RNA. Since the affinity of rifampin is much stronger for the bacterial enzyme than for the human enzyme, the human cells are unaffected at therapeutic doses.

Major antibiotics and their issues

Each type of antibiotic has a specific application in medicine and can serve as a useful model for exploring the various mechanisms by which antibiotics exert their effects. The following list mainly focus on the penicillins and cephalosporins, imipenem, the antituberculosis antibiotics, and the agents aztreonam, bacitracin, and vancomycin.

Penicillins

The penicillins have a unique structure, a β-lactam ring that is responsible for their antibacterial activity. The β-lactam ring interacts with proteins in the bacterial cell responsible for the final step in the assembly of the cell wall. The penicillins can be divided into two groups: the naturally occurring penicillins (penicillin G, penicillin V, and benzathine penicillin) and the semisynthetic penicillins. The semisynthetic penicillins are produced by growing the mold *Penicillium* under conditions whereby only the basic molecule (6-aminopenicillanic acid) is produced. By adding certain chemical groups to this molecule, several different semisynthetic penicillins are produced that vary in resistance to degradation by β-lactamase (penicillinase), an enzyme that specifically breaks the β-lactam ring, thereby inactivating the antibiotic. In addition, the antibacterial spectrum of activity and pharmacological properties of the natural penicillins can be changed and improved by these chemical modifications. The addition of a β-lactamase inhibitor, such as clavulanic acid, to a penicillin dramatically improves the effectiveness of the antibiotic. Several naturally occurring inhibitors have been isolated, and others have been chemically synthesized.

The naturally occurring penicillins remain the drugs of choice for treating streptococcal sore throat, tonsillitis, endocarditis caused by some streptococci, syphilis, and meningococcal infections. Several bacteria, most notably *Staphylococcus*, developed resistance to the naturally occurring penicillins, which led to the production of the penicillinase-resistant penicillins (methicillin, oxacillin, nafcillin, cloxacillin, and dicloxacillin). The use of several of these agents, however, has been severely limited by resistance; methicillin is no longer used, because of the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA). To extend the usefulness of the penicillins to the treatment of infections caused by gram-negative rods, the broad-spectrum penicillins (ampicillin, amoxicillin, carbenicillin, and ticarcillin) were

developed. These penicillins are sensitive to penicillinase, but they are useful in treating urinary tract infections caused by gram-negative rods as well as in treating typhoid and enteric fevers. The extended-spectrum agents (mezlocillin and piperacillin) are unique in that they have greater activity against gram-negative bacteria, including *Pseudomonas aeruginosa*, a bacterium that often causes serious infection in people whose immune systems have been weakened. They have decreased activity, however, against penicillinase-producing *Staphylococcus aureus*, a common bacterial agent in food poisoning. The penicillins are the safest of all antibiotics. The major adverse reaction associated with their use is hypersensitivity, with reactions ranging from a rash to bronchospasm and anaphylaxis. The more serious reactions are uncommon.

Cephalosporins

The cephalosporins have a mechanism of action identical to that of the penicillins. However, the basic chemical structure of the penicillins and cephalosporins differs in other respects, resulting in some difference in the spectrum of antibacterial activity. Modification of the basic molecule (7-aminocephalosporanic acid) produced by *Cephalosporium acremonium* resulted in **four generations** of cephalosporins. The first-generation cephalosporins (cefazolin, cephalothin, and cephalexin) have a range of antibacterial activity similar to the broad-spectrum penicillins described above. For instance, they are effective against most staphylococci and streptococci as well as penicillin-resistant pneumococci. The second-generation cephalosporins (cefamandole, cefaclor, cefotetan, cefoxitin, and cefuroxime) have an extended antibacterial spectrum that includes greater activity against additional species of Gram-negative rods. Thus, these drugs are active against *Escherichia coli* and *Klebsiella* and *Proteus* species (though several strains of these organisms have developed resistance). Cefamandole is active against many strains of *Haemophilus influenzae* and *Enterobacter*, while cefoxitin is particularly active against most strains of *Bacteroides fragilis*. Second-generation cephalosporins have decreased activity, however, against Gram-positive bacteria. The third-generation cephalosporins (ceftriaxone, cefixime, and ceftazidime) have increased activity against the Gram-negative organisms compared with the second-generation agents. Most *Enterobacter* species are susceptible to these drugs, as are *H. influenzae* and various species of *Neisseria*. The antibacterial spectrum of the fourth-generation compounds (cefepime) is similar to that of the third-generation drugs, but the fourth-generation drugs have more resistance to β-lactamases. Like the penicillins, the cephalosporins are relatively nontoxic. Because the structure of the cephalosporins is similar to that of penicillin, hypersensitivity reactions can occur in penicillin-hypersensitive patients.

Imipenem

Imipenem is a β-lactam antibiotic that works by interfering with cell wall synthesis. It is highly resistant to hydrolysis by most β-lactamases. This drug must be given by intramuscular injection or intravenous infusion because it is not absorbed from the gastrointestinal tract. Imipenem is hydrolyzed by an enzyme present in the renal tubule; therefore, it is always administered with cilastatin, an inhibitor of this enzyme. Neurotoxicity and seizures have limited the use of imipenem.

Antituberculosis antibiotics

Isoniazid, ethambutol, pyrazinamide, and ethionamide are synthetic chemicals used in treating tuberculosis. Isoniazid, ethionamide, and pyrazinamide are similar in structure to nicotinamide adenine dinucleotide (NAD), a coenzyme essential for several physiological processes. Ethambutol prevents the synthesis of mycolic acid, a lipid found in the tubercule bacillus. All these drugs are absorbed from the gastrointestinal tract and penetrate tissues and cells. An isoniazid-induced hepatitis can occur, particularly in patients 35 years of age or older. Cycloserine, an antibiotic produced by *Streptomyces orchidaceus*, is also used in the treatment of tuberculosis. A structural analog of the amino acid D-alanine, it interferes with enzymes necessary for incorporation of D-alanine into the bacterial cell wall. It is rapidly absorbed from the gastrointestinal tract and penetrates most tissues quite well; high levels are found in urine. Rifampin, a semisynthetic agent, is absorbed from the gastrointestinal tract, penetrates tissue well (including the lung), and is used in the treatment of tuberculosis. Rifampin administration is associated with several side effects, mostly gastrointestinal in nature. The drug can turn urine, feces, saliva, sweat, and tears red-orange in colour.

Aztreonam, bacitracin, and vancomycin

Aztreonam is a synthetic antibiotic that works by inhibiting cell wall synthesis, and it is naturally resistant to some β-lactamases. Aztreonam has a low incidence of toxicity, but it must be administered parenterally. Bacitracin is produced by a special strain of *Bacillus subtilis*. Because of its severe toxicity to kidney cells, its use is limited to the topical treatment of skin infections caused by *Streptococcus* and *Staphylococcus* and for eye and ear infections. Vancomycin, an antibiotic produced by *Streptomyces orientalis*, is poorly absorbed from the gastrointestinal tract and is usually given by intravenous injection. It is used for the treatment of serious staphylococcal infections caused by strains resistant to the various penicillins. Its use against MRSA led to the emergence of vancomycin-resistant *Staphylococcus aureus* (VRSA).

Antibiotic resistance

A problem that has plagued antibiotic therapy from the earliest days is the resistance that bacteria can develop to the drugs. An antibiotic may kill virtually all the bacteria causing a disease in a patient, but a few bacteria that are genetically less vulnerable to the effects of the drug may survive. These go on to reproduce or to transfer their resistance to others of their species through processes of gene exchange. With their more vulnerable competitors wiped out or reduced in numbers by antibiotics, these resistant strains proliferate. The end result is bacterial infections in humans that are untreatable by one or even several of the antibiotics customarily effective in such cases. The indiscriminate and inexact use of antibiotics encourages the spread of such bacterial resistance. Researchers are continually working to discover new antibiotics as a means of overcoming antibiotic resistance. Some potentially effective compounds that have been discovered include certain bacterial toxins and antimicrobial peptides. Novel treatment strategies, such as combining synergistic antibiotics to boost the killing of bacteria, are also under investigation. It may be possible to introduce compounds into bacterial populations that effectively resensitize the bacteria to existing antibiotic drugs. Antimicrobial resistance (AMR) in cultured fish has resulted from the continuous use of antibiotics in aquaculture. Eventually, it is increasing the possibility of AMR-related factors' transfer to the whole environment. Countries with climate change issues have the highest chance of AMR risks, including human health hazards with varied bioavailability in aquatic systems depending on their environmental impacts. Consequently, sustainable solutions to minimize this antibiotic usage with increased system resilience have recently become a significant concern.

Pesticides

Pesticides are chemicals that are applied to agricultural land, and other public areas to kill undesirable organisms. Pesticides in water resources adversely affect both the ecosystems and humans. Those materials have been thought as probable mutagens as they comprise constituents to trigger deviations in DNA. The word "pesticides" is a complex word that encompasses all compounds that are applied to destroy or regulate pests; this includes insecticides (insects), herbicides (weeds) and fungicides (fungi). Although the amount of pesticides in practice is quite large almost 2 million metric tons of active ingredient, it is likely that greater use will be associated with a few pesticide products. These 2 million tons of pesticides can be divided into 47.5% herbicides, 29.5% insecticides, 17.5% fungicides and 5.5% other pesticides. Although the practice of pesticides is low to zero in conventional and

subsistence farming in Asia and Africa, the environmental, water quality and public health effects of pesticides excessive and inappropriate uses are recognized worldwide.

Pesticides classification

Pesticide is a general word that describes numerous groups of insecticides, fungicides, herbicides, garden chemicals, household disinfectants and rodenticides that are operated to both destroy and protect from pests. These pesticides vary in their chemical and physical properties from one class to another. For that reason, it is praiseworthy to categorize them depending on their properties and study their particular groups. A synthetic pesticide is man-made chemicals, and does not exist in nature. They are classified into several groups based on their use. Currently, there are three widely held method of pesticides' classification recommended by Drum (1980). These three common approaches of pesticides classes encompass: (i) the chemical structure of the pesticide, (ii) the entry mode, and (iii) the action of pesticide and the organisms they kill. Chemical pesticides are classified into four types depending onto their sources: carbamate, organophosphate, organochlorine, and pyrethroid pesticides. On the other hand, there is another class of pesticides named biopesticides, which are naturally occurring or naturally derived materials especially from living organisms such as plants, fungi, bacteria, etc. Biopesticides are divided into three major groups: biochemical pesticides, microbial pesticides, and plant incorporated protectants.

Pesticide categories

Pesticides are classified based on the target pest object and are given special names to reflect their activities. The category names for these pesticides come from the Latin word *cide* (means killer), which is used after the name of the target pest. The pesticide categories based on target pests can be summarized in ghe following Table.

Table. Pesticide name and its type and target pests.

Type of pests	**Pesticides Example**	**Target pests/Function**
Avicides	Avitrol (aminopyridine)	Kill birds
Acaricides	Bifenazate	Kill mites that feed on plants and animals
Attractant	Pheromones	Attracts wide range of pests
Algaecides	Copper sulfate	Control or kill growth of algae
Bactericides	Copper complexes	Kill bacteria or acts against bacteria

Type of pests	Pesticides Example	Target pests/Function
Biopesticide	Bacillus thuringiensis	Wide range of organisms
Bait	Anticoagulants	Wide range of organisms
Desiccants	Boric acid	Act on plants by drying their tissues
Defoliant	Tribufos	Removes plant foliage
Fungicides	Azoxystrobin, Chlorothalonil	Kill fungi (including blights, mildews, molds, and rusts)
Fumigant	Aluminum phosphide	Wide range of organisms
Herbicides	Atrazine, glyphosate, 2,4-D	Kill weeds and other plants that grow where they are not wanted
Insecticides	Aldicarb, Carbaryl, imidacloprid	Kill insects and other arthropods
Insect growth regulator	Diflubenzuron	Insects
Lampricides	Trifluromethyl	Target larvae of lampreys which are jawless fish latching on vertebrate fish in rivers
Larvicides	Methoprene	Inhibits growth of larvae
Molluscicides	Metaldehyde	Inhibit or kill molluscs i.e. snails usually disturbing growth of plants
Moth balls	Dichlorobenzene	Stop any damage to cloths by moth larvae or molds
Nematicides	Aldicarb, Ethoprop	Kill nematodes that act as parasites of plants
Ovicides	Benzoxazin	Inhibits the growth of eggs of insects and mites
Piscicides	Rotenone	Act against fishes
Plant growth regulator	Gibberellic acid, 2,4-D	Regulates plant growth

Type of pests	Pesticides Example	Target pests/Function
Predacide	Strychnine	Mammal predators
Repellents	Methiocarb	Repel pests by its taste or smell, vertebrates and invertebrates
Rodenticides	Warfarin	Control mice and other rodents
Silvicides	Tebuthiuron	Acts against woody vegetation
Termiticides	Fipronil	Kill termites
Virucides	Scytovirin	Act against viruses

Chemical structure of pesticides

The most widespread and appropriate method for classifying insecticides depends on their chemical composition and description of the active ingredients. It is a type of classification that provides evidence of efficacy, chemical and physical properties of special pesticides. Depending on the chemical composition, pesticides are classified into 4 main categories: organiochlorine, organic phosphorous, carbamate, pyrethrin and pyrethroid.

a) Organochlorine pesticides (OCPs)

Organochlorines are stable chemicals that are very persistent in the environment and have the potential to accumulate in adipose tissue. In humans, these compounds or their metabolites mostly work at the level of the central nervous system altering enzymatic nerve membranes and electrophysiological properties, which leads to changes in the kinetics of the flow of K^+ and Na^+ through the nerve cell membrane and may cause symptoms such as acute poisoning death and seizures from apnea. Structurally, organochlorines fall into five classes: (1) DDT and its analogs including DDT and dichlorodiphenyldichloroethylene (DDE); Dichlorodiphenyldichloroethane (DDD) (2) hexachlorocyclohexane (HCH), such as lindane; (3) cyclodienes including aldrin, dieldrin, endrin (sometimes referred to as "drins" in the literature), heptachlor, chlordane, and endosulfan; (4) toxaphene; and (5) mirex and chlordecone . The field half-life time of some organochlorines such as DDT, DDE and DDD) is 15 years, while for aldrin and toxaphene is 365 and 9 days respectively. The acute toxicity of most OCPs generally occurs at concentrations that are higher than those considered environmentally realistic so death under natural conditions may be slow and often is seen as a general wasting away or chronic illness. The lipophilic and persistent nature of most OCPs can

lead to long-term storage in adipose tissue, followed by a release into the circulatory system during harsh environmental conditions. This can cause delays from the time of first exposure to the onset of effects. DDT can remain in the human body for 50 years or more.

b) Organophosphate pesticides

Some esters derived from phosphoric acid are known as organophosphate pesticides. These esters form are working in humans on the central nervous system by blocking the enzyme acetylcholine. This enzyme manages the amount and levels of the neurotransmitter acetyl cholinesterase, which disturbs the nerve impulse by the serene phosphorylation of the OH group in the active site of the enzyme. Intoxication symptoms are coma, dizziness, nausea, headache, cramps, convulsions, loss of reactions, and even death.

c) Carbamate pesticides

Some organic ester compounds derived from dimethyl N-methyl carbamic acid are used as herbicides, insecticides, nematicides and fungicides, and named as carbamates. Carbamates such as thiobencarb, propoxur, molinate, disulfiram (Antabuse), pyridostigmine, methiocarb and carbaryl are widely used in both cats and dogs. The carbamate compounds toxicity varies according the molecular structure, but in general they have shorter duration than that of organophosphates and organochlorines, and the latter inhibits acetyl cholinesterase.

d) Pyrethroid pesticides

Pyrethroids are natural insecticides derived from the pyrethrum extracts of chrysanthemum flowers known as pyrethrin found in Kenya. It works on the central nervous system, which causes fluctuations in the dynamics of sodium cation channels in the membrane of the nerve cell, which leads to an increase in the time of opening of the sodium channels. The sodium cation stream extends across the membrane in both vertebrates and insects.

Effects of pesticides in aquatic ecosystems

The application of pesticides may lead to contamination of the aquatic environment through several ways including: spray drift, runoff, and leaching. The transfer of pesticides sourced from agricultural land may be harmful to terrestrial and aquatic ecosystems. Pesticides can affect fishes in a direct way. Small fishes seem to be affected more than larger ones. There are also some indirect toxic effects of pesticides on fishes through decreasing fish's food sources (algae and plankton), changing food habits and deteriorating the quality of aquatic habitat. Some pesticides (e.g. herbicides) may reduce the abundance of primary producers thus ultimately decrease the primary and secondary consumers. The primary consumers such as zooplankton are severely affected by some organochlorine pesticides. In addition, other insecticides may also adversely affect micro-crustaceans.

Formaldehyde

Formaldehyde (systematic name methanal) is a naturally occurring organic compound with the formula CH_2O and structure H-CHO. The pure compound is a pungent, colourless gas that polymerises spontaneously into paraformaldehyde, hence it is stored as an aqueous solution (formalin), which is also used to store animal specimens. It is the simplest of the aldehydes (R-CHO). The common name of this substance comes from its similarity and relation to formic acid.

Forms

Formaldehyde is more complicated than many simple carbon compounds in that it adopts several diverse forms. These compounds can often be used interchangeably and can be interconverted.

Molecular formaldehyde. A colorless gas with a characteristic pungent, irritating odor. It is stable at about 150 °C, but polymerizes when condensed to a liquid.

1,3,5-Trioxane, with the formula $(CH_2O)_3$. It is a white solid that dissolves without degradation in organic solvents. It is a trimer of molecular formaldehyde.

Paraformaldehyde, with the formula $HO(CH_2O)nH$. It is a white solid that is insoluble in most solvents.

Methanediol, with the formula $CH_2(OH)_2$. This compound also exists in equilibrium with various oligomers (short polymers), depending on the concentration and temperature. A saturated water solution, of about 40% formaldehyde by volume or 37% by mass, is called "100% formalin".

Uses

Industrial applications

Formaldehyde is a common precursor to more complex compounds and materials. In approximate order of decreasing consumption, products generated from formaldehyde include urea formaldehyde resin, melamine resin, phenol formaldehyde resin, polyoxymethylene plastics, 1,4-butanediol, and methylene diphenyl diisocyanate. The textile industry uses formaldehyde-based resins as finishers to make fabrics crease-resistant. Formaldehyde is also a precursor to polyfunctional alcohols such as pentaerythritol, which is used to make paints and explosives.

Disinfectant and biocide

An aqueous solution of formaldehyde can be useful as a disinfectant as it kills most bacteria and fungi (including their spores). It is used as an additive in vaccine manufacturing to

inactivate toxins and pathogens. Formaldehyde releasers are used as biocides in personal care products such as cosmetics.

Tissue fixative and embalming agent

Formaldehyde preserves or fixes tissue or cells. The process involves cross-linking of primary amino groups. Formaldehyde is also used as a denaturing agent in RNA gel electrophoresis, preventing RNA from forming secondary structures. A solution of 4% formaldehyde fixes pathology tissue specimens at about one mm per hour at room temperature.

Drug testing

Formaldehyde and an 18 M (concentrated) sulfuric acid makes Marquis reagent - which can identify alkaloids and other compounds.

Photography

In photography, formaldehyde is used in low concentrations for the process C-41 (color negative film) stabilizer in the final wash step, as well as in the process E-6 pre-bleach step, to make it unnecessary in the final wash.

Safety

In view of its widespread use, toxicity, and volatility, formaldehyde poses a significant danger to human health. In 2011, the US National Toxicology Program described formaldehyde as "known to be a human carcinogen". However, concerns are associated with chronic (long term) exposure by inhalation as may happen from thermal or chemical decomposition of formaldehyde-based resins and the production of formaldehyde resulting from the combustion of a variety of organic compounds (for example, exhaust gases). As formaldehyde resins are used in many construction materials, it is one of the more common indoor air pollutants. At concentrations above 0.1 ppm in air, formaldehyde can irritate the eyes and mucous membranes. Formaldehyde inhaled at this concentration may cause headaches, a burning sensation in the throat, and difficulty breathing, and can trigger or aggravate asthma symptoms. In the residential environment, formaldehyde exposure comes from a number of routes; formaldehyde can be emitted by treated wood products, such as plywood or particle board, but it is produced by paints, varnishes, floor finishes, and cigarette smoking as well. For most people, irritation from formaldehyde is temporary and reversible, although formaldehyde can cause allergies and is part of the standard patch test series. People who suffer allergic reactions to formaldehyde tend to display lesions on the skin in the areas that have had direct contact with the substance, such as the neck or thighs (often due to formaldehyde released from permanent press finished clothing) or dermatitis on the face.

MAXIMUM LIMITS / MRL OF CHEMICAL HAZARDS/CONTAMINANTS IN FISH AND FISH PRODUCTS AS PER FSSR AND GLOBAL REGULATORY SCENARIO

Contamination of food may pose a serious risk to human health. Moreover, in some cases they may also have a negative impact on the quality of the food or feed. Food and feed can become contaminated by various causes and processes. Potential food safety hazard of environmental chemical contaminants (heavy metals, pesticides, and industrial chemicals) residues in farm-raised and wild caught seafood products are of great concern. The sources of these contaminants are wide-ranging. Some of these contaminants may have been manufactured for industrial or agriculture use and if released to the environment, they may enter the food chain. Other environmental contaminants, such as heavy metals (e.g., arsenic, cadmium, chromium, nickel, and lead) are naturally present in the environment, for example in rocks and soils. Contaminants can be transported to aquatic environments via municipal wastewater discharges and surface runoff from agricultural fields fertilized with animal manure and/or treated with pesticides. These contaminants may accumulate in fish and depending on the chemical's type and amount they can cause human health problems (e.g., developmental issues, carcinogenic or mutagenic effects). The hazard is commonly associated with exposure over a prolonged period of time (chronic exposure). Illnesses related to a single exposure (consumption of one fish meal) are very rare.

When there are indications that health hazards may be involved with consumption of food that is contaminated, it is necessary that a risk assessment should be undertaken. Depending on the assessment of the problems and the possible solutions, it may be necessary to establish MLs or other measures to control the contamination of food and feed. The Codex maximum level (ML) for a contaminant in a food or feed commodity is the maximum concentration of that substance recommended by the CAC to be legally permitted in that commodity. A Codex guideline level (GL) is the maximum level of a substance in a food or feed commodity which is recommended by the CAC to be acceptable for commodities moving in international trade. When the GL is exceeded, governments should decide whether and under what circumstances the food should be distributed within their territory or jurisdiction.

The traces pesticides leave in treated products or those left by veterinary drugs in animals are called "residues". The amounts of residues found in food must be safe for consumers and must be as low as possible. Codex sets MRLs for all food and animal feed.

The MRLs for all crops and all pesticides are publicly available on the Codex website. In the case of pesticide residues, a maximum residue limit (MRL) is the highest level of a pesticide residue that is legally tolerated in or on food or feed when pesticides are applied correctly in accordance with Good Agricultural Practice. And in the case of veterinary drug residues, the maximum residue limit (MRL) is the maximum concentration of residue legally tolerated in a food product obtained from an animal that has received a veterinary medicine. Codex Alimentarius Commission has given the General Standard for Contaminants and Toxins in Food and Feed (CXS 193-1995, revised in 2009, amended in 2019).

Chemical contaminants in seafood

Seafood is a rich source of nutrients and highly preferred among populations as a protein source. It is a highly traded commodity across nations. Some of the major food safety concerns linked to seafood are

presence of Ciguatera toxin in reef dwelling finfish

Veterinary Drug Residues in aquaculture products

Pesticide Residues in inland fishes

Heavy Metals in cephalopods and Pelagic Migratory Fishes

Polyaromatic Hydrocarbons (PAHs) in smoked products

Polychlorinated biphenyls in fishery products

Apart from the above-mentioned concerns which are mostly global, there are regional issues like use of adulterants like formaldehyde to retard decomposition process, ammonia to mask spoilage, use of un-approved additives (preservatives), and high level of pesticides in dry fish. The RASFF portal of EU indicates alert notifications due to presence of veterinary drug residues, heavy metals, histamine, foreign bodies, biotoxin, defective packaging, incorrect labelling, improper health certificate, unapproved colour and additives and organoleptic aspects. In recent months most of the rejections from Japan had been due to presence of furazolidone (AOZ) and Ethoxyquin in shrimp.

FOOD SAFETY AND STANDARDS (FOOD PRODUCTS STANDARDS AND FOOD ADDITIVES) REGULATIONS, 2011

The regulation covers the requirement of different food products. In the case of fish and fish products, it covers the vertical standards for frozen shrimp, frozen lobsters, frozen squid, frozen finfish, frozen fish fillets, dried shark fins, salted fish/dried salted fish, canned fishery products, frozen cephalopods, smoked fishery products, ready –to-eat finfish or shell fish curry in retortable pouches, sardine oil, edible fish powder, fish pickle, frozen minced fish meat, freeze dried prawns (shrimps), frozen clam meat, live and raw bivalve molluscs, fish sauce, quick

frozen fish sticks (fish fingers), fish portions and fish fillets - breaded or battered and fresh & quick frozen raw scallop products.

FOOD SAFETY AND STANDARDS (CONTAMINANTS, TOXINS AND RESIDUES) REGULATIONS, 2011

As per the regulation, "Crop contaminant" means any substance not intentionally added to food, but which gets added to articles of food in the process of their production (including operations carried out in crop husbandry, animal husbandry and veterinary medicine), manufacture, processing, preparation, treatment, packing, packaging transport or holding of articles of such food as a result of environmental contamination. FSSR (2011) covers metal contaminants, Naturally Occurring Toxins, Antibiotic and other pharmacologically active substances, Polychlorinated biphenyls (PCBs) and Polycyclic Aromatic Hydrocarbon (PAH) compounds in Fish and Fishery Products, biotoxins and histamine.

- **Metal contaminant**

The main threat among metal contaminants is cadmium (Cd), arsenic (As), lead (Pb) and mercury (Hg). Toxic metals above normal level affects the quality, safety and marketability of seafood. They are "Cumulative poisons" as they can lead to progressive and irreversible accumulation in body. Metallic elements having atomic weight higher than 40.04 and specific density > 5g/ cm3. The table given below show the permissible limits as per different regulations and as per FSSR (2011).

Heavy metals	Permissible limit (mg/Kg)			
	EU	USFDA	Codex	India
Mercury	*Fishery products* -0.5 *Certain fishes*- 1	*All fishes(methyl mercury)* - 1	*Fishes* – 0.5 *Predatory fishes* - 1	*Fishes* – 0.5 *Predatory fishes* - 1
Cadmium	*Crustaceans* – 0.5 *Bivalves* – 1 *Cephalopods* – 1 *Fishes* – 0.05 to 0.1	*Crustaceans* –3 *Bivalves* –4	*Bivalves* –2 *Cephalopods* – 2	*Fish* – 0.3 *Crustaceans* – 0.5 *Bivalves* –2 *Cephalopods* – 2
Lead	*Crustaceans* – 0.5 *Bivalves* – 1 *Cephalopods* – 1 *Fishes* – 0.2to 0.4	*Crustaceans* – 1.5	*Fish* - 0.3	*Fish* – 0.3 *Crustaceans* – 0.5 *Bivalves* – 1.5 *Cephalopods* – 1
Arsenic	*NIL*	*Crustaceans* – 76 *Bivalves* – 86	*NIL*	*Fish* – 76 *Crustaceans* – 76 *Bivalves* – 86

FSSR (2011) also specify the limit for Methyl Mercury (Calculated as the element): 0.25 ppm.

- **Naturally Occurring Toxins (NOTS)**

Saffrole in Fish Preparations and Fish Products:10 ppm

- **Antibiotic and other pharmacologically active substances**

Antibiotics with MRL for Fish and Fish Products are

Ampicillin: 0.01 ppm in Fish

Amoxicillin: 0.05 ppm (Fish Fillet and Muscle)

Cloxacillin: 0.01 ppm (All fish)

Chlortetracycline/Oxytetracycline/Tetracycline: 0.2 ppm (Prawn and Shrimp)

Flumequine: 0.5 ppm (Trout)

Sulfadiazine: 0.01 ppm (All fish)

Sulfanilamide: 0.01 ppm (All fish)

Sulfaguanidine: 0.01 ppm

- **Polychlorinated biphenyls (PCBs) and Polycyclic Aromatic Hydrocarbon (PAH) compounds in Fish and Fishery Products**

Name of the contaminants	Article of food	Limit
Polychlorinated biphenyls (Sum of PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180)	Inland and Migratory Fish	2.0 ppm
Polychlorinated biphenyls (Sum of PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180)	Marine Fish, Crustaceans and molluscs	0.5 ppm
Benzo(a)pyrene	Smoked Fishery Products	5.0 ppb

Name of the Insecticide	Food	Maximum Residue Limit (MRL) in mg/kg
Carbaryl	Fish	0.2
Quinalphos	Fish	0.01

Tolerance limit of 0.01 mg/kg shall apply in cases of pesticides for which MRL have not been fixed.

- **Biotoxin**

Sl. No.	Name of the contaminants	Article of food	Limit (µg/kg)
(1)	(2)	(3)	(4)
1.	Paralytic Shellfish Poison (PSP)	Bivalve Molluscs	80 µg/100g (Saxitoxin Equivalent)
2.	Amnesic Shellfish Poison (ASP)	Bivalve Molluscs	20 µg/g (Domoic acid equivalent)
3.	Diarrhetic shellfish poison (DSP)	Bivalve Molluscs	160 µg of Okadaic acid equivalent/Kg
4.	Azaspiracid poison (AZP)	Bivalve Molluscs	160 µg of azaspiracid equivalent/Kg
5.	Brevetoxin (BTX)	Bivalve Molluscs	200 mouse units or equivalent/Kg]

- **Histamine**

S. No.	Product Category	Applicable to	Histamine Level
1.	Raw/Chilled/Frozen Finfish	Species with high amount of free histidine (Listed fish species with potential to cause histamine fish poisoning)	n=9, c=2; m=100 mg/kg, M=200 mg/kg
2.	Thermally Processed Fishery Products		n=9, c=2; m=100 mg/kg, M=200 mg/kg
3.	Smoked fishery products		n=9, c=2; m=100 mg/kg, M=200 mg/kg
4.	Fish Mince/Surimi and analogues		n=9, c=2; m=100 mg/kg, M=200 mg/kg
5.	Battered and breaded fishery products		n=9, c=2; m=100 mg/kg, M=200 mg/kg
6.	Other Ready to Eat fishery products		n=9, c=2; m=100 mg/kg, M=200 mg/kg
7.	Other value added fishery products		n=9, c=2; m=100 mg/kg, M=200 mg/kg
8.	Other fish based products		n=9, c=2; m=100 mg/kg, M=200 mg/kg
9.	Dried/ Salted and Dried fishery products		n=9, c=2; m=200 mg/kg, M=400 mg/kg
10.	Fermented Fishery products		n=9, c=2; m=200 mg/kg, M=400 mg/kg
11.	Fish Pickle		n=9, c=2; m=200 mg/kg, M=400 mg/kg

n : Number of units comprising the sample
c : Maximum allowable number of defective sample units
m : Acceptable level in a sample
M : Specified level when exceeded in one or more samples would cause the lot to be rejected

Satisfactory, if the following requirements are fulfilled:

1. the mean value observed is ≤ m
2. a maximum of c/n values observed are between m and M
3. no values observed exceed the limit of M,

Unsatisfactory, if the mean value observed exceeds m or more than c/n values are between m and M or one or more of the values observed are >M.

- **Formaldehyde**

  Gazette Notification of Food Safety and Standards (Food Products Standards and Food Additives) First Amendment Regulations, 2023 related to standards for limits of naturally occurring formaldehyde in freshwater and marine fish. The limit of naturally occurring formaldehyde shall not exceed the limit as given below for different fish species

Group & Species	**mg/kg, Max.**
Group - I (Marine)	
All finfishes (including Barracuda, Billfishes, Bombay Duck, Bullseyes, Catfishes, Croakers, Eels, Filefishes and Puffers, Flat fishes, Goatfishes, Groupers (Rock Cods), Half Beaks and Full Beaks, Horse Mackerel, Leather Jacket (Queen Fish), Mackerel, Mullets, Other Carangids, Other Clupeoids, Anchovies, Other Perches, Pigface Breams, Pomfrets, Ribbon Fish, Sardines, Seer Fishes/Spanish Mackerel, Silver Bellies/Biddies, Snappers, Tarpons, Threadfin Breams, Threadfins, Tuna and Bonitos, White Fish and any other commercial varieties), **elasmobranchs, crustaceans and molluscs except those under Group III & IV**	4.0
Group – II (Freshwater Origin)	
Finfishes (including Indian Major Carps, Minor Carps, Exotic Carps, Freshwater Catfishes, Snakeheads/Murrels, Tilapia, Trout and all other freshwater fin fishes), crustaceans and molluscans	
Group – III (Marine)	
Lizard fishes and any other marine fishes not covered under Group I	8.0
Group – IV (Frozen Stored marine fish products)	
All frozen stored marine fish products	100”;

Sample Preparation/Processing for fishery products

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

Procedure for thawing:

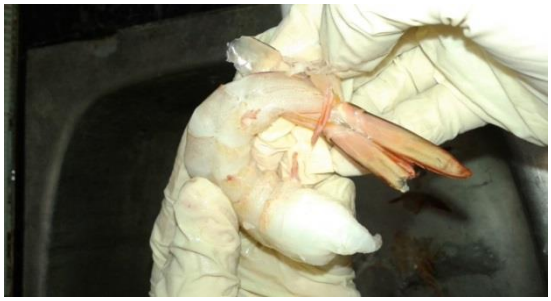
The sample unit is thawed by enclosing it in a film type bag and immersing in water at room temperature (not greater than 35°C). The complete thawing of the product is determined by gently squeezing the bag occasionally so as not to damage the texture of the shrimp, until no hard core or ice crystals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



IMPORTANCE OF HANDLING, STORAGE AND INTERMEDIATE QUALITY CHECKS OF ANALYTICAL STANDARDS

In analytical chemistry, a standard solution is a solution containing a precisely known concentration of an element or a substance. And standards are materials containing a known concentration of an analyte. There are two categories of analytical standards: primary standards and secondary standards

Primary standards: Primary standards are ultrapure compound that serves as the reference material. Hey are also known as primary calibrators or primary reference materials

Secondary standards: A standard established by comparison with a primary standard and also known as working standard/in-house reference material

Analytical standards are compounds of high purity and known concentration to be used as a calibration standard for a given assay. While, Reference materials (RM) are such materials, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process. Reference material characterized by a metrologically valid procedure for one or more specified properties, accompanied by a reference material certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability is known as Certified Reference Material (CRM). Whereas, Internal standards (IS) are those substances, similar to the analyte which is added in a constant amount to the blank, the standards, and the samples for nullifying the matrix effect during sample analysis.

Certified Reference Materials (CRMs)

CRMs are those controls/ standards which helps to provide a benchmark to deliver accurate and comparable results to the testing laboratory. They are used to validate the results by ensuring the quality and metrological traceability of products; through validation of analytical measurement methods as well as for the calibration of instruments. During the procurement of CRM various factors needs to be considered by testing laboratories *viz.,*

- ✓ Identification of a reputed vendor/approved manufacturer or supplier,
- ✓ Shelf life / expiry period of the CRM,
- ✓ Traceability,
- ✓ Packing size / net weight of CRM, and
- ✓ Price of the CRM.

Based on all these aspects it is recommended to purchase the most economical CRM with longest expiry period.

Traceability

The International Vocabulary of Basic and General Terms in Metrology defines traceability as the property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties. Traceability is a property of measurement, not of a device/instrument. For example, the indications on a calibrated thermometer is traceable to National Institute of Standards and Technology (NIST), not a thermometer itself.

Check list at the time of receipt of CRM

During the receipt of CRM, the laboratory is recommended to check the following things to ensure the proper receipt of CRM

1. Check seal of the container and the postage storage conditions (ambient / cold chain)
2. Weight / volume of CRM
3. Certificate of analysis (CoA)for purity claimed as per the quotation / purchase order (PO)
4. Expiry date of the CRM as per (CoA) & as per the PO
5. Recommended storage conditions, and
6. Record the total weight of the bottle containing the CRM

Immediately after receipt the CRM should be labelled with unique laboratory code (uniquely identifying standard from neat standard to dilutions), document the receipt of the standard with traceability, and keep the records with the details of CRM received such as name, unique code, purity, lot number, quantity received, gross weight, date received, and date of expiry.

The storage should be done at the recommended temperature as mentioned in the CoA (in the deep freezer at or below -20 ºC temperature). The CRM container can be put in a centrifuge tube, keep in a respective numbered storage box and that can be kept at the recommended temperature as mentioned in the CoA. It is also recommended to keep stock standards, and dilutions including mixed standards separately in order to avoid any contamination. CRMs should be provided with controlled access, documented standard usage

through appropriate records, monitoring of storage temperature (refrigerator/deep freezer) on daily basis and proper maintenance of monitoring records.

Preparation of stock and intermediate solution

Each stock solution should be labelled with unique code, name, concentration, date preparation, solvent, and initials of preparer and expiry date of the solution. For Intermediate dilutions also the records should be maintained with amount of stock standard used, volume made up with solvent, solvent used, lot number of used stock standard, purity of standard, final concentration of solution obtained, date of preparation, expiry and location of storage. During the preparation of stock solution, the purity of RM as well as the salt associated with the standard should be included as purity & salt correction in the calculation for intended concentration.

For example, if a stock solution of 1000mg/L of 10ml needed to be prepared from a RM 'X" with 98.5% purity, the purity correction calculation can be done as follows:

Required purity=100%

Actual purity =98.5%

So purity correction = (required purity/actual purity) * volume to be prepared *i.e.*

$(100/98.5)\times 10=10.15$

i.e. 10.15mg in 10ml gives 1000mg/L.

If the purity correction is applied, the preparation of 1000mg/L will result in an actual concentration of 985 mg/L instead of 1000mg/L, since the purity diminishes by 1.5% as per CoA (purity provided is 98.5%). To compensate that purity correction is required.

Salt correction:

Purity correction should be accompanied by salt correction, if any salt is associated with the compound of interest. In that case, the molecular mass provided will be including the mass of the salt also. For example, if the molecular mass of "X Std" is 481.5 g/mol and the "X" stands for HCl, then the calculation of 1000mg/L 10ml stock solution should include the salt correction as follows:

Molecular mass of compound of interest (Subtract the mass of HCl from the total mass) = 481.5-36.5=445

Salt correction for 1000mg/L 10ml stock solution preparation

= (481.5/445) ×10.15=10.98 mg in 10ml →1000mg/L stock solution

Intermediate Quality Checks (IQC)

A periodic quality check performed for every reference standard while it is still within calibration date to ensure that is still in good condition is known as Intermediate quality check (IQC). The term "good condition" indicates that the error or drift that it encounters is within acceptable limits within a given period or given situation. IQC is a type of calibration quality control that is implemented internally; as well as it is a planned activity in which the frequency of implementation should be laid out. It may include the 'functionality check' of the standard also. Functionality check is carried out to check and verify the functionality (the quality of being suited to serve a purpose well) of the reference standard in combination with the measurement results.

As per ISO 17025:2017, Clause number 6.4.2 states that when the laboratory uses equipment outside its permanent control, it shall ensure that the requirements for equipment of this document are met. Under the clause 6.4.10, which states that "when intermediate checks are necessary to maintain confidence in the performance of the equipment, these checks shall be carried out according to a procedure". The clause number 7.7.1 (Ensuring the Validity of Results) it is stated that "the laboratory shall have a procedure for monitoring the validity of results. This monitoring shall be planned and reviewed and shall include, where appropriate, but not be limited to: (e). intermediate checks on measuring equipment"

ISO 9001:2015 Clause 7.1.5 b- Monitoring and measuring resources- also states about IQC as "The organization shall retain appropriate documented information as evidence of fitness for purpose of the monitoring and measurement resources."

IQC should be performed under certain situations as follows:

- If the standards are exposed to a different environmental conditions different from that of CoA recommended storage and handling conditions for a period, then immediately after that IQC should be performed
- In order to monitor the validity of results, after many usages, to ensure that it is still in confidence while waiting for its next calibration due date
- To ensure continuing fitness for its purpose

- To maintain the confidence

Steps to perform Intermediate Quality Checks

- Before performing IQC the standard should be stabilized to laboratory testing conditions from its storage conditions sufficient time
- Prepare a checklist or a form to document and list the needed details about the intermediate check, *viz*.
- The identification of the Reference standard to be used for cross-checking.
- The name of the standard that will undergo intermediate check,
- Brand, Model & Serial No.
- Temperature and humidity
- Date of functionality check
- Name and signature of person who performed the functionality check
- The tolerance to determine pass and fail status

Intermediate Quality Checks can be performed through different methods

Method 1:

- Choose a test point or nominal value.
- Perform at least 3 trials.
- Analyze the result and calculate the standard deviation
- Determine the acceptance criteria
- Have it approved and verified by an authorized person.

Method 2:

- By comparing two instruments with the same parameters, one is a standard and the other is a UUC (Unit Under Calibration/Unit Under Test) to check any differences or unacceptable error

For example: Prepare working standards, WS1 & WS2- with same concentration from stock solution & intermediate dilutions. Analyze the equipment responses- R1 & R2 from two different instruments. Find out the relative percent difference using the following formula

$[(R1 - R2)/[(R1 + R2)/2]]\times 100$ if $R_1 > R_2$

$[(R2 - R1)/[(R1 + R2)/2]]\times 100$ if $R_2 > R_1$

Acceptance Criteria:

Acceptance criteria is used to evaluate the goodness and fitness of an analytical method for its intended purpose. In general, there is no fixed acceptance criteria, because, different instruments need different methods of assessment. Whatever method is chosen for determining the acceptance criteria, for that records should be maintained for standardization and audit purposes. Acceptance criterion can be determined by various ways as follows:

- By determining the error and comparing it with the manufacturer specifications for accuracy or tolerance details
- By determining the drift and using the limits of the control charts up to 3 standard deviations. In this case the standard deviation for determining the limits should be taken from the method validation data set
- Using guides where a tolerance limit is provided for specific instruments. For example, ISO Guide

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 20th 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.

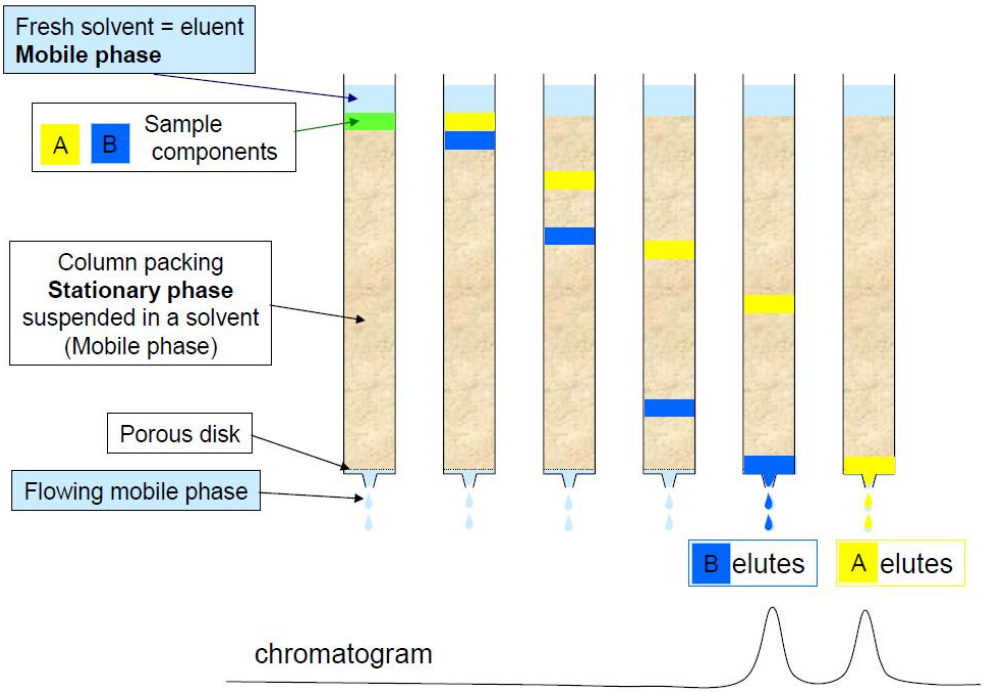


Figure 1: Chromatographic separation

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.

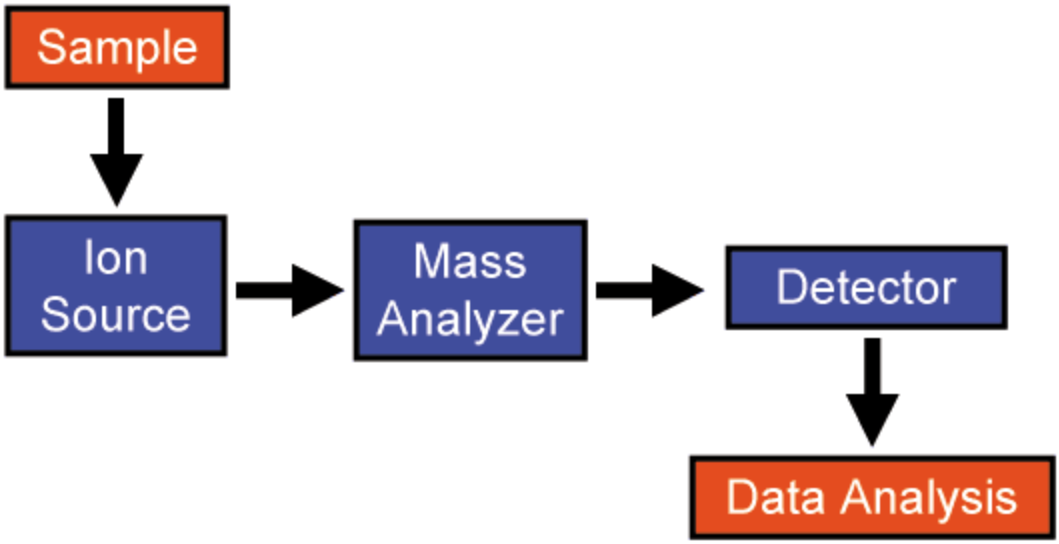


Figure 2: Basis components of a mass spectrometer

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 pressure and 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.

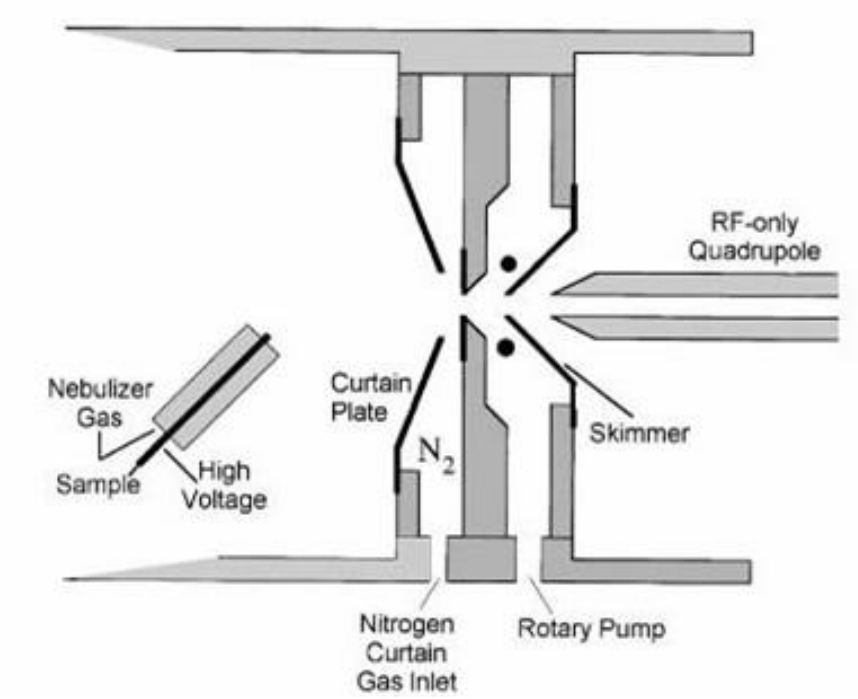


Figure 3. Diagram of API interface

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 best over a broader range of different analytes. Compounds with higher polarity and molecular weights can only be analysed by ESI.

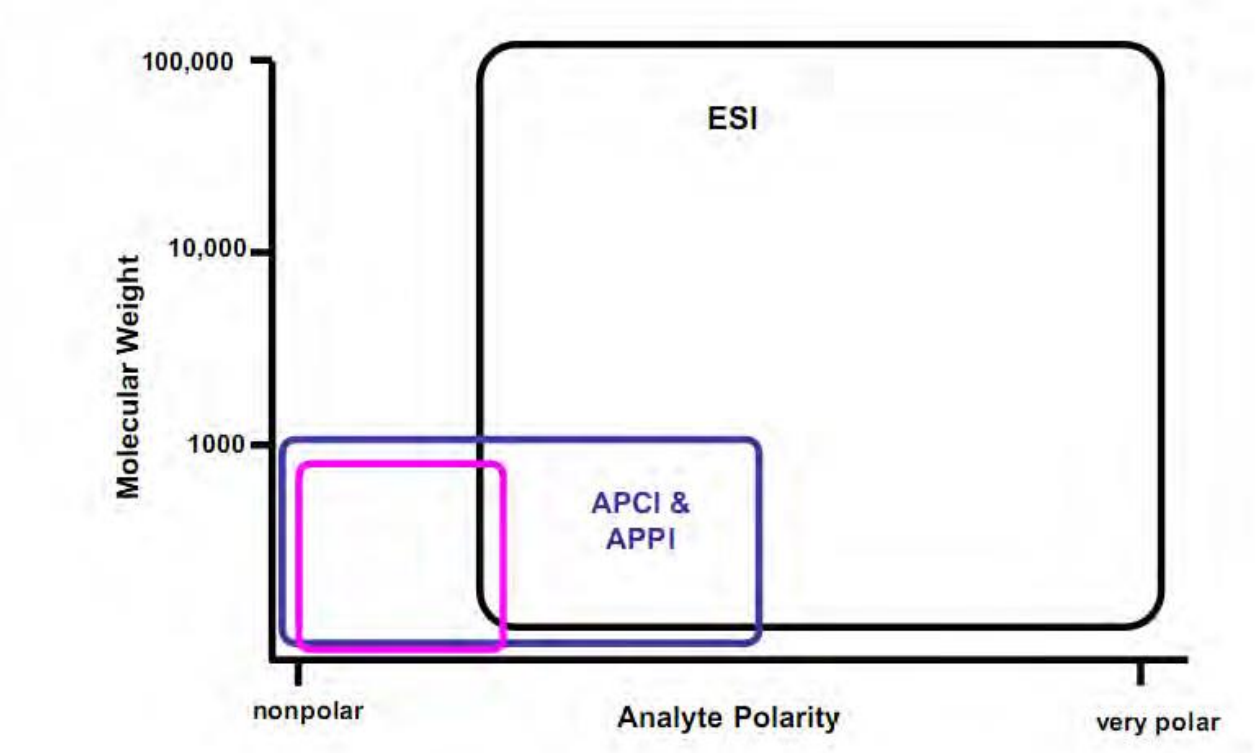
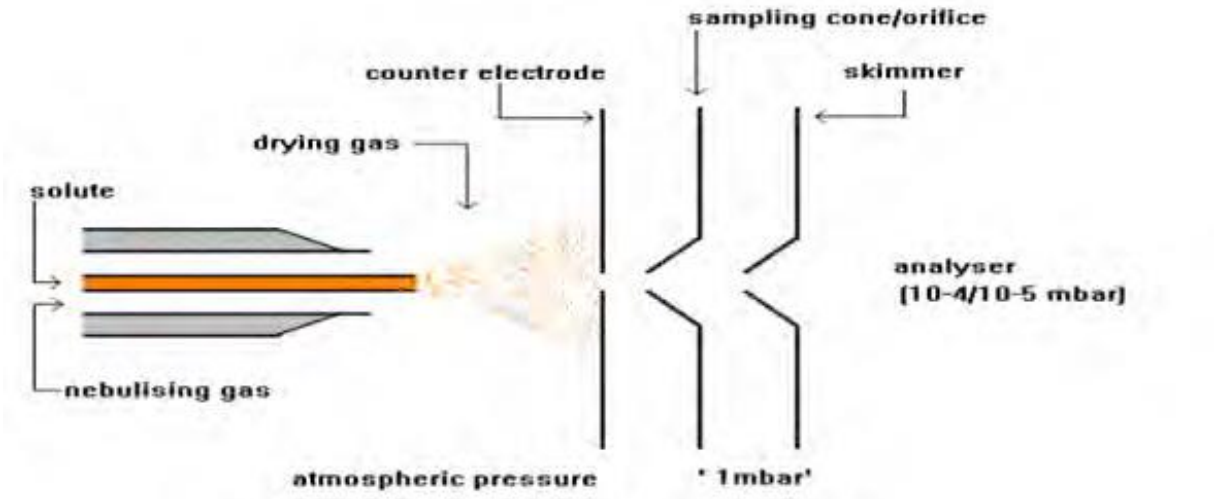


Figure 4. Representation of various ionization techniques such as ESI, APCI, and APPI as a function of compound polarity and molecular weight.

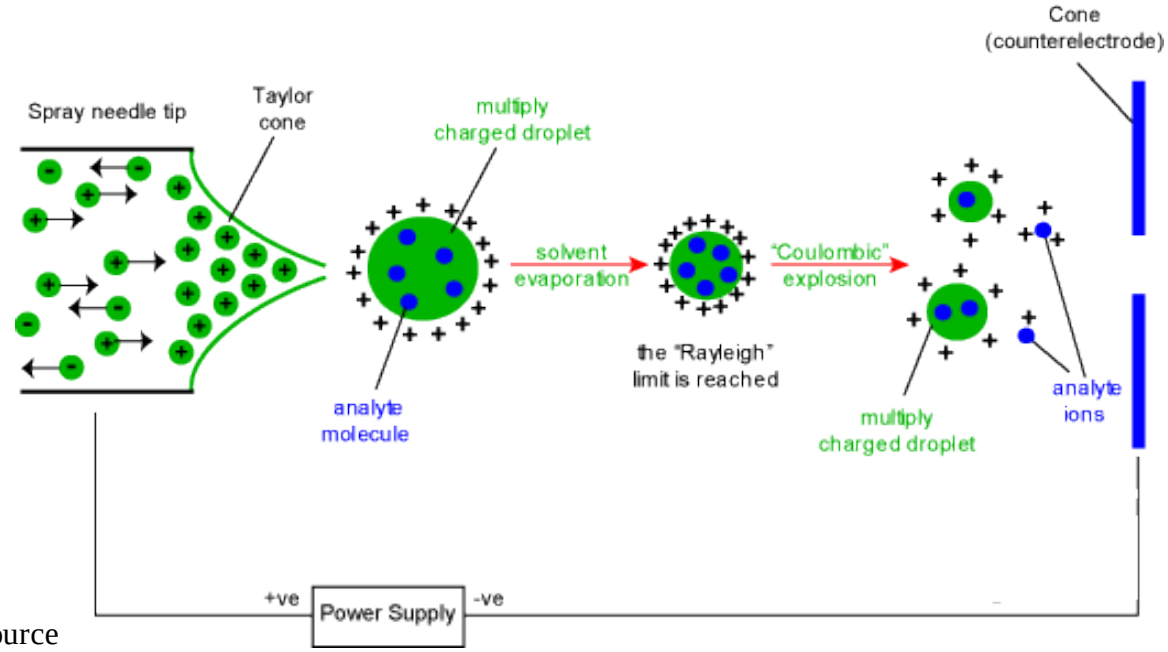
ESI is generally accomplished by forcing the LC mobile phase containing the analyte through a small capillary into an electric field of high positive or negative electrical potential typically of the order of 3-5 kV depending on whether positive ionization (higher voltages) or negative ionization (lower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

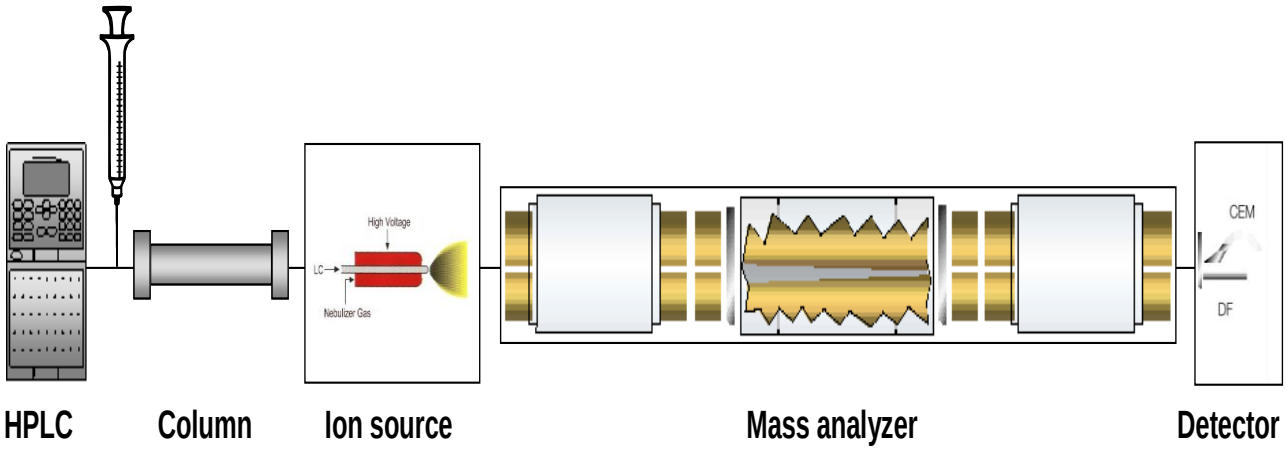


Figure 7: Ideal HPLC with mass analyzer (Golden standard for quantitation)

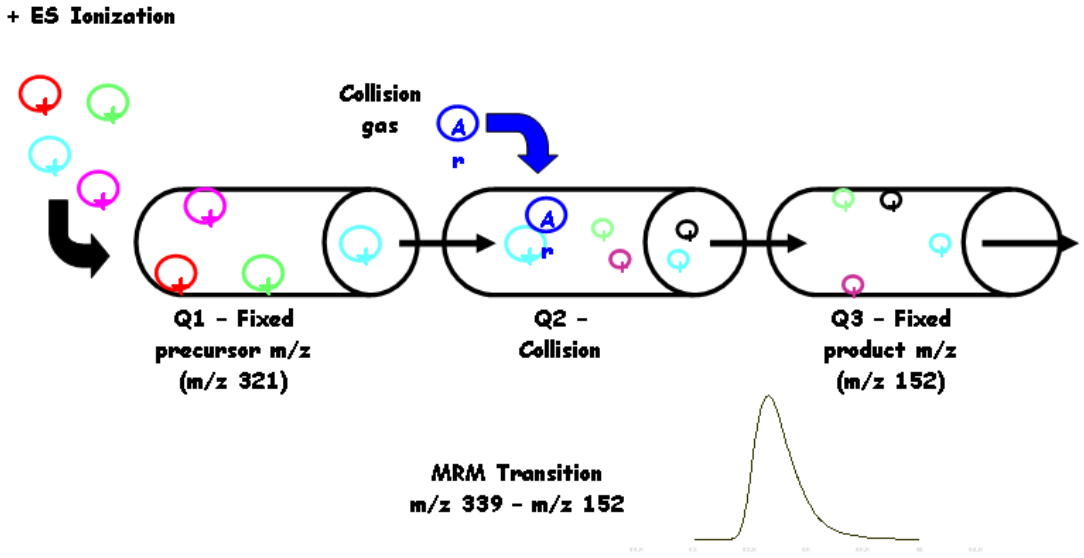


Figure 8: Fragmentation in SRM/MRM

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 anemia 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℃ approximately) and intermediate standard solutions prepared in methanol is generally stable for 6 months if stored in the refrigerator (1-5℃ 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℃) 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 samples frozen (< -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 @ 4^0C 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).

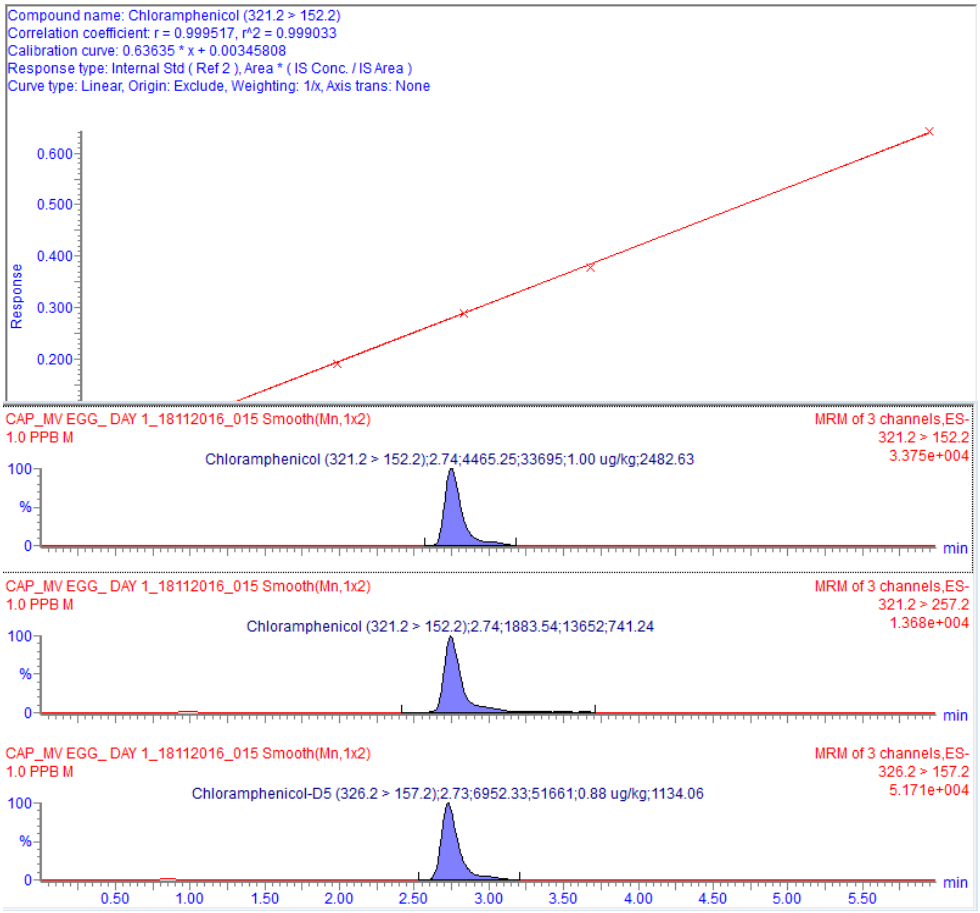


Figure 9: Representative chromatogram with calibration curve for Chloramphenicol

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/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= 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. • Keep in well-closed containers away from ignition sources. • Avoid contact or prolonged exposure to vapors. • Work in fume hood. • Keep away from flame or heat. Wear personal protective equipment, avoid skin contact.
Acetonitrile, Methanol	Flammable	
Formic acid	Corrosive, Caustic	

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.

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 NP SEM: - 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 samples frozen (< -10 to -18°C) prior to analysis.

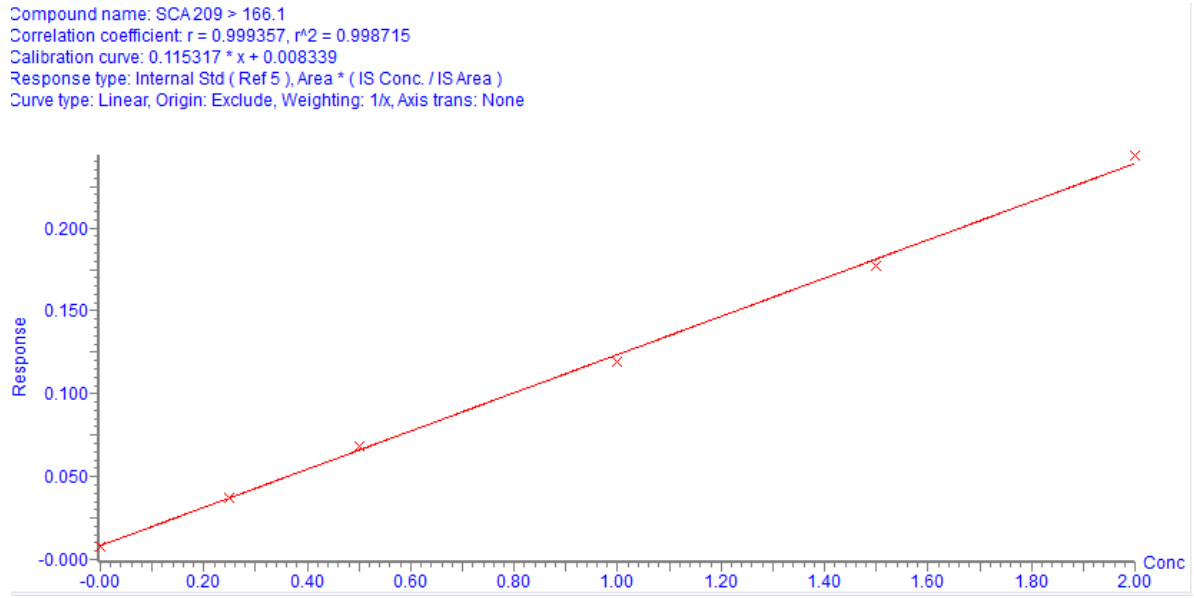
Extraction Procedure

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 8000 rpm 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 8000 rpm 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).



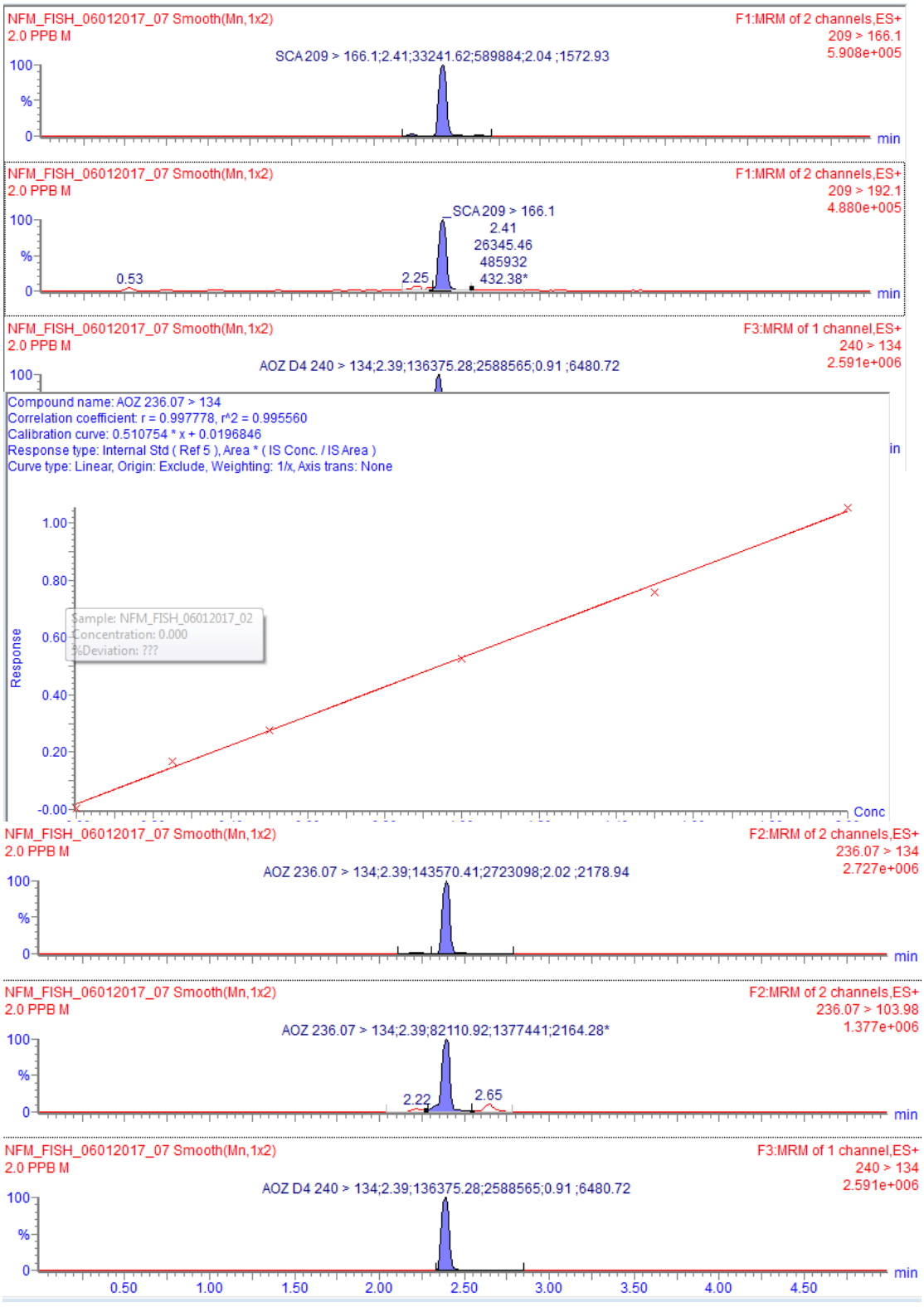


Figure 10: Representative chromatograms and calibration curve for Nitrofuran metabolites

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. • Keep in well-closed containers away from ignition sources. • Avoid contact or prolonged exposure to vapors. • Work in fume hood. • Keep away from flame or heat. Wear personal protective equipment, avoid skin contact.
Acetonitrile, Methanol	Flammable	
Formic acid	Corrosive, Caustic	

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.

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.

Table 1: UPLC gradient for the elution of target compounds

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

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 samples frozen (< -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).

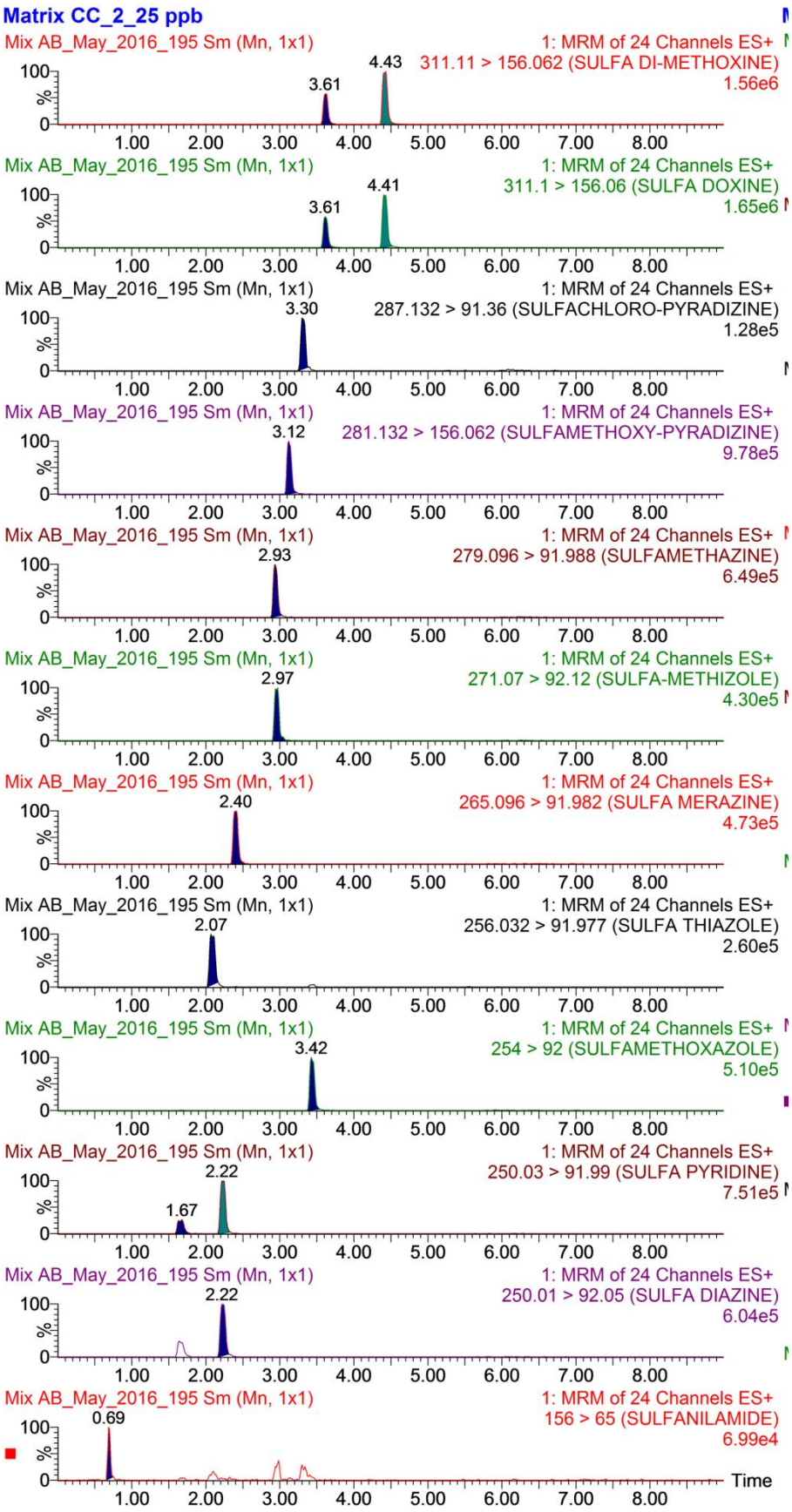


Figure 12: Representative chromatograms for Multi residues

The order of Injection shall be as given below:

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

Calculations

For Quantitation of each compound of interest

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

    - $\text{Normalized Response Component 1} = \frac{\text{Response of Component 1}}{\text{Response of Internal Standard}}$
- Generate a linear curve fit to each analyte in standard curve using normalized response to concentration in tissue (µg/kg or ppb).
- 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

- Choose a standard or recovery containing the analyte of interest.
- 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
- 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 %.
- Calculate the ratio of the response of product ion #2 to product ion #1 in the standard or recovery for the analyte of interest

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

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. • Keep in well-closed containers away from ignition sources.
Acetonitrile, Methanol	Flammable	• Avoid contact or prolonged exposure to vapors. • Work in fume hood. • 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 Garrido Frenich, 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

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 (CCα)
h) Detection Capability (CCβ)
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. In case 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 – CCα:** ***Decision limit (CCα) means the limit at and above which it can be concluded with a specific error probability of α that a sample is non-compliant.***

*Two ways of establishing CC*α 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**α** 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 [α error is equivalent to 1%].
- In the case of substances with established permitted limit [MRL] CCα 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) 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 [α error is equivalent to 5%].

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

- For calculation of CCα (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 CCα (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 (α error=5%).

  Note: The CCα established should ideally be 1/3 of MRPL and CCα 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 CCα 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 ß.***

*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 CCα and CCβ (for MRPL/banned substances) must be verified by blank samples which are fortified with the respective concentration of CCα and CCβ if the figures obtained are **lower than <1/3rd 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 CCα 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 CCα 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 CCα 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).

ANALYSIS OF MULTICLASS ANTIBIOTICS IN FISH AND FISH PRODUCTS USING LC- MS/MS

Analytical Steps

1. Weigh accurately 5 g of sample into 50 ml centrifuge tubes
2. Add 10 ml of 0.1% formic acid in acetonitrile, 10 ml 0.05M EDTA and 5 ml n-Hexane
3. Vortex for 1 minute
4. Shake in multitude shaker incubator at 150 rpm for 30 minutes
5. Centrifuge at 4000 rpm for 10 minutes
6. **Clean up using HLB Prime cartridge**
7. Conditioning of the Oasis Prime HLB 3cc cartridge with 1mL acetonitrile: water (1:1)
8. Pass 2 mL of supernatant sample followed by washing with 0.5ml acetonitrile: water (1:1)
9. Collect 2.5ml sample in to the 15 ml centrifuge tube
10. Filter through 0.2 micron RC filter in to LC- MS vials.

Instrument conditions

LC-MS/MS - AB Sciex 6500 QTRAP system with Exion LC and auto sampler.

Column - Raptor C18 2.7µm; 100 x 2.1mm column and the injection volume was 2µl.

Time, min	Flow Rate, mL /min	% Mobile Phase A (0.1% HCOOH in H_2O)	% Mobile Phase B (0.1%HCOOH in MeOH)
0.5	0.5	95	5
7	0.5	5	95
8	0.5	5	95
9	0.5	95	5
10	0.5	95	5

Scan type - Scheduled Multiple Reaction Monitoring (MRM)

MRM detection window 45sec & target scan time 0.7sec; curtain gas is 40psi, CAD gas 12 and ion spray voltage is 5500psi. Source temperature operated at 350^0C and Ion Source Gas 1 (nebulizer gas) 55 and Ion Source Gas 2 (heater gas) 50.

Group	Compounds	Q1 Mass (m/z)	Q3 Mass (m/z)	DP	CE	CXP	EP	RT min
β lactams	Amoxicillin	398.00	381.00	10.00	15.00	4.00	10.00	2.81
			349.20	10.00	22.00	10.00	10.00	
	Ampicillin	382.20	223.00	50.00	18.00	10.00	10.00	3.31
			333.20	50.00	23.00	10.00	10.00	
	Cloxacillin	436.20	277.00	117.00	18.30	16.10	10.00	5.42
			160.00	117.00	17.00	14.00	10.00	
Tetracyclines	Tetracycline	445.20	410.10	82.00	27.00	4.00	10.00	2.77
			154.10	82.00	40.00	4.00	10.00	
	Oxytetracycline	461.20	201.10	75.00	54.00	10.00	10.00	2.84
			426.10	75.00	23.00	10.00	10.00	
	Chlortetracycline	479.20	462.00	42.00	24.00	8.00	10.00	3.43
			444.00	42.00	28.00	8.00	10.00	
	Doxycycline	445.00	428.00	35.00	25.00	8.00	10.00	4.08
			410.00	35.00	30.00	7.00	10.00	
	4 epi Tetracycline	445.10	410.20	71.00	27.00	20.00	10.00	2.32
			428.20	71.00	21.00	16.00	10.00	
	4 epi Chlortetracycline	479.00	462.00	42.00	24.00	10.00	10.00	2.76
			197.00	42.00	56.30	10.00	10.00	
	4 epi Oxytetracycline	461.00	443.10	39.00	17.63	10.00	10.00	2.66
			426.20	39.00	26.00	10.00	10.00	
	Trimethoprim	291.10	230.20	160.00	32.00	4.00	10.00	2.54
			261.20	160.00	35.00	4.00	10.00	

	Sulfacetamide	215.00	156.00	49.00	14.00	13.00	10.00	1.32
			92.00	49.00	29.00	9.00	10.00	
	Sulfadiazine	251.10	108.00	100.00	33.00	4.00	10.00	1.76
			156.10	100.00	25.00	7.00	10.00	
	Sulfathiazole	256.10	156.10	110.00	21.00	4.00	10.00	2.16
			92.10	108.00	42.00	4.00	10.00	
	Sulfapyridine	250.10	156.10	100.00	25.00	8.00	10.00	2.27
			108.10	100.00	38.00	8.00	10.00	
	Sulfamerazine	265.10	156.00	93.00	25.00	4.00	10.00	2.42
			108.10	93.00	35.00	4.00	10.00	
	Sulfamethoxypyridazine	281.10	155.90	101.00	24.00	4.00	10.00	2.97
			92.10	101.00	45.00	4.00	10.00	
	Sulfaethoxypyridazine	295.00	156.00	90.00	18.00	15.00	10.00	3.66
			108.00	90.00	32.00	12.00	10.00	
	Sulfamethoxazole	254.10	156.10	85.00	23.00	7.00	10.00	3.20
			92.10	85.00	40.00	7.00	10.00	
	Sulfaquinoxaline	301.10	156.10	97.00	25.00	4.00	10.00	4.14
			92.10	97.00	46.00	4.00	10.00	
	Sulfadimethoxine	311.10	156.10	94.00	26.00	4.00	10.00	4.01
			92.10	93.00	50.00	4.00	10.00	
	Sulfadoxine	311.10	140.10	105.00	39.00	4.00	10.00	3.35
			65.00	105.00	72.00	4.00	10.00	
	Sulfanilamide	156.00	108.00	41.00	15.00	10.00	10.00	1.31
			92.00	41.00	19.00	10.00	10.00	
	Sulfachlorpyridazine	285.00	156.00	66.00	23.00	5.00	10.00	3.09
			108.10	66.00	40.00	5.00	10.00	
Fluroquinolones	Ciprofloxacin	332.10	231.10	94.00	54.00	4.00	10.00	2.93
			288.20	94.00	26.00	4.00	10.00	
	Enrofloxacin	360.30	342.20	105.00	31.00	4.00	10.00	3.01

			316.40	105.00	28.00	4.00	10.00	
	Norfloxacin	320.00	302.00	60.00	27.00	20.00	10.00	2.80
			231.00	60.00	51.00	19.00	10.00	
	Danofloxacin	358.20	340.20	100.00	30.00	4.00	10.00	2.98
			314.10	100.00	24.00	4.00	10.00	
	Difloxacin	400.10	382.10	90.00	29.06	18.22	10.00	3.08
			356.10	90.00	25.77	24.16	10.00	
	Sarafloxacin	386.00	368.00	62.00	30.00	6.00	10.00	3.16
			342.00	62.00	26.00	11.00	10.00	
	Flumequine	262.10	202.10	87.00	47.00	14.00	10.00	4.76
			174.10	87.00	56.00	13.00	10.00	
	Ofloxacin	362.00	261.00	65.00	47.00	10.00	10.00	2.74
			317.90	65.00	36.00	10.00	10.00	
Quinolones	Oxolinic acid	262.10	244.00	68.00	27.00	15.00	10.00	4.12
			216.10	68.00	41.00	15.00	10.00	
	Nalidixic acid	233.10	215.20	83.00	20.00	4.00	10.00	4.70
			187.10	83.00	35.00	4.00	10.00	
Albendazole	Albendazole	266.00	234.00	50.00	25.40	13.00	10.00	4.95
			191.00	50.00	42.00	12.30	10.00	
	Albendazole sulfone	298.20	266.10	70.00	27.00	10.00	10.00	3.96
			159.10	70.00	50.00	10.00	10.00	
	Albendazole sulfoxide	282.20	240.10	52.00	19.00	10.00	10.00	3.63
			208.10	52.00	32.00	10.00	10.00	
	ABZ 2 amino sulfone	240.10	198.10	81.00	27.00	10.00	10.00	2.09
			133.20	81.00	39.00	10.00	10.00	

Q1 Mass - Precursor Ion [M+H]+; Q3 Mass – Product Ion; DP - Declustering Potential; CE - Collision Energy; CXP - Collision cell Exit Potential Cell; EP – Entrance Potential; RT – Retention Time

NITROFURAN METABOLITES IN FISH AND FISH PRODUCTS (CATRIDGE METHOD)

Analytical Steps

1. Weigh 2 g of homogenized sample into 50 mL centrifuge tubes
2. Vortex & keep it for 10 minutes
3. Add 300 µL100 mM 2-NBA, vortex and keep it for 30 min.
4. Add 10 ml 0.2M HCl,
5. Keep for 16 hrs incubation at 37℃/150 rpm **OR** keep for Microwave Digestion for 2hrs
6. Cool to RT & add 300 µL of 0.3M TSP.
7. Dilute it with H_2O (10 mL)
8. Adjust the pH with 2 M NaOH
9. Centrifuge at 8000 rpm for 10 minutes and collect the supernatant
10. **Clean up using HLB Prime cartridge**
11. Conditioning of the Oasis Prime HLB 3cc cartridge with 6 mL Ethyl acetate + 6 mL Methanol + 6 mL H_2O
12. Apply the sample followed by washing with 3mL H_2O+3mL 30% methanol in water
13. Elute with 6 mL Ethyl Acetate
14. Evaporate and reconstitute with 1ml Acetonitrile: H_2O (50:50)
15. Filter through 0.2-micron RC filter in to LCMS vials

Instrument conditions

LC-MS/MS - AB Sciex 6500 QTRAP system with Exion LC and auto sampler.

Column –Acquity UPLC® BEH C18 1.7 µm; 2.1 x 100 mm; Injection volume: 2 µL

LC Gradient Program

Time min	Flow Rate ml/min	% Mobile Phase A (H_2O)	% Mobile Phase B (ACN)
1	0.4	95	5
3	0.4	70	30
6	0.4	30	70
7	0.4	30	70
8	0.4	95	5
9	0.4	95	5

MRM Parameters:

Scheduled Multiple Reaction Monitoring (MRM) scan type operated in Electron Spray Ionization mode in positive polarity used for the detection of AOZ, AMOZ, AHD, SEM. Optimized curtain gas is 40psi, CAD gas 8 psi and ion spray voltage is 5500 psi. Source temperature operated at 550^{o}C and Ion Source Gas 1 (nebulizer gas) 40 and Ion Source Gas 2 (heater gas) 60.

Name	**Q1**	**Q3**	**DP (volts)**	**CE (volts)**	**CXP (volts)**	**EP**	**RT(min)**
SC 1	209.10	166.10	66.00	15.00	30.00	10.00	3.8
SC 2	209.10	192.10	66.00	15.00	16.00	10.00	3.8
AOZ1	236.10	134.00	61.00	17.00	10.00	10.00	4.1
AOZ2	236.10	104.20	61.00	31.00	18.00	10.00	4.1
AMOZ 1	335.20	291.20	46.00	17.00	16.00	10.00	4.2
AMOZ 2	335.20	100.20	46.00	39.00	8.00	10.00	4.2
AHD 1	249.10	134.00	66.00	17.00	12.00	10.00	3.8
AHD 2	249.10	104.10	66.00	29.00	18.00	10.00	3.8
AMOZD4	340.20	296.40	45.00	19.00	10.00	10.00	4.2
AOZD4	240.10	134.00	24.00	17.00	10.00	10.00	4.0
AHD13C3	252.00	134.00	70.00	16.00	10.00	10.00	3.8
SC13C15N2	212.10	168.10	80.00	14.00	10.00	10.00	3.7

Q1 Mass - Precursor Ion [M+H]+; Q3 Mass – Product Ion; DP - Declustering Potential; CE - Collision Energy; CXP - Collision cell Exit Potential Cell; EP – Entrance Potential; RT – Retention Time

NITROFURAN METABOLITES IN FISH AND FISH PRODUCTS (CLEANUP METHOD)

Analytical Steps

1. Weigh 2 g of homogenized sample into 50 mL centrifuge tubes
2. Vortex and keep for 10 minutes
3. Add 300 µL 100 mM 2-NBA, vortex and keep it for 30 min.
4. Add 10 mL 0.2M HCl,
5. Keep for 16 hrs incubation at 37℃/150rpm **OR** keep for Microwave Digestion for 2hrs
6. Cool to RT & Add 300 µL of 0.3M TSP.
7. Dilute it with H_2O (5 mL)
8. Adjust the pH with 2 M NaOH
9. Add 10mL Ethyl acetate & Vortex for 5 minutes
10. Centrifuge at 8000 rpm for 10 minutes & collect the supernatant
11. Keep it in freezer (-20°C) for 3 hrs
12. Centrifuge at 4000rpm for 5 minutes & clean up using (150mg) $MgSO_4$ + (50mg) C_{18} +(25mg) Zsep + (25mg) PSA
13. Centrifuge at 8000 rpm for 10minutes
14. Evaporate and reconstitute in 1mL ACN: H_2O (50:50)
15. Filter through 0.2-micron RC filter in to LCMS vials

Instrument conditions

LC-MS/MS - AB Sciex 6500 QTRAP system with Exion LC and auto sampler.

Column –Acquity UPLC® BEH C18 1.7 µm; 2.1 x 100 mm; Injection volume: 2 µL

LC Gradient Program

Time min	Flow Rate ml/min	% Mobile Phase A (H_2O)	% Mobile Phase B (ACN)
1	0.4	95	5
3	0.4	70	30

6	0.4	30	70
7	0.4	30	70
8	0.4	95	5
9	0.4	95	5

MRM Parameters:

Scheduled Multiple Reaction Monitoring (MRM) scan type operated in Electron Spray Ionization mode in positive polarity used for the detection of AOZ, AMOZ, AHD, SEM. Optimized curtain gas is 40psi, CAD gas 8 psi and ion spray voltage is 5500 psi. Source temperature operated at 550^{o}C and Ion Source Gas 1 (nebulizer gas) 40 and Ion Source Gas 2 (heater gas) 60.

Name	**Q1**	**Q3**	**DP (volts)**	**CE (volts)**	**CXP (volts)**	**EP**	**RT(min)**
SC 1	209.10	166.10	66.00	15.00	30.00	10.00	3.8
SC 2	209.10	192.10	66.00	15.00	16.00	10.00	3.8
AOZ1	236.10	134.00	61.00	17.00	10.00	10.00	4.1
AOZ2	236.10	104.20	61.00	31.00	18.00	10.00	4.1
AMOZ 1	335.20	291.20	46.00	17.00	16.00	10.00	4.2
AMOZ 2	335.20	100.20	46.00	39.00	8.00	10.00	4.2
AHD 1	249.10	134.00	66.00	17.00	12.00	10.00	3.8
AHD 2	249.10	104.10	66.00	29.00	18.00	10.00	3.8
AMOZD4	340.20	296.40	45.00	19.00	10.00	10.00	4.2
AOZD4	240.10	134.00	24.00	17.00	10.00	10.00	4.0
AHD13C3	252.00	134.00	70.00	16.00	10.00	10.00	3.8
SC13C15N2	212.10	168.10	80.00	14.00	10.00	10.00	3.7

Q1 Mass - Precursor Ion [M+H]+; Q3 Mass – Product Ion; DP - Declustering Potential; CE - Collision Energy; CXP - Collision cell Exit Potential Cell; EP – Entrance Potential; RT – Retention Time

ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBONS IN FISH USING GC-MS/MS

Analytical Steps

1. Take 10 g homogenized tissue in a 50 ml centrifuge tube. Add 10 ml distilled water to it and vortex for 1 min.
2. Add 10 ml of ethyl acetate and vortex for 1 min.
3. Add 6 g of $MgSO_4$ and 2 g of sodium chloride and vortex for 3 min. Centrifuge at 4000 rpm for 5 min. Collect the supernatant in 15 ml tube and cool at -20 ℃ for 30 min.
4. Collect 2 ml supernatant, add 50 µl isooctane as keeper and evaporate to dryness in a nitrogen evaporator system at 40 ℃.
5. **Clean up using Silica Cartridge**
6. Reconstitute the residue in 1 ml Hexane and load in a silica SPE cartridge previously equilibrated with 4 ml Hexane: DCM (3:1) and 3 ml Hexane. Elute the SPE cartridge with 10 ml Hexane: DCM (3:1)
7. Add 2 ml ethyl acetate and 100 µl Isooctane as keeper and evaporate in nitrogen evaporator system at 40 ℃.
8. Reconstitute the residue in 1 ml ethyl acetate. Filter through PTFE (0.2 µm) Syringe filter and collect in a vial for analysis.

OVEN PROGRAMME

Rate (^oC/min)	Temperature (^oC)	Hold Time (min)
	60.0	2.00
25.0	150.0	0.00
3.0	200.0	0.00
8.0	290.0	4.00
8.0	310.0	1.00

PTV PROGRAMME

	Rate (^oC/s)	**Temperature(^oC)**	**Time (min)**	**Flow (ml/min)**
Injection			0.01	20.0
Evap.	14.5	90	0.08	30.0
Transfer	5.00	300	25.00	
Cleaning	14.5	330	10.00	80.0

ANALYSIS OF POLY CHLORINATED BIPHENYLS IN FISH USING GC-MS/MS

Analytical Steps

1. Take 5 g homogenized tissue in a 50 ml centrifuge tube
2. Add 10 ml distilled water to it and vortex for 1 min
3. Add 15 ml of acetonitrile (1% acetic acid), 2ml n-Hexane and vortex for 1 min.
4. Add 6 g of $MgSO_4$, 2 g of sodium acetate and vortex for 3 min. Centrifuge at 4,000 rpm for 5 min. Keep the supernatant at -20 ℃ for 30 min.
5. Centrifuge at 4,000rpm for 5 min and take 1 ml of cooled acetonitrile supernatant. Add 150 mg anhydrous $CaCl_2$ and centrifuge at 10,000 rpm for 10 min.
6. **Clean up using dSPE**
   Add 1.5 ml supernatant to another eppendorf tube containing 50 mg Z-Sep, 150 mg C18, 150 mg $CaCl_2$ and 150 mg $MgSO_4$. Vortex for 1 min, followed by centrifugation at 10,000 rpm for 10 min.
7. Collect 1 ml supernatant, add 100 µl toluene as keeper and evaporate to dryness in a nitrogen evaporator system at 40 ℃.
8. Reconstitute with 1 ml ethyl acetate. Filter 1 ml with PTFE (0.2 µm syringe filter and transfer to a vial for GC-MS/MS analysis

Instrument conditions:

GC-MS/MS: Thermo Scientific, TSQ 8000 EVO (MS), Trace 1310 (GC)

Software: Tracefinder 3.3 SP1 EFS and XCalibur

Column: Agilent column DB5MS-UI (Part No.122-5532UI) 30 m x 0.250 mm x and 0.25 mm

Injection mode/ Volume: PTV-LVI/ 5 µl

MRM Transitions

Compounds	Q1 mass (m/z)	Q2 mass (m/z)	Collision Energy (CE)
PCB 101	254	184	30
PCB 101	325.9	254	20
PCB 101	325.9	255.9	25
PCB 138	360	287.9	25
PCB 138	360	289.8	25
PCB 138	360	324.9	10
PCB 153	357.84	287.88	25
PCB 153	359.9	289.7	30
PCB 153	361.9	289.9	35
PCB 180	323.9	253.8	30
PCB 180	391.81	321.84	25
PCB 180	393.9	323.8	30
PCB 28	256	150.1	50
PCB 28	256	151.1	40
PCB 28	256	186	25
PCB 52	292	220	25
PCB 52	292	222	25
PCB 52	292	257	10

OVEN PROGRAMME

Rate (^{o}C/min)	Temperature (^{o}C)	Hold Time (min)
	60.0	2.00
25.0	150.0	0.00
3.0	200.0	0.00
8.0	290.0	4.00
8.0	310.0	1.00

PTV PROGRAMME

	Rate (^{o}C/s)	Temperature(^{o} C)	Time (min)	Flow (ml/min)
Injection			0.01	20.0
Evap.	14.5	90	0.08	30.0
Transfer	5.00	300	25.00	
Cleaning	14.5	330	10.00	80.0

ANALYSIS OF MULTICLASS PESTICIDE RESIDUE IN FISH USING LC-MS/MS

Analytical Steps

1. Take 5 g homogenized tissue in a 50 ml centrifuge tube
2. Add 5 ml distilled water to it and vortex for 1 min
3. Add 10 ml of acetonitrile (1% acetic acid) and vortex for 1 min
4. Add 6 g of MgSO4, 2 g of sodium acetate and vortex for 3 min. Centrifuge at 4000 rpm for 5 min. Keep the supernatant at -20 ℃ for 30 min
5. Centrifuge at 4000rpm for 5 min and collect the supernatant
6. **Clean up using HLB prime Cartridge**
7. Condition HLB prime cartridge with 2 ml of 1% acetic acid in acetonitrile. Pass 2 ml of the supernatant through the cartridge followed by 0.5 ml acetonitrile
8. Collect eluent in a 15 ml centrifuge tube
9. Filter the extract with 0.2 µm RC membrane syringe filter and transfer to a vial for
10. LC-MS/MS QTRAP analysis

Instrument conditions

LC-MS/MS – AB Sciex 6500 QTRAP system with Exion LC and auto sampler. Column- Kinetex C18 2.6 µm;50 x 4.6 mm column at a flow rate of 0.6 ml/min and the injection volume was 10 µl

MRM Transitions

Compounds	Q1 Mass (m/z)	Q3 Mass (m/z)	DP (volts)	CE (volts)	CXP (volts)
Carbaryl 1	202.10	145.00	71.00	15.00	6.50
Carbaryl 2	202.10	127.00	71.00	39.00	3.00
Carbofuran 1	222.10	165.00	16.00	17.00	10.00
Carbofuran 2	222.10	123.00	16.00	29.00	6.00

Emamectin benzoate b1a (1)	886.50	158.00	106.00	49.00	10.00
Emamectin benzoate b1a (2)	886.50	82.10	106.00	107.00	6.00
Imidacloprid 1	256.00	209.10	55.00	21.00	11.00
Imidacloprid 2	256.00	175.10	55.00	29.00	8.00
Linuron 1	249.10	182.10	61.00	21.00	10.00
Linuron 2	249.10	160.00	61.00	25.00	10.00
Carbaryl D7	209.10	152.30	80.00	11.30	12.00
Monocrotophos 1	224.00	127.00	41.00	23.00	10.00
Monocrotophos 2	224.00	98.00	41.00	20.00	10.00
Emamectin benzoate b1b (1)	872.60	158.20	75.00	41.00	10.00
Emamectin benzoate b1b (2)	872.60	302.30	75.00	39.00	10.00
Emamectin benzoate b1b (3)	872.60	126.10	75.00	80.00	10.00
Quinalphos 1	299.00	147.00	160.00	47.00	10.00
Quinalphos 2	299.00	109.00	160.00	47.00	10.00
Dichlorovos 1	221.00	109.00	39.00	29.00	10.00
Dichlorovos 2	221.00	127.00	39.00	29.00	10.00
Propoxur 1	210.00	168.00	20.00	9.50	10.00
Propoxur 2	210.00	153.20	20.00	8.40	10.00

Time (mins)	**Flow rate (ml/min)**	**% Mobile phase A (H2O)**	**% Mobile phase B (ACN)**
	0.6	90	10
0.50	0.6	90	10
3.00	0.6	40	60
10.00	0.6	10	90
14.00	0.6	2	98
15.00	0.6	2	98
16.00	0.6	90	10

ANALYSIS OF MULTICLASS PESTICIDE RESIDUE IN FISH USING GC-MS/MS

Analytical Steps

1. Take 5 g homogenized tissue in a 50 ml centrifuge tube
2. Add 10 ml distilled water to it and vortex for 1 min
3. Add 15 ml of acetonitrile (1% acetic acid), 2 ml n-Hexane and vortex for 1 min
4. Add 6 g of $MgSO_4$, 2 g of sodium acetate and vortex for 3 min. Centrifuge at 4,000 rpm for 5 min. Keep the supernatant at -20 ℃ for 30 min
5. Centrifuge at 4,000rpm for 5 min and take 1 ml of cooled acetonitrile supernatant. Add 150 mg anhydrous $CaCl_2$ and centrifuge at 10,000 rpm for 10 mins.
6. **Clean up using dSPE**
7. Add 1.5 ml supernatant to another eppendorf tube containing 50 mg Z-Sep, 150 mg C18, 150 mg $CaCl_2$ and 150 mg $MgSO_4$. Vortex for 1 min, followed by centrifugation at 10,000 rpm for 10 min.
8. Collect 1 ml supernatant, add 100 µl toluene as keeper and evaporate to dryness in a nitrogen evaporator system at 40 ℃.
9. Reconstitute with 1 ml Ethyl acetate. Filter 1 ml with PTFE (0.2 µm) syringe filter and transfer to a vial for GC-MS/MS analysis

Instrument conditions:

GC-MS/MS: Thermo Scientific, TSQ 8000 EVO & 9000 EVO (MS), Trace 1310 & 1300 (GC)

Software: Tracefinder 3.3 and XCalibur

Column: Agilent column DB5MS-UI (Part No.122-5532UI) 30 m x 0.250 mm x and 0.25 mm

Injection mode/ Volume: PTV-LVI/ 5 µl

OVEN PROGRAMME

Rate (°C/min)	Temperature (°C)	Hold Time (min)
	60.0	2.00
25.0	150.0	0.00
3.0	200.0	0.00
8.0	290.0	4.00
8.0	310.0	1.00

PTV PROGRAMME

	Rate (°C/s)	Temperature(°C)	Time (min)	Flow (ml/min)
Injection			0.01	20.0
Evap.	14.5	90	0.08	30.0
Transfer	5.00	300	25.00	
Cleaning	14.5	330	10.00	80.0

DERIVATIZATION OF FORMALDEHYDE FOR GC-MS/MS ANALYSIS, AND PURIFICATION OF REAGENTS

Derivatization is the process of "chemically modifying" a compound to produce a new compound which has properties that are suitable for analysis using a GC. The modified analyte is known as derivative and will have a similar or closely related structure, but not the same as the original non-modified chemical compound. The derivatization process is carried out

- To Improve chromatographic behavior or detectability
- To Impart Volatility
- To reduce in column absorption
- To improve detectability
- To accentuate differences among the compounds, and
- To permit analysis of compounds not directly amenable to analysis due to, inadequate volatility or stability

During derivatization process there will be "substitution of active hydrogens" on the compound to be derivatized with a variety of functional groups. These functional groups impart the desired characteristics to the compound while eliminating the adverse effects. The general formula for the reaction is as follows:

R_1-AH + R_2-D → R_1-AD+ R_2-H

Where,

atom "A" = Oxygen, Sulfur, Nitrogen or similar atoms

atom "D" = Functional group on the derivatization reagent

Derivatization reagents should be selected on the basis of various properties such as

- Production of more than 95% of derivatives
- Lack of structural or molecular alterations
- Lack sample loss
- Non – interacting derivatives, and
- Stable derivatives with time

There are different types of derivatization

- Alkylation -replacement of active hydrogen by an aliphatic or aliphatic-aromatic (e.g., benzyl) group in process referred to as "ESTERIFICATION
- Silylation - Replacement of "active hydrogen" by a silyl group reduces the polarity of the compound
- Acylation - An acyl group is introduced to an organic compound
- Chiral Derivatization - Involves reaction of an enatiomeric molecule with an enantiomerically pure Chiral Derivatizing Agent (CDA) to form two "diastereomeric" derivatives that can be separated in this case using GC.

Derivatization of formaldehyde is an alkylation process. Commonly used derivatization reagent for aldehydes or ketones is 2.4-Dinitrophenylhydrazine (DNPH). DNPH reacts with aldehyde and produce bright, yellow-to-orange crystalline 2,4-dinitrophenylhydrazone (2,4-DNP) and water molecules as products. Since the reaction results in the formation of water molecules, it is a kind of condensation process. The process improves the molecular mass of the formaldehyde and thus imparts greater sensitivity and selectivity during detection in GC-MS/MS.

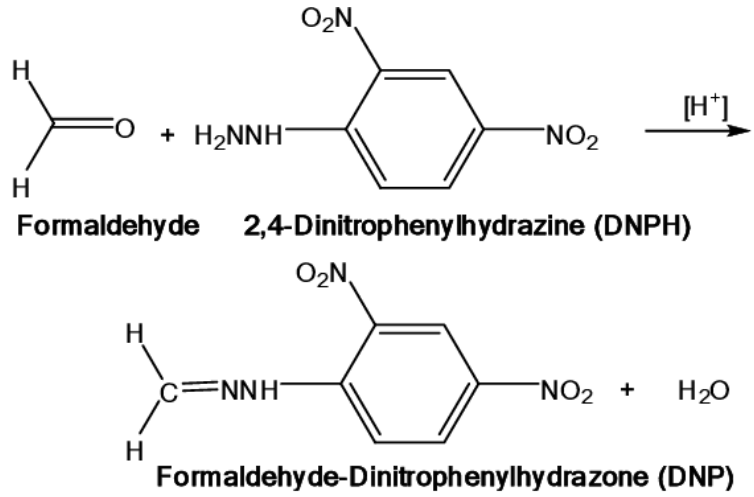


Figure 1. Schematic diagram of formaldehyde derivatization with 2,4-DNPH

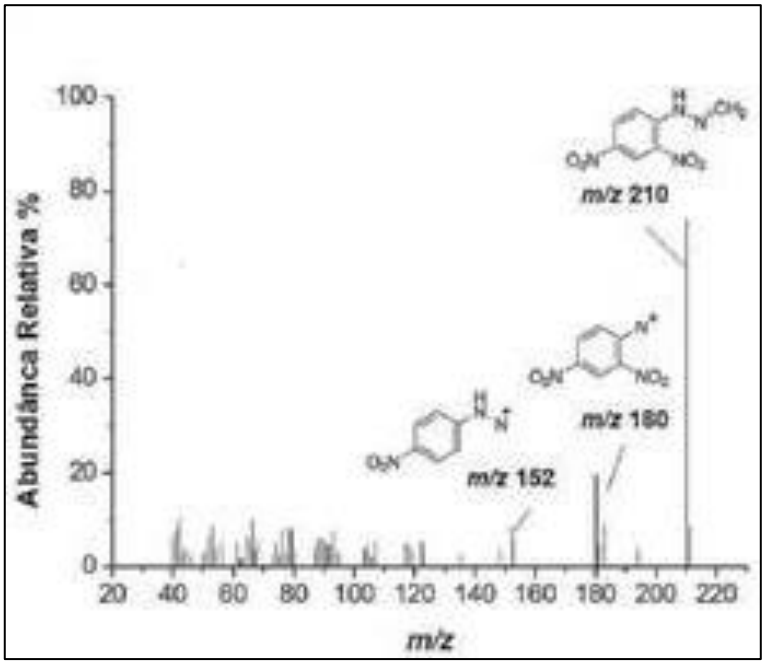


Figure 2: Mass spectrum of 2,4-DNPH derivatized formaldehyde

DNPH derivatization of formaldehyde is a reversible nucleophilic reaction process. Hence, the type, dosage and purity of chemicals as well as the temperature used for derivatization process is critical to ensure complete derivatization of the formaldehyde originally present in the sample. The proposed derivatization process use orthophosphoric acid for the preparation of DNPH working solution instead of glacial acetic acid/ sulphuric acid/hydrochloric acid which are very common acids used in analytical laboratories. If glacial acetic acid is used, the derivatization process is found to be incomplete; at the same time sulphuric acid and hydrochloric acid provides complete derivatization but with the increase of the equivalent volume of the acid. These strong acids are also having strong dehydration ability, resulting in high water content of the reaction medium which intern forces the derivatization reaction to reverse. Due to these reasons, the procedure for determination of free fromaldehyde in fish uses orthophosphoric acid for the preparation of DNPH working solution.

Purity of derivatization reagent is another critical factor which affects the performance of derivation reaction process as well as accuracy of the confirmatory method. In order to remove the unwanted impurities/contaminating substances present and to get pure crystals of DNPH, recrystallization is carried out prior to the derivatization process. It is a kind of purification method for reagents. The general methods of reagent purification are

- Sublimation
- Crystallization, and
- Distillation

During recrystallization process, an impure solid *i.e*. 2,4-DNPH is mixed with hot solvent (acetonitrile) to form a saturated solution, this solution is then cooled down to room temperature, capped in brown bottle and stored overnight at 4℃. During that storage, the solubility of the compound decreases, pure crystals grow from solution which was collected and used for working solution preparation for derivatization of formaldehyde.

The temperature used is another critical factor which affects the performance of derivation reaction process. That is because, if the temperature used is below 40°C, the reaction substrate molecules in the medium move slowly, leading in to an incomplete derivatization process. Moreover, the dehydration process requires endothermic conditions. When the temperature is 40℃-65℃, the reaction will gradually be strengthened; when it reaches 60°C-65°C, a relatively gentle zone appears with the exhaustion of the reaction substrate; If the temperature is raised further that will cause evaporation of solvent, along with the loss of volatile compounds, and results in decreased detection sensitivity. Due to these reasons, the current method for determination of free formaldehyde in fish applies a temperature of 40°C during DNPH derivatization of formaldehyde.

DETERMINATION OF FREE FORMALDEHYDE IN FISH

Scope	The method is applicable to- ● Raw/ chilled/ frozen finfish (09.2.1)
Caution	1) The DNPH must be recrystallize to get pure DNPH crystals. Purity of DNPH affects the derivatization reaction performance. 2) Appropriately dilute the extract before injection into GC-MS/MS. Higher concentration injection will lead to carry over and contamination of GC column.
Principle	Free formaldehyde in fish is extracted by aqueous extraction and derivatized with 2,4-dinitrophenylhydrazine (DNPH). The derivatized formaldehyde is identified and quantified using GC-MS/MS in MRM mode.
Apparatus/ Instruments	1) Laboratory Tissue Grinder 2) Balances (precisions 0.1 g & 0.001 g) 3) Refrigerated centrifuge (capable of centrifugal force of 8000 g) 4) Centrifuge tubes (plastic with closing caps) 5) Pipettes (ranges 20 µl to 200 µl, 100 µl to 1000 µl, 5000 µl & 10000 µl) 6) Vortex mixer 7) pH paper/pH meter 8) Shaking Incubator (40 ˚C ± 1˚C) with dark cover or equivalent) 9) Refrigerator (5 ˚C ± 3 ˚C) 10) Freezer (capable of temperatures < -18 ˚C) 11) Needles (20 G 0.9 mm disposable) 12) Filters (0.2 µm disposable, PTFE/ PP) 13) Syringes (2 ml, disposable) 14) Glass autosampler vial (2 ml with insert (200 µl) & cap) 15) GC Capillary Column (DB-5MS 30m, 0.250mm, 0.25µm or equivalent; temperature 60^0 c to 325^0 c) 16) Gas Chromatograph – tandem mass spectrometer.
Materials and reagents	1) Formaldehyde in water (CRM) 2) Formaldehyde d_2(Internal standard) 3) Deionized Water 4) Sodium Hydroxide (1.0 N)

	5) Glacial Acetic Acid 6) 2,4 Dinitrophenyl hydrazine 7) Acetonitrile 8) Orthophosphoric Acid (85% in water) 9) Ethyl Acetate (HPLC grade) 10) Dispersive cleanup kit (Mg SO_4, C18, PSA)
Preparation of reagents	*1) NaOH-1N* 100 g in 1000 ml of water *2) Acetate Buffer-Dilute 64.3 ml of 0.1 N NaOH and 5.7 ml glacial Acetic acid to 900 ml with organic free reagent water. Dilute to 1 liter with organic-free reagent water. Adjust the pH to 4.93±0.02 if needed.* 1) *Recrystallization of 2,4 dinitrophenyl hydrazine* *-DNPH should be recrystallized prior to use by dissolving 10g of 2,4 DNPH in 100ml hot analytical grade acetonitrile to form saturated solution. After complete dissolution, the solution was cooled to room temperature, capped in brown bottle and stored overnight at 4^0c for crystallization. The solvent is decanted and the crystals were collected after drying under gentle stream of nitrogen.* 5) DNPH working solution: *150 mg of 2,4 DNPH Crystals were accurately weighed, dissolved in 49.5 ml of acetonitrile and mixed with 0.5 ml of orthophosphoric acid (85%)* 6) *Formaldehyde in water CRM solution* = 55.3 mg/l 7) *Internal standard* (IS) formaldehyde D2= 1000 mg/l-Dissolve 10 mg of formaldehyde D2 in 10 ml of HPLC/GC grade Ethyl acetate. The solution is stable for one year if kept at a temperature of 5 ± 3 °C
Sample preparation	1)Homogenize the sample by grinding in a laboratory grinder mixer. 2)Transfer a test portion consisting of 2 g ± 0.1 g of homogenate to a centrifuge tube. 3)Add 40 ml of Acetate buffer in 2 g of fish (sample) in the centrifuge tube and mix and adjust pH 5 using pH paper, then sonicate for 30 min. 4)After complete homogenization, centrifuge at 8000 rpm for 10 min at 4 °C. 5)Collect 10 ml of supernatant in a graduated centrifuge tube, then add type-1 water to made up to 20 ml, adjust the pH to 5 with orthophosphoric acid.

<table>
<tr><td></td><td>6) Derivatization: Add 6 ml of 2,4 DNPH in the centrifuge tube, vortex and place in a shaking incubator for 1 h, at 150 rpm in the dark at 40 °C.
7) After derivatization extract with 10 ml of HPLC/GC grade ethyl acetate, vortex for 10 mins then centrifuge for 8000 rpm for 10 mins. Repeat the ethyl acetate extraction steps twice and pull all the supernatant.
8)Clean up: Add 2 ml of the pulled ethyl acetate extract to the dispersive clean up tube containing (150 mg $MgSO_4$, 25 mg C18 and 25 mg PSA) and vortex for 2 min then centrifuge at 12000 rpm for 10 min.
9)After centrifugation, filter the supernatant with 0.22µm (PTFE) syringe filter.
10)Dilute the samples appropriately with ethyl acetate as per the sensitivity of the instrument used and spiking concentration of matrix fortified standards. Add equal volume of internal standard solution to each vial before injection.</td></tr>
<tr><td>Method of analysis</td><td>Detection and estimation by GC- MS/MS:
GC conditions:
● Injection volume: 1 µl (constant temperature splitless, preferably in a PTV injector)
● GC Oven Programs
<table><tr><td>Rate(^{0}c/min)</td><td>Temperature(^{0}c)</td><td>Hold Time(min)</td></tr><tr><td>0</td><td>150.0</td><td>3.00</td></tr><tr><td>25.0</td><td>290.0</td><td>3.00</td></tr><tr><td>15.0</td><td>310.0</td><td>1.00</td></tr></table>● Injection mode: PTV, CT Splitless
● Carrier mode : Constant flow
● Inlet
Temperature : 290 °C
Split flow : 50.0 ml/min
Split less time : 1.00 min
● Carrier gas flow: 1.200 ml/min
MRM Conditions:</td></tr>
</table>

<table>
<tr><td></td><td>
<table>
<tr><th>Compound</th><th>Precursor ion</th><th>Product ion</th><th>Collision energy</th></tr>
<tr><td>Formaldehyde 1</td><td>210</td><td>78</td><td>10</td></tr>
<tr><td>Formaldehyde 2</td><td>210</td><td>122</td><td>10</td></tr>
<tr><td>Formaldehyde D3</td><td>213</td><td>125.1</td><td>5</td></tr>
</table>

Matrix fortified calibration samples: Weigh 2 g blank tissue each in six 50 ml centrifuge tubes. Prepare two sets of six tubes. Spike the tubes at 0, 1, 2, 4, 8, and 16 ppm level for one set and at 0, 10, 20, 40, 80, 160 ppm level in another set. The different range of calibration is required to cover fresh fish where formaldehyde content might be low, and for high concentration ranges for deliberately adulterated samples. Prepare the samples in the same way as mentioned in the sample preparation protocol and use them as matrix fortified calibration standards. Multiplication with dilution factor won’t be necessary unless different dilution is used for a particular sample.
</td></tr>
<tr><td>Calculation with units of expression</td><td>Area ratio of Formaldehyde quantifier ion to formaldehyde internal standard is plotted against different calibration concentration and a linear regression equation is formed. The concentration in sample is calculated through the instrument software using the calibration curve. One transition is used as quantifier transition and the other transition is used as qualifier transition. Other than quantifier and qualifier transitions, ion ratio is considered for unambiguous identification.</td></tr>
<tr><td>Reference</td><td>EPA METHOD 8315A</td></tr>
<tr><td>Approved by</td><td>Scientific Panel on Methods of Sampling and Analysis</td></tr>
</table>

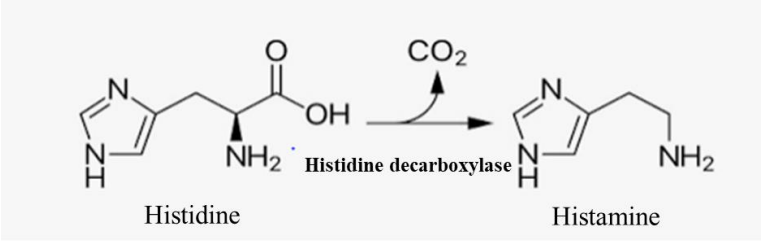
ANALYSIS OF HISTAMINE IN FISH AND FISHERY PRODUCTS

Introduction

Histamine is formed as a result of time-temperature abuse of certain fish species and can cause illness to the consumer. The illness is closely linked to the growth of bacteria capable of producing histidine decarboxylase enzyme to develop histamine in those fish. Food safety and standards regulation (FSSR) of India had set the maximum limit for histamine at 200 mg/kg for fishery products with an exception in a fish pickle, dried/salted, and fermented fish which is set at 400 mg/kg. Histamine-related illness in seafood is also known as scombrotoxin poisoning, which is primarily associated with the consumption of fish such as tuna, mackerel, bonito, etc. Other fish, such as mahi mahi, bluefish, marlin, anchovy, etc. can also cause scombroid fish poisoning. Originally, the illness was termed "scombroid poisoning" because of its association with fish in the families Scombridae and Scomberesocidae. However, other species of fish are now known to cause the illness and hence the terms "histamine poisoning" and "histamine fish poisoning" have also been applied to the illness. However, biogenic amines other than histamine are also known to have associated with the illness.

Process of histamine formation

Histidine decarboxylase enzyme is produced by certain bacteria during their growth. This enzyme reacts with histidine present in larger quantities in some fish than in others. The result is the formation of scombrotoxin (histamine).



Histamine-forming bacteria can grow and produce histamine over a wide temperature range. The formation of histamine is more rapid, however, at high-abuse temperatures (e.g. 21.1°C or higher) than at moderate-abuse temperatures (e.g. 7.2°C). Once the enzyme histidine decarboxylase is present in the fish, it can continue to produce histamine in the fish even if the bacteria are not active. The enzyme can be active at or near refrigeration temperatures. The enzyme remains stable while in the frozen state and may be reactivated very rapidly after thawing.

Symptoms of histamine poisoning

Symptoms of scombrotoxin poisoning include tingling or burning in or around the mouth or throat; rash or hives on the upper body; drop in blood pressure; headache; dizziness; itching of the skin; nausea; vomiting; diarrhea; asthmatic-like constriction of the air passage; heart palpitation; and respiratory distress. Symptoms usually occur within a few minutes to a few hours of consumption and last from 12 hours to a few days.

Source of histamine forming bacteria

The kinds of bacteria that are associated with histamine development are commonly present in the saltwater environment. They naturally exist on the gills, on external surfaces, and in the gut of live, saltwater fish, with no harm to the fish. Upon death, the defense mechanisms of the fish no longer inhibit bacterial growth in the muscle tissue, and histamine-forming bacteria may start to grow, resulting in the production of histamine. Evisceration and removal of the gills may reduce, but not eliminate, the number of histamine forming bacteria. The potential for histamine formation is increased when the scombrotoxin-forming fish muscle is in direct contact with the enzyme-forming bacteria.

Histamine formation during the processing of fish

The direct contact of fish with enzyme-forming bacteria occurs when the fish are processed (e.g. filleting) and can be particularly problematic when the surface-to volume ratio of the exposed fish muscle is large, such as in minced tuna for salads. Even when such products are prepared from canned or pouch retorted fish, recontamination can occur during salad preparation, especially with the addition of raw ingredients. The mixing in of the bacteria throughout the product and the high surface-to volume ratio can result in substantial histamine formation if time and temperature abuse occurs. At least some of the histamine-forming bacteria are halotolerant (salt tolerant) or halophilic (salt loving). Some are more capable of producing histamine at elevated acidity (low pH). As a result, histamine formation is possible during processes such as brining, salting, smoking, drying, fermenting, and pickling until the product is fully shelf-stable. Refrigeration can be used to inhibit histamine formation during these processes. A number of the histamine-forming bacteria are facultative anaerobes that can grow in reduced oxygen environments. As a result, reduced oxygen packaging (e.g., vacuum packaging, modified atmosphere packaging, and controlled atmosphere packaging) should not be viewed as inhibitory to histamine formation. Histamine is water soluble (dissolves in water) and would not be expected in significant quantity in products such as fish oil that do not have

a water component. However, histamine could be present in products such as fish protein concentrate that are prepared from the muscle or aqueous (water-based) components of fish tissue.

Fish species having potential to cause histamine poisoning

Sl. No.	Family	Scientific Name	Common Name
1.	Carangidae	*Alectis indica*	Indian Threadfish
		Alepes spp.	Scad
		Atropus atropos	Cleftbelly trevally
		Carangoides bartholomaei	Yellow Jack
		Carangoides spp.	Trevally
		Caranx crysos	Blue runner
		Caranx spp.	Jack/Trevally
		Decapterus koheru	Koheru
		Decapterus russelli	Indian scad
		Decapterus spp.	Scad
		Elagatis bipinnulata	Rainbow Runner
		Megalaspis cordyla	Horse Mackerel/Torpedo Scad
		Nematistius pectoralis	Roosterfish
		Oligoplites saurus	Leather Jacket
		Pseudocaranx dentex	White trevally
		Scomberoides commersonnianus	Talang queenfish
		Scomberoides spp.	Leather Jacket/Queen Fish
		Selene spp.	Moonfish
		Seriola dumerili	Greater/Japanese Amberjack or RudderFish
		Seriola lalandi	Yellowtail Amberjack
		Seriola quinqueradiata	Japanese Amberjack
		Seriola rivoliana	Longfin Yellowtail
		Seriola spp.	Amberjack or Yellowtail
		Trachurus capensis	Cape Horse Mackerel
		Trachurus japonicas	Japanese Jack Mackerel

		Trachurus murphyi	Chilean Jack Mackerel
		Trachurus novaezelandiae	Yellowtail Horse Mackerel
		Trachurus spp.	Jack Mackerel/Horse Mackerel
		Trachurus trachurus	Atlantic Horse Mackerel
		Uraspis secunda	Cottonmouth jack
2.	Chanidae	*Chanos chanos*	Milkfish
3.	Clupeidae	*Alosa pseudoharengus*	Alewife
		Alosa spp.	Herring
		Amblygaster sirm	Spotted Sardinella
		Anodontostoma chacunda	Chacunda gizzard shad
		Brevoortia patronus	Gulf Menhaden
		Brevoortia spp.	Menhaden
		Brevoortia tyrannus	Atlantic Menhaden
		Clupea bentincki	Araucanian herring
		Clupea harengus	Atlantic herring
		Clupea pallasii pallasii	Pacific herring
		Clupea spp.	Pichard/Shad/Herring
		Dorosoma spp.	Gizaard Shad
		Ethmalosa fimbriata	Bonga Shad
		Ethmidium maculatum	Pacific Menhaden
		Etrumeus sadina	Red-eye round herring
		Harengula spp.	Sprat/Herring
		Harengula thrissina	Pacific flatiron herring
		Hilsa spp.	Shad
		Nematolosa spp.	Gizzard Shad
		Opisthonema libertate	Pacific thread herring
		Opisthonema spp	Thread Herring
		Opisthopterus tardoore	Tardoore
		Sardina pilchardus	European Pilchard
		Sardinella aurita	Round Sardinella
		Sardinella gibbosa	Gold stripe Sardinella
		Sardinella longiceps	Indian Oil Sardine
		Sardinella maderensis	Madeiran Sardinella

		Sardinella spp.	Sardine
		Sardinops sagax	South American Pilchard
		Sardinops spp.	South American Pilchard
		Spratelloides gracilis	Silver-stripe round herring
		Tenualosa ilisha	Hilsa shad
		Tenualosa spp.	Shad
4	Coryphaenidae	*Coryphaena hippurus*	Mahi-Mahi /Dolphin fish
5	Engraulidae	*Anchoa* spp.	Anchovy
		Anchoviella spp.	Anchovy
		Cetengraulis mysticetus	Pacific anchoveta
		Engraulis capensis	Southern African anchovy
		Engraulis encrasicolus	European anchovy
		Engraulis japonicus	Japanese anchovy
		Engraulis ringens	Peruvian anchovy
		Engraulis spp.	Anchovy
		Stolephorus spp.	Anchovy
6	Istiophoridae	*Istiompax indica*	Black Marlin
		Istiophorus albicans	Atlantic sailfish
		Istiophorus platypterus	Indo-Pacific sailfish
		Kajikia albida	Atlantic white marlin
		Kajikia audax	Striped Marlin
		Makaira mazara	Indo-Pacific blue marlin
		Makaira spp.	Marlin/Sailfish
		Tetrapturus spp.	Marlin/Spearfish
		Tetrapturus spp.	Spearfish
7	Mugilidae	*Mugil cephalus*	Flathead Grey Mullet
8	Pristigasteridae	*Ilisha* spp.	Ilisha/Pellona
		Pellona ditchella	Indian pellona
9	Scombridae	*Acanthocybium solandri*	Wahoo
		Auxis spp.	Bullet Tuna/Frigate Tuna
		Cybiosarda elegans	Leaping Bonito
		Euthynnus affinis	Little tuna or Kawakawa
		Euthynnus spp.	Bonito

		Gasterochisma melampus	Butterfly kingfish
		Grammatorcynus spp.	Short Mackerel
		Gymnosarda unicolor	Dogtooth tuna
		Katsuwonus pelamis	Skipjack Tuna
		Orcynopsis unicolor	Plain Bonito
		Rastrelliger brachysoma	Short Mackerel
		Rastrelliger kanagurta	Indian Mackerel
		Sarda spp	Bonito
		Scomber australasicus	Blue mackerel
		Scomber japonicas	Chub mackerel
		Scomber scombrus	Atlantic mackerel
		Scomber spp.	Mackerel
		Scomberomorus cavalla	King Mackerel
		Scomberomorus commerson	Narrow-barred Spanish mackerel
		Scomberomorus guttatus	Indo-Pacific king mackerel/Spotted Spanish Mackerel
		Scomberomorus niphonius	Japanese Spanish mackerel
		Scomberomorus spp.	Spanish Mackerel
		Scomeromorus lineolatus	Streaked seerfish
		Thunnus alalunga	Albacore Tuna
		Thunnus albacares	Yellowfin Tuna
		Thunnus atlanticus	Blackfin Tuna
		Thunnus maccoyi	Southern bluefin tuna
		Thunnus obesus	Bigeye Tuna
		Thunnus orientalis	Pacific bluefin tuna
		Thunnus spp.	Tuna
		Thunnus thynnus	Atlantic bluefin tuna
		Thunnus tonggol	Longtail Tuna
10	Xiphiidae	*Xiphias gladius*	Swordfish

Source: Food Safety and Standards (Contaminants, Toxins and Residues) Regulations, 2011

Limits of histamine level in fish and fishery products

S. No.	Product Category	Applicable to	Histamine Level
1.	Raw/Chilled/Frozen Finfish	Species with high amount of free histidine (Listed fish species with the potential to cause histamine fish poisoning)	n=9, c=2; m=100 mg/kg, M=200 mg/kg
2.	Thermally Processed Fishery Products		n=9, c=2; m=100 mg/kg,M=200 mg/kg
3.	Smoked fishery products		n=9, c=2; m=100 mg/kg,M=200 mg/kg
4.	Fish Mince/Surimi and analogues		n=9, c=2; m=100 mg/kg, M=200 mg/kg
5.	Battered and breaded fishery products		n=9, c=2; m=100 mg/kg,M=200 mg/kg
6.	Other Ready to Eat fishery products		n=9, c=2; m=100 mg/kg,M=200 mg/kg
7.	Other value added fishery products		n=9, c=2; m=100 mg/kg,M=200 mg/kg
8.	Other fish based products		n=9, c=2; m=100 mg/kg,M=200 mg/kg
9.	Dried/ Salted and Dried fishery products		n=9, c=2; m=200 mg/kg, M=400mg/kg
10.	Fermented Fishery products		n=9, c=2; m=200 mg/kg, M=400 mg/kg
11.	Fish Pickle		n=9, c=2; m=200 mg/kg, M=400mg/kg

Source: Food Safety and Standards (Contaminants, Toxins and Residues) Regulations, 2011

Where,

n: Number of units comprising the sample

c: Maximum allowable number of defective sample units m: Acceptable level in a sample

M: Specified level when exceeded in one or more samples would cause the lot to be rejected

Satisfactory, if the following requirements are fulfilled:

1. the mean value observed is ≤ m

2. a maximum of c/n values observed are between m and M

3. no values observed exceed the limit of M,

Unsatisfactory, if the mean value observed exceeds m or more than c/n values are between m and M or one or more of the values observed are >M.

Control of histamine formation in fish

Freezing may inactivate some of the enzyme forming bacteria. Both the enzyme and the bacteria can be inactivated by cooking. However, once histamine is produced, it cannot be eliminated by heat (including retorting) or freezing. After cooking, recontamination of the fish with the enzyme-producing bacteria is necessary for additional histamine to form. For these reasons, histamine development is more likely in raw, unfrozen fish but should not be discounted in other product forms of scombrotoxin-forming fish species. Rapid chilling of scombrotoxin-forming fish immediately after death is the most important element in any strategy for preventing the formation of scombrotoxin (histamine), especially for fish that are exposed to warm waters or air, and for tuna which generate heat in their tissues. Some recommendations are:

a. Fish exposed to air or water temperatures above 28°C should be placed in ice, or in refrigerated seawater, ice slurry, or brine of 4°C or less, as soon as possible after harvest, but not more than 6 hours from the time of death; or
b. Fish exposed to air and water temperatures of 28°C or less should be placed in ice, or in refrigerated seawater, ice slurry, or brine of 4°C or less, as soon as possible after harvest, but not more than 9 hours from the time of death; or

c. Fish that are gilled and gutted before chilling should be placed in ice, or in refrigerated seawater, ice slurry, or brine of 4℃ or less, as soon as possible after harvest, but not more than 12 hours from the time of death; or
d. Fish that are harvested under conditions that expose dead fish to harvest waters of 18℃ or less for 24 hours or less should be placed in ice, or in refrigerated seawater, ice slurry, or brine of 4℃ or less, as soon as possible after harvest, but not more than the time limits listed above, with the time period starting when the fish leave the 18°C or less environment.

Detection of histamine in fish

The most common method preferred for the determination of histamine is High Pressure Liquid Chromatography (HPLC). Chemical testing is an effective means of detecting the presence of histamine in fish muscles. However, the variability in histamine levels between fish and within an individual fish can be large. For this reason, a sampling plan has been provided by the FSSR of India for the analysis of histamine in fish. The number of samples 'n' (i.e., scombrotoxin forming fish) necessary to make a judgment about a lot depends on the anticipated variability, but should not be fewer than 09 samples per lot, unless the lot contains less than 09 fish, in which case a sample should be collected from each fish.

Principle

The sample is extracted by mixing with Perchloric acid. Pre-column derivatization is performed using dansyl chloride. The biogenic amines and the components in the solution are separated by HPLC with an appropriate column, using UV detection. Histamine mass concentration is calculated from the peak area ratio of histamine and internal standard with a calibration curve.

Reagents and Materials

- Acetone
- Acetonitrile
- Toluene
- Water, HPLC grade
- Water, distilled water, or equivalent
- Nitrogen gas
- Perchloric Acid
- Saturated Sodium Carbonate solution
- Dansyl Chloride Solution

- L- Proline
- Histamine stock solution
- Internal standard (IS) 1,7-diaminoheptane stock solution

Sample preparation

A pre-determined weight of the fish sample is taken and homogenized in perchloric acid in presence of 1,7-diaminoheptane. Then the sample is centrifuged for 5 min at 4°C. The supernatant is collected into a tube and mixed with sodium carbonate solution and Dansyl chloride solution. This solution is vortexed and incubated in the dark at 60 °C. After incubation, the tube is cooled and L-proline solution is added to it. The sample is vortexed and placed in the tube in dark for 15 min. Then toluene is added to this solution and kept in the freezer. The upper organic phase is transferred into a new tube and dried it in the fume hood with a stream of nitrogen. It is then re-suspended with acetonitrile/water (60/40 volume fraction) and vortexed. The solution is filtered in a glass autosampler vial and the autosampler is filled with the sample vails.

Preparation of standard samples

Concentration of Histamine [mg/kg]	Amount of Standard spiked for calibration [µl]
0	0
11	4.4
25	10
50	20
100	40
250	100
500	200

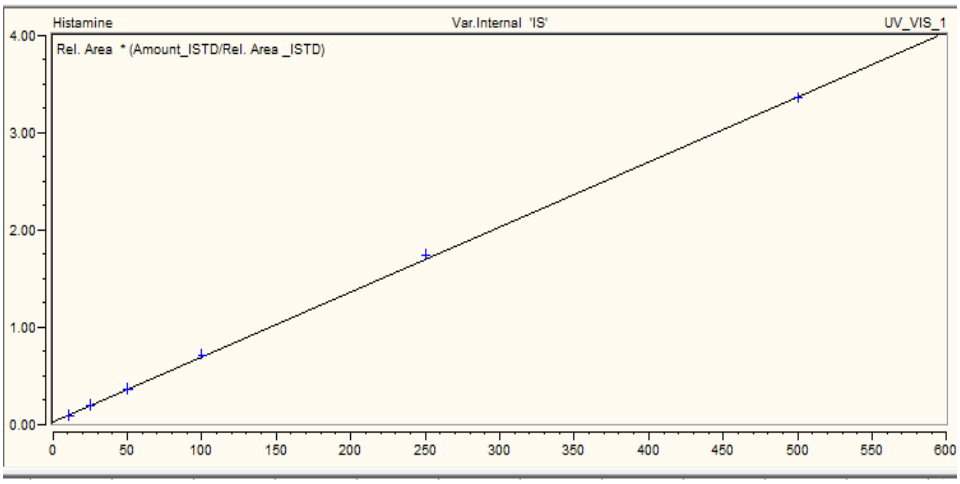


Fig 1. Calibration Curve

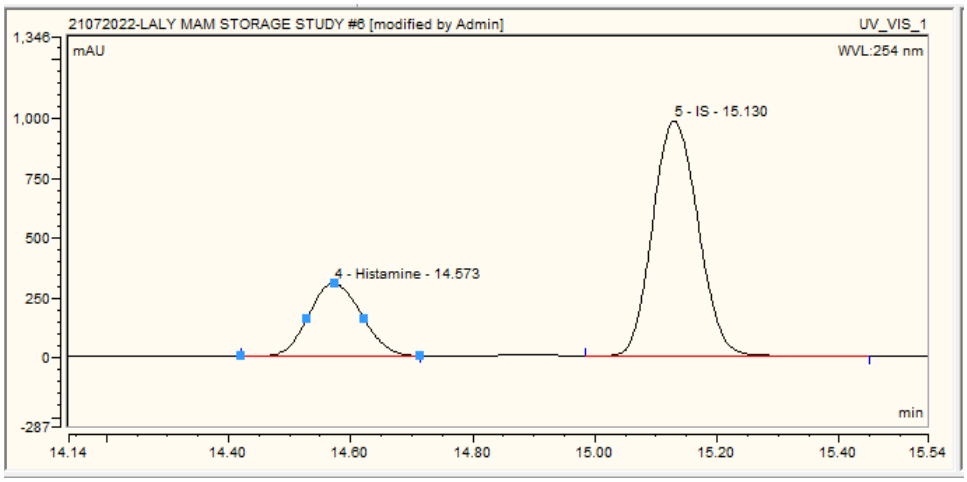


Fig 2. Peak of Histamine & IS at 50 mg/kg

Fig 3. Ultra High Pressure Liquid Chromatography (UHPLC)

Results expression

The estimated concentration of histamine in the fish sample is expressed as 'X' mg/kg.

Where,

X = calculated value of histamine

References

Food and Drug Administration. (2021). Fish and fishery products hazards and controls guidance. US Department of Health and Human Services Food and Drug Administration Center for Food Safety and Applied Nutrition, 4th Ed, p 113-152.

FSSAI, I. (2011). Food Safety and Standards (Contaminants, Toxins and residues) Regulations, 2011. Ministry of Health and Family Welfare, India, (August, 1 2011), 2.

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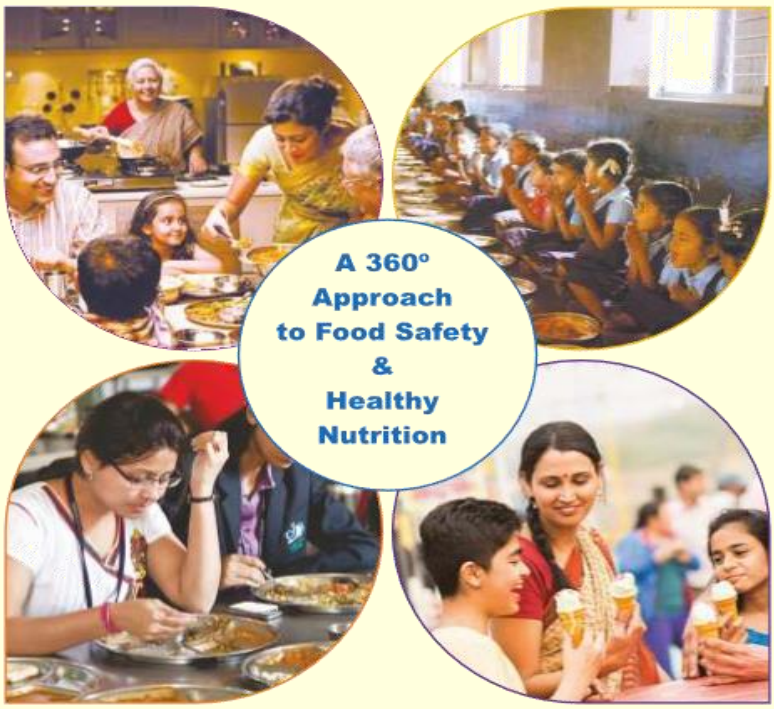
Inspiring Trust, Assuring Safe & Nutritious Food

Build a new India, Healthy & Happy India



A Bouquet of Initiatives for Citizens Guidance and Behaviourial Change

to prevent food borne infections and diseases and for complete nutrition for citizens everywhere at all times.

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