A DISSERTATION ON

"DETECTION OF BETA-LACTAM(Penicillin-G) IN MILK PRODUCTS"

SUBMITTED TO THE

DEPARTMENT OF BIOSCIENCES

INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILMENT

FOR THE DEGREE OF

MASTER OF SCIENCE IN BIOCHEMISTRY

BY

SARFRAZ

M.SC BIOCHEMISTRY (IV SEMESTER)

DEPARTMENT OF BIOSCIENCE

INTEGRAL UNIVERSITY, LUCKNOW

UNDER THE SUPERVISION OF

MRS.NIDHI

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LCMS/MS

FARELABS PVT LTD, GURGAON

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TO WHOM IT MAY CONCERN

This is to certify that **Sarfraz** student of M. Sc. Biochemistry (1V semester), Integral University has completed his four months dissertation work entitled "**Detection of beta-lactam(penicillin-G) in milk products**" successfully. He has completed this work from the guidance of **MRS. NIDHI (head of department lcms/ms)**. The dissertation was a compulsory part of his M. Sc. degree.

I wish him good luck and bright future.

Dr. Snober S. Mir,

Head

Department of Biosciences

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31st May 2022

TRAINING CERTIFICATE

This is to certify that **Mr. Sarfraz,** S/o Mr. Abdul Wahid from **Integral University Lucknow, MSc Biochemistry,** has successfully completed his dissertation on **"Detection of beta lactam (antibiotics) in skimmed milk product."** from 17th January 2022 to 31st May 2022 at **FARE Labs Pvt. Ltd.** and has been awarded excellent grade basis of his performance and the project report submitted.

He has accomplished the training successfully. We have found him sincere and devoted during the training.

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DECLARATION

I, **SARFRAZ**, certify that the work embodied in the training report **"Detection of beta lactam(antibiotic) in milk products"** to be submitted to the Master of Science in Biochemistry of Integral University, Lucknow, Uttar Pradesh, India is original and is the result of analysis carried out by me under the supervision of **Mrs. NIDHI** Head of LCMS/MS and GCMS/MS Department, FARE Labs Pvt. Ltd. for the time period of January, 2022 to June, 2022. The matter embodied in Master of Science thesis has not been submitted for the award of any other degree/ diploma.

I declare that I have faithfully acknowledged and referred to the research workers wherever their works have been cited in the text. I further certify that I have not wilfully lifted up some other's work, paragraph, text data, results, etc. reported in journals, books, magazines, reports, dissertations thesis, etc., or available at web sites and included them in this M.Sc. thesis and cited as my own work. I have completed all pre submission requirement as per the University rules.

SARFRAZ

ACKNOWLEDGEMENT

*The submission of my Master's thesis gave me immense pleasure, satisfaction and unique sense of accomplishment. I convey my humble gratitude to God and then my parents and my other family members for their prayers, love, care and sacrifices for educating and preparing me for my future. They have made this thesis a reality by having faith in me and by instilling in me the courage to strive and achieve higher goals in life**.** With the help, counselling and support of many people, this report has finally completed. At this point I would like to express our sincere gratitude to **Dr. Meenakshi Tripathi (Quality Manager)** for the great supervision, for the patience she always had despite her many other duties and responsibilities, and for providing good working place.*

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Thanking everyone

Sarfraz

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LIST OF ABBREVIATIONS

MRL	Maximum residue limit
PBP	Penicillin binding protein
ATCC	American type culture collection
MIC	Minimum inhibitory concentration
IM	Intramuscular
GC	Gas chromatography
LC	Liquid chromatography
HPLC	High performance liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectroscopy
ACN	Acetonitrile
EDTA	Ethylene diamine tetraacetic acid

INTRODUCTION

INTRODUCTION

Antibiotics are essential drugs that are regarded the best way to treat human infections. However, their effectiveness is jeopardized by their widespread and inappropriate usage, not just in medicine but also in agriculture. Antibiotics are used at therapeutic levels in veterinary medicine to treat illnesses and prevent infection. Subtherapeutic doses are also utilized to improve feed efficiency, boost growth, and avoid disease. Antibiotic use frequently results in medication residues that can be discovered in varying concentrations in animal-derived products such as milk or meat. It is prohibited to havemedication or antibiotic residues in food that exceed the limit amount established by various public agencies around the world (Kempe *et.al*, 2000).

Antibiotic residues in foodstuffs produced from animals treated with veterinary medical goods might cause (a) allergic or toxicological reactions in consumers, (b) selective pressure for antibiotic resistant strains, and, finally, (c) technological issues in the manufacture of fermented foods (5, 6). Regulation 2377/90 establishes the maximum residue limit (MRL) for a variety of veterinary medications in foods of animal origin in order to address the foregoing concerns (7). Raw ex-farm milk is periodically tested for the presence of antibiotics in order to provide consumers with safe and high-quality goods (8). Medicines that have a B- Lactam ring in their molecular structure are known as beta lactam drugs. And the major function of these B-Lactam medicines is to suppress the bacterial microorganism's cell wall formation. Bacteria, on the other hand, can develop an ind o resistance to Beta Lactam medicines by secreting! The enzyme -lactamase attacks the!-lactam ring.Overcome this adversity! Antibiotics containing -lactams are frequently prescribed. Inhibitors of -lactamase, such as clavulanic acid.

The monocyclic β-lactams (or monobactam) course speaks to the only basic stage among these antibiotics. The monocyclic stage of this b-lactam was separated from Chromobacterium violaceum **.** Pename is a class of β-lactam antibiotics with azabicycloheptane ring system, carboxylic acid moiety of C3, and sulfur atom at position 1. The archetypal and best-known penam is penicillin G (PNG or benzylpenicillin) isolated from Penicillium chrysogenum. Beta-lactam antibiotics act by structurally mimicking the d-alanine-d-alanine motif of bacterial cell wall peptidoglycan. They inhibit bacterial

transpeptidases that catalyze the cross-linking of peptidoglycans through the formation of isopeptide bonds.

Peptidoglycan is a component of the bacterial cell wall that protects cells from external stress while also maintaining osmotic stability. The breakdown and resynthesis of cell walls are essential for bacterial cell growth and division. PBPs, which are key peptide cross-linking enzymes required for peptidoglycan formation, are the main target of -lactams. The serine nucleophile attacks the lactam carbonyl in the presence of a -lactam, resulting in a stable acyl-enzyme complex. This process compromises the bacterial cell wall's integrity, reducing the potential for growth and division while also decreasing protection from osmotic and tensile stress. The L,D-transpeptidases are a second key mode of action that is unique to carbapenem-lactams. Faropenem's activity against mycobacteria, for example, is attributed to the creation of L,D transpeptidase covalent adducts.

The penicillin binding protein (PBP) BlaR is a sign transduction membrane protein, which induces the synthesis of β-lactamase. The C-terminal area of BlaR (BlaR-CTD) protein is located within the extracellular place, which acts as a drug binding web site (Kerff, et.al, 2003).. BlaR-CTD is first of all the sensor area of a penicillin receptor this is acylated by means of penicillin. This protein can discover and bind to a selection of β-lactam antibiotics (Duval, et.al, 2003; Golemi-Kotra, et.al, 2003). In the energetic site of the protein, STYK, serine is a key amino acid (AA) that can take part within the binding of β-lactams (Zapun, et.al, 2008). BlaR-CTD protein has been used to locate the β-lactam antibiotics residues within the receptor-primarily based screening assay, which include a organic fluid method the use of colloidal gold classified receptor protein (Li, et.al, 2020) and a receptor-primarily based enzyme related immune-sorbent assay developed via our lab, in which BlaR-CTD from B. Licheniformis ATCC14580 become immobilized at the plate (Peng, et.al, 2013).

REVIEW OF LITERATURE

Review of Literature

β-lactam antibiotics are the maximum broadly used antimicrobial marketers inside the prevention and treatment of bacterial infectious diseases in animals, and are useful within the manage of mastitis in dairy cows, urethral diseases, gastroenteritis and respiratory infections in other animals. In addition, they can be used as feed components to prevent diseases in cattle and chicken, through the inhibition of bacterial cellular walls. They play an critical role in killing pathogenic microorganisms, and have the blessings of sturdy antimicrobial capacity, bactericidal outcomes **(**Cherian, et.al, 2018)low toxicity and huge spectrum (Ronquillo, et.al, 2017). However, because of the flawed use, abuse and non-compliance of the withdrawal length, the lifestyles of veterinary drug residues in animal derived ingredients together with milk and animal tissues poses a risk to human health, the ecological environment and meals safety (Baynes, et.al, 2016). Further, the abuse of antibiotics may motive allergic reactions, microbial resistance and the overall decline of immunity (Muhammad, et.al,2016). The types of beta lactam are: Benzathine, Benzathine penicillin G.**,** Benzathine penicillin V.**,** Phenoxymethylpenicillin (penicillin V)**,** Procaine penicillin, Pheneticillin.

Two penicillin G molecules react with diphenylethylene diamine to generate benzathine penicillin. Beta-hemolytic streptococci (groups A, B, C, G, H, L, and M), as well as Treponema pallidum and Treponema carateum, are all susceptible to it. Streptococcus pyogenes has never been found to be resistant to benzathine penicillin. When utilising benzathine penicillin, this activity explains the indications, mechanism of action, administration, side effects, and contraindications. Two penicillin G molecules react with diphenylethylene diamine to generate benzathine penicillin. Beta-hemolytic streptococci (groups A, B, C, G, H, L, and M), as well as Treponema pallidum and Treponema carateum, are all susceptible to it. Streptococcus pyogenes has never been found to be resistant to benzathine penicillin.

Indications Approved by the FDA:

1. Acute glomerulonephritis is a kind of glomerulonephritis that affects the
2. Infections of the respiratory tract
3. Chorea and rheumatoid arthritis

4. Heart problems caused by rheumatoid arthritis

5. Other venereal diseases, such as syphilis

The mechanism of action of Beta-lactam antimicrobials include benzathine penicillin. Antimicrobials known as beta-lactams are bactericidal. During the active multiplication stage, this antibiotic inhibits the manufacture of the cell wall peptidoglycan. It inhibits peptidoglycan transpeptidase in bacteria. This causes an osmotically unstable cell wall, which leads to cell wall lysis, bacterial cell disintegration, and bacterial cell death.

Administration

The injectable suspension of benzathine penicillin is injected intramuscularly, rather than intravenous, intraarterial, or subcutaneously. To reduce the pain associated with the injection, the medicine should be warmed to room temperature before use, and it should not be administered near an artery or nerve. Adults should inject the buttocks' upper outer quadrant. Administer the injection to the mid-lateral muscle of the thigh, not the gluteal region, in children under the age of two, and rotate the injection site on subsequent doses. The opaque and viscous benzathine penicillin has a low solubility. As a result, the antibiotic is slowly released and degraded to penicillin G at the injection site. The drug's concentration in the blood remains lower but for a longer period of time due to sluggish absorption and hydrolysis. Adults have detectable drug concentrations for 14 days after a 1.2 million unit injection, and often longer. Syringes of 600,000 units/1 mL, 1.2 million units/2 mL, or 2.4 million units/4 mL antimicrobials are commonly used. The antibacterial should be kept refrigerated between 36 and 46 degrees Fahrenheit (2 and 8 degrees Celsius) and should never be frozen. The following are the dosage recommendations:

Adult dosages:

Group A streptococci pharyngitis/tonsillitis (1.2 million units IM x 1).

Mild to moderate group A streptococci in the upper respiratory tract that are responsive to low, extended doses of benzathine penicillin (1.2 million units intramuscularly [IM] x 1).

Secondary glomerulonephritis prevention (prophylaxis for people who have had acute glomerulonephritis) (1.2 million units IM every four weeks or 600,000 units IM twice monthly).

Rheumatic fever secondary prevention (prophylaxis) (1.2 million units IM every 3 to 4 weeks or 600,000 units IM twice monthly).

Syphilis:

Primary, secondary, or latent for less than one year (2.4 million units IM x 1, may repeat dose x 1 after one week in pregnant patients); latent for more than one year (2.4 million units IM x 1, may repeat dose x 1 after one week in pregnant patients); latent for more than one year (2.4 million units IM x 1, may repeat dose x 1 after one week in (2.4 million units IM weekly for three weeks)

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Children and Infants:

Syphilis: Primary, secondary, and early latent less than 1 year (50,000 units/kg/dose IM x 1, maximum 2.4 million units/dose); late latent greater than 1 year (50,000 units/kg/dose IM weekly for 3 doses, maximum 2.4 million units/dose); late latent greater than 1 year (50,000

units/kg/dose IM weekly for 3 doses, maximum 2.4 million units/dose); late latent greater than 1 year (50,000 units/kg/dose IM weekly

Negative Effects:

Patients tolerate benzathine penicillin well, with the most common complaint being injection pain. Hypersensitivity responses are among the other side effects. Patients having a history of penicillin hypersensitivity, as well as those with asthma, hay fever, allergies, or urticaria, are more susceptible to hypersensitivity reactions. The type-I, IgE-mediated reaction, often known as anaphylaxis, is a dangerous and/or lethal hypersensitivity event. Urticarial skin rash, itching, wheezing, dyspnea, nausea, vomiting, and diarrhoea are common symptoms of this type-I hypersensitivity reaction, which can lead to hemodynamic instability and mortality. Any previous anaphylactic reaction or serious skin reaction to any penicillin (for example, Steven-Johnson syndrome or Toxic Epidermal Necrosis) is a contraindication for usage. Patients with anaphylactic shock have been reported in the past or severe skin reactions from cephalosporins or carbapenems will have a form of cross-reactivity reaction that will cause serious adverse events when given any penicillin medicine, however these reports are thought to be far lower than previously thought. The other side effect is a superinfection, which can occur after continuous use. After 2 months of antibiotic treatment, a superinfection can occur. C. difficile-associated diarrhoea and pseudomembranous colitis are two of these potentially lethal illnesses.

Penicillin G:

Penicillin G is made from Penicillium chrysogenum fermentation. It is usually given intravenously as a sodium, potassium, benzathine, or procaine salt due to its limited oral bioavailability. Penicillin G is a beta-lactam antibiotic that kills gram-positive bacteria such as nonpenicillin resistant streptococcal, staphylococcal, and enterococcal species. It also has modest efficacy against gramme negative anaerobic organisms and some gramme positive anaerobic organisms, as well as gramme negative aerobes such Neisseria species. Penicillin G, on the other hand, is rarely used to treat these infections.

Penicillin G is contraindicated in those who are hypersensitive to penicillins, cephalosporins, and carbapenems and in those known to be hypersensitive to procaine or benzathine if these

salt forms are to be administered. Some commercially available penicillin procaine preparations contain sulfites that can cause allergic reactions. Also, some commercially available preparations of penicillin G contain tartrazine that may cause allergic reactions in susceptible individuals.Penicillin G is a spirochete antibiotic that is often used to treat syphilis. It's also used to treat skin and skin structure infections, as well as meningitis and endocarditis. Penicillin resistance is seen in a variety of species.

Therapeutics:

Penicillin G is effective against gram-positive bacteria such as Streptococcus pyogenes (group A), Streptococcus pneumoniae, and Streptococcus viridans strains. Resistant Streptococcus pneumoniae, on the other hand, has emerged and is common in some locations. In rare cases, heavy dosages of penicillin can be used to overcome resistance. Staphylococcus aureus that does not produce penicillinase can be treated with penicillin G. Taking care of Gram-positive bacteria require larger dosages of penicillin G than Gram-negative bacteria. Peptostreptococcus, Peptococcus, and certain Clostridium anaerobic bacteria Penicillin G-resistant strains are also common. Penicillin G is effective against a variety of bacteria. Gram-negative organisms that do not produce penicillinase, such as Neisseria meningitidis, Haemophilus influenzae, and Neisseria gonorrhoeae. The Enterobacteriaceae family is resistant to antibiotics. Treatment with penicillin G. Penicillin G's anaerobic action against Gram-negative bacteria varies. It's up and running.

Infections, adults:

Dose chosen will be dependent on the indication and causative organism. Higher doses should be employed for more severe infections, and a dosage adjustment is required with renal insufficiency. Penicillin G procaine doses are 600,000 to 1.2 million units daily for approximately 10–14 days. Penicillin G procaine should be administered intramuscularly. It cannot be given intravenously. The penicillin G benzathine dose is 1.2 million units/day as a deep intramuscular injection. It cannot be given intravenously.

Infections, children:

Higher doses should be used for more severe infections. Neonate dosing differs based on age. Dosage adjustment required with renal insufficiency.

Endocarditis:

The dose selected is dependent on the organism. Entococcus endocarditis requires 18–30 million units, whereas Viridans streptococci and Streptococcus bovis dosing ranges from 12–18 million units and is dependent on MICs to penicillin. Use in combination with gentamicin. Dosage adjustment required with renal insufficiency.

Negative Effects:

Gastrointestinal problems, rash, and hypersensitivity reactions are the most common side effects observed with penicillin G. Nausea, vomiting, anorexia, diarrhoea, and gastritis are all gastrointestinal side effects. Clostridium difficile colitis is a type of colitis caused by Clostridium difficile. Penicillin G therapy has been reported to be effective. Rash, angioedema, and other hypersensitivity reactions include Stevens-Johnson syndrome, toxic epidermal necrolysis, erythema multiforme, and serum reactions that are similar to sickness Penicillin G treatment can potentially cause anaphylaxis. Eosinophilia, hemolytic anaemia, leukopenia, neutropenia, agranulocytosis, and thrombocytopenia are all hematologic responses. There have been a few occurrences of acute interstitial nephritis. reported. Penicillin G causes hallucinations, disorientation, and tiredness in the brain. coma, convulsions, and dysphasia With higher doses, these neurotoxic consequences are more common. In patients with renal impairment, this can go as high as 20 million units per day. Benzathine or Benzathine Hoigne's syndrome is characterised by the usage of procaine penicillin. by strange neurologic responses and behaviour When penicillin is used, the Jarisch-Herxheimer response ensues. G is a drug that is used to cure syphilis. It happens 2–12 hours after starting penicillin G medication. Headache, fever, chills, sweating, sore throat, and changes in vital signs are all symptoms of this reaction. signs. It is most common in people with primary and secondary syphilis in the United States. System of Hospital Formulary (2001).

Procaine Penicillin:

Procaine penicillin is an injectable antibiotic that also acts as a local anaesthetic. The treatment of all stages of syphilis, mild to moderate pneumococcal pneumonia, and as an adjuvant in the treatment of diphtheria with intramuscular (IM) antitoxin are among the

indications for use. Additional indications include, but are not limited to, the treatment of Listeria monocytogenes infections, as well as infections caused by numerous Treponema and Actinomyces species, as well as scarlet fever, rat-bite fever, and tonsillitis. As part of the interprofessional team, this activity outlines the indications, mechanism of action, methods of administration, significant adverse effects, contraindications, monitoring, and toxicity of procaine penicillin so that providers can direct patient therapy in treating infections for which it is indicated. Procaine penicillin is an injectable antibiotic that also acts as a local anaesthetic. The treatment of all stages of syphilis, mild to moderate pneumococcal pneumonia, and as an adjuvant in the treatment of diphtheria with intramuscular (IM) antitoxin are among the indications for use. While procaine penicillin has been characterised as an anthrax therapy and post-exposure prophylaxis regimen, it is not the first choice. Additional indications include, but are not limited to, the treatment of Listeria monocytogenes infections, as well as infections caused by numerous Treponema and Actinomyces species, as well as scarlet fever, rat-bite fever, and tonsillitis. Procaine penicillin is not recommended for the treatment of gonorrhoea, and the manufacturer discourages its usage in this manner.

Mechanism of Action:

The beta-lactam penicillin component of procaine penicillin exerts its bactericidal effects by inhibiting cell wall production. The drug's activity is due to the drug's binding to penicillin-binding proteins, which are naturally occurring proteins in the target organism. Once occupied, these proteins impede the completion of peptidoglycan production, which is required for the formation of the bacterial cell wall. Cell lysis and bacterial cell death occur as a result of the cessation of cell wall formation mixed with the continuous action of native cell wall autolytic enzymes. Penicillin resistance is generally caused by bacteria-produced penicillinase. While this resistance phenomena appears in a variety of organisms, it is worth noting that no resistance by Streptococcus pyogenes has been reported to far. The procaine component is an amino ester local anaesthetic that produces local anaesthesia by acting on the fast sodium channel. The purpose of this anaesthetic additive is to alleviate the pain of IM injection in large enough doses to achieve therapeutic penicillin concentrations.

Negative Effects:

Many of procaine penicillin's most dangerous side effects occur as a result of unintentional intravascular injection. These include cardiac conduction disturbances and neurologic consequences, such as tonic-clonic seizures, as well as persistent neurovascular damage in the form of disorders like transverse myelitis and gangrene, which necessitate the amputation of the digital and even proximal extremities. Necrosis and sloughing at the injection site, as well as transitory psychological instability, have also been reported as side effects. The latter is most commonly noticed when very high doses, such as 4.8 million units IM, are administered. Confusion, combativeness, convulsions, anxiety, and a sensation of imminent death are all symptoms of Hoigne syndrome, a procaine-related illness. Symptoms of Hoigne syndrome last 15 to 30 minutes. As a matter of course, resolution will occur. The focus of treatment is on symptom management. Anaphylactoid reaction, Jarisch-Herxheimer reaction, hemolytic anaemia, superinfection at the injection site, interstitial nephritis, and acute widespread exanthematous pustulosis are some of the less commonly reported side effects (AGEP). AGEP is a poorly understood illness that has been documented two to three weeks after starting therapy and manifests as a fever and a desquamating rash. Because penicillin is secreted in breast milk, nursing women must exercise extreme caution. There has been no evidence of teratogenesis in animal models, and penicillin has never been demonstrated to harm a human foetus in the past. Because there are no randomised trials to show that procaine penicillin is safe during pregnancy, the manufacturer suggests only using it if absolutely necessary.

CHROMATOGRAPHY-

Chromatography is a laboratory technique for the separation of a mixture. The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus affect the separation.

Types of chromatography: -

- **Gas chromatography (GC)**

Gas chromatography is a separation technique in which the molecules are separated based on their retention time depending on the affinity of the molecules to the stationary phase.

- **Reverse-phase chromatography**

Reverse-phase chromatography is a liquid chromatography technique where the separation of molecules is achieved through hydrophobic interaction between the liquid mobile phase and the stationary phase.

- **Liquid Chromatography (LC)**

Liquid chromatography (LC) is a separation method used to isolate the personal additives of a mixture. This method includes mass switch of a pattern thru a polar cell section and non-polar desk bound section. This form of chromatography employs a liquid mobile phase. Liquid-solid chromatography utilizes a solid stationary phase, and the major mechanism of retention is adsorption. Popular adsorbents are silica and alumina, which both retain polar compounds. If a polar mobile phase is used, the solutes are rapidly swept from the bed. Thus, the preferred mobile phase is a nonpolar or slightly polar solvent. Liquid-liquid chromatography employs liquid mobile and stationary phases. High-performance liquid chromatography uses small particles with molecules bonded to their surface to give a thin film that has liquid-like propertiSeveralr of bonding agents are available. A nonpolar molecule can be bonded to the solid and a polar mobile phase usedis . This method is termed reverse-phase liquid chromatography. The partition coefficient depends on the identity of both mobile and stationary phases. In this case, however, the number of stationary phases is limited, while there is a large number of liquids and combinations of them used for the mobile phase. Mobile phases of constant composition are called isocratic

- **High-Performance Liquid Chromatography (HPLC)**

High-performance liquid chromatography (HPLC), also known as high-pressure liquid chromatography, is an advanced type of LC. HPLC is amenable to a wide range of

applications, such as pharmaceuticals and food analysis. It is especially useful for low or non-volatile organic compounds, which cannot be handled with gas chromatography.

Mass Spectrometry (MS)

Mass spectrometry (MS) ionizes atoms or molecules to facilitate their separation and detection according to their molecular hundreds and charges (mass to fee ratio). MS is utilized in diverse applications, e.g., biochemical and atomic physics.

The basic precept of MS -A mass spectrometer generates a couple of ions from the pattern beneath investigation, it then separates them in step with their unique mass-to-fee ratio (m/z), after which the relative abundance of every ion type. The first step withinside the mass spectrometric evaluation of compounds is the manufacturing fuel line segment ions of the compound, essentially through electron ionization. This molecular ion undergoes fragmentation. Each number one product ion derived from the molecular ion, in turn, undergoes fragmentation, and so on. The ions are separated withinside the mass spectrometer in step with their mass-to-fee ratio and are detected in share to their abundance. A mass spectrum of the molecule is as a result produced. It shows the bring about the shape of a plot of ion abundance as opposed to he mass-to-fee ratio. Ions offer facts regarding the character and the staff in their precursor molecule. In the spectrum of a natural compound, the molecular ion, if present, seems at the very best of and offers the molecular mass of the compound. Among the various specific types of mass analyzers, those that locate software in LC-MS structures are the quadrupole, time-of-flight (TOF), ion traps, and hybrid quadrupole-TOF (QTOF) analyzers.

Components of MS

The tool includes three foremost additives:

1**. Ion Source**: For generating gaseous ions from the substance being studied.

2. **Analyzer**: For resolving the ions into their traits mass additives are in line with their mass-to- e ratio.

3. Detector System: For detecting the ions and recording the relative abundance of every of the resolved ionic species. In addition, a pattern advent device is vital to confess the samples

to be studied to the ion supply whilst preserving the excessive vacuum requirements (~10-6 to 10-eight mm of mercury) of the technique; and a computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries.

4. Detection: a detector is used to decide the species and amount of every ion. 25 In the prevailing study, the following column and tool situations had been optimized for quantitative evaluation of general aflatoxins peanuts via way of means of Liquid Chromatography-Tandem Mass Spectrometry.

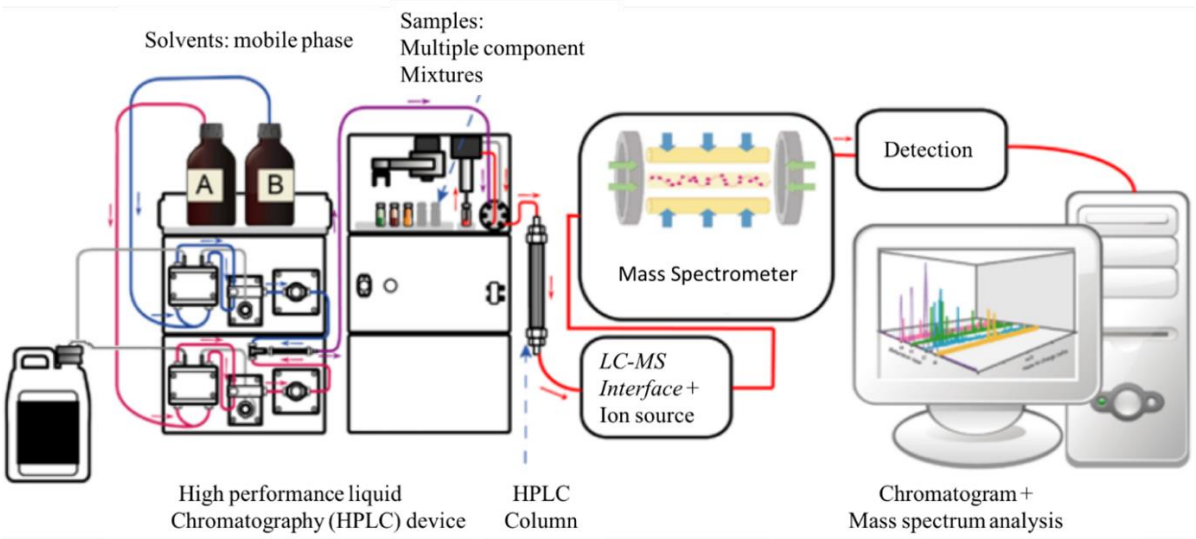


Fig: LC-MS/MS

Pump

The basic design and operation of the reciprocating-piston pump are illustrated by the single-piston pump. The key components are a piston, pump seal, pump head, and a couple of check valves. The piston, usually made of sapphire, is driven back and forth in the pump head by a rotating motor. Various means have been conceived to convert the rotary motion of the motor into the bidirectional movement of the piston. Most commonly, this is done by a cam pressing against one end of the piston to push it into the pump head and a spring to push the piston back out. It is a polymer ring that fits around the piston, and a small lip forms a liquid-tight seal against the piston with the aid of spring and liquid pressure. A pair of ruby check valves with sapphire seats are mounted on the top and bottom of the pump head. The check valves control the direction of flow through the pump. On the intake stroke, the piston is

withdrawn, which creates a low-pressure area inside the pump head. This allows the outlet check valve to close and the inlet check valve to open so that the mobile phase flows in to fill the pump head. On the delivery stroke, the piston moves into the pump head, and the inlet check valve is closed as the pressure increases. When the pressure inside the pump head exceeds the pressure in the column, the outlet check valve opens and the mobile phase flows to the column. When all is working well, this simple pump design is quite reliable.

Sampler

The total-volume injection autosampler features a pressure tolerance of 130 MPa as well as the world's fastest sample injection (10 seconds), which dramatically reduces the total cycle time. It includes auto pre-treatment and overlapping functions as standard, and an optional loop-injection method configuration to minimize delay volume. With the reduction of the needle contact area, special coatings, surface treatments, and a new needle seal, the reaches a new level of low carryover performance, which is especially beneficial for LCMSMS analysis. In addition, the included sample cooler features a dehumidifier function for storing samples at a constant temperature between 4°C and 40°C.

Column

LC separates the components of a sample based on the differences in their affinity or retention strength for the stationary phase and mobile phase. ... The sample (in purple) is injected into the LC column and gets separated into 2 analyte bands (red and blue) and gets eluted from the column.

Detector (MS/MS)

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio (m/z) of charged particles (ions). Although there are many different kinds of mass spectrometers, all of them make use of electric or magnetic fields to manipulate the motion of ions produced from an analyte of interest and determine their m/z. The basic components of a mass spectrometer are the ion source, the mass analyzer, the detector, and the data and vacuum systems. The ion source is where the components of a sample introduced in an MS system are ionized using electron beams, photon beams (UV lights), laser beams, or corona discharge. In the case of electrospray ionization, the ion source moves ions that exist in the

liquid solution into the gas phase. The ion source converts and fragments the neutral sample molecules into gas-phase ions that are sent to the mass analyzer. While the mass analyzer applies the electric and magnetic fields to sort the ions by their masses, the detector measures and amplifies the ion current to calculate the abundances of each mass-resolved ion. Inordinate a mass spectrum that a human eye can easily recognize, the data system records, processes, stores, and displays data on a computer. The mass spectrum can be used to determine the mass of the analytes, their elemental and isotopic composition, or to elucidate the chemical structure of the sample. MS is an experiment that must take place in the gas phase and under a vacuum (1.33 * 10−2 to 1.33 * 10−6 pascal). Therefore, the development of devices facilitating the transition from samples at a higher pressure and in condensed phase (solid or liquid) into a vacuum system has been essential to developing MS as a potent tool for the identification and quantification of organic compounds and peptides. MS is now in very common use in analytical laboratories that study the physical, chemical, or biological properties of a great variety of compounds.

OBJECTIVE

OBJECTIVES

1. **To analyze and detect the beta lactam(penicillin-G) in milk product.**
2. **Characterization of beta lactam using LC-MS/MS.**

MATERIAL AND METHOD

Material and Method

Drugs are commonly used for the prevention & treatment of bacterial growth mainly because of their high and broad-spectrum activity. Because of these harmful effects, different countries have established MRLs (Maximum Residual Limits) for these antibiotics in food of animal origin products. There are several techniques available for determination and quantification of these Drugs; however, due to insufficient sensitivity and poor selectivity posed by very complex matrix, the targeted MRPL's cannot be measured using those techniques. The use of LC-MS/MS has shown good selectivity and sensitivity to quantify the Residue at the required MRL levels in complex food matrices.

Requirements-

Apparatus: Analytical balance (accuracy 0.1 mg), Vortex mixer, Glass centrifuge tube size 15 ml ,Polypropylene centrifuge tube size 50 ml, Vibrating Shaker, Refrigerator centrifuge ,,Measuring Cylinder, Disposable 0.2 µm syringe filter ,Disposable 2 ml syringe, Amber vial size 2 ml with insert spring tube, Eppendrof tubes, HPLC column : Zorbax Eclipse XDB-C18 (1.8µm, 3.0x 100mm), Nitrogen evaporator, Dispenser , Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS): Adjustable pipettes: 10-100 µl and 100-1000 µl with disposable tips, Mobile phase and sample filtration unit.

Reagents and Glassware: Millipore water, Formic Acid (LCMS grade), Acetonitrile (LCMS grade) purity 99.9%, Reference standards, PSA, C18, Acetic Acid, Sodium Chloride, Magnesium Sulfate, Ethyl Acetate, Volumetric flask-10ml , Glass tubes, Measuring Cylinder: 500 ml, Auto-sampler Vials.

Procedure:

1. Mobile Phase

Preparation A: 0.1 % formic acid in water: Take one litre of water in a mobile phase bottle add 1000 µL of formic acid. Shake vigorously & degas in ultra sonicator bath for 5 min at room temperature and it is stable for 5 days.

2. Mobile Phase B

Preparation B: 0.1 % formic acid in ACN:

Take one liter of Acetonitrile in a mobile phase bottle and add 1000 µL of formic acid. Shake vigorously & degas in ultra sonicator bath for 5 min at room temperature and it is stable for 5 days.

Preparation of Stock standards (1000 mg/Kg): Transfer 10 mg or 0.01g of standard into 10 ml volumetric flask and dissolve in LCMS grade Acetonitrile. Make up to 10 mL .Label with name of the standard, Concentration, date of preparation, date of expiry .The stock standard solution is stable up to the expiry of date mentioned in certified of analysis.

Preparation of Intermediate standard mixture solution (10mg/kg): Pipette out 100µL of each Antibiotic Standard from Stock solution (1000 mg/Kg) in to 10 mL volumetric flask and make up with 0.1 % ACN: H2O (80: 20) and Label it as Mix of 10 mg/Kg.

Preparation of working standard dilution for calibration curve: Prepare the following working standard as below using acetonitrile, water (20:80) containing 0.1 % formic acid as diluents:

Intermediate standard conc.(mg/L)	Volume of Working standard (µL)	Solvent volume (µL)	Final volume (mL)	Final conc. (mg/L)	Label
100	100	900	1	10	WS1
10	100	900	1	1	WS2
1	100	900	1	0.1	WS3

Preparation of Sample:

- Weigh 2.0 +/- 0.05 g of milk sample into polypropylene centrifuge tube.
- Add 2 ml EDTA solution (0.1 M, PH 4.0).
- Vortex properly and add 8 ml Acetonitrile then again vortex and shake vigorously.
- Centrifuge the samples for 5 min at 4000 rpm.

- Take 5 ml layer and evaporate up to dryness under nitrogen evaporator.
- Reconstitute with 1 ml of 0.2% Acetic acid in 98:2 Water: Acetonitrile.

Sample weight (gm)	Standard of WS volume (µL)	Final concentration (µg / Kg)	Label
5	150(WS2)	150	CC6
5	100(WS2)	100	CC5
5	75(WS2)	75	CC4
5	50(WS2)	50	CC3
5	25(WS2)	25	CC2
5	100(WS3)	10	CC1

Batch Organization:

Prior to start the analysis condition the appropriate analytical column with 30 column volume of mobile phase 98:2 composition of Eluent A and Eluent B.

1. Blank: There is a Matrix blank per batch of samples. The Matrix blank should give no peaks meaning that the Signal to noise ratio (S/N) should be less than 3.

2. **Determination & Confirmation:** Process the samples with Quantization mode provided in Mass Hunter supplied by Agilent. The concentration of the unknown has to be calculated from the equation using regression analysis using weighing factor (1/x). The sample is confirmed for the presence of the test analyte if its retention time agrees within 2 % of that of standard and ion ratios of the analyte agree within 25 % of that of standard.

3. Spike Recovery: For every batch a control sample spiked with standards shall be run. The analysis of the spiked sample should be carried out in the sample procedure as the normal sample. The spike recovery is calculated using the formula:

$$\% \text{ Recovery } R=(S-U) \times V \times 100/W \times Csa$$

Where,

S= measured concentration of an analyte in the matrix spike sample result

U= Measured concentration of an analyte in the unspiked sample.

Csa= Spiking level

V= Volume of extract made up

W= weight/volume of sample taken.

4. Batch Sequence:

S. No	QC Point	Criteria	Run
1.0	Solvent Blank	-	1
2.0	Matrix calibration standards(CC1 to CC6)	$R^2 \geq 0.9950$	1
3.0	Reagent Blank	-	1
4.0	Samples 1 to 6	-	Each 1
5.0	Spike sample/Check std	Check Recovery/Repeatability	-
6.0	Samples 1 to 6	-	Each 1
7.0	Spike sample/Check std	Check Recovery/Repeatability	-

5. CHROMATOGRAPHY CONDITION:

LC Conditions:	
Column used	Zorbax Eclipse XDB-C18 (1.8µm, 2.1x 50mm)
Mobile phase	1. Mobile Phase A- 5 milli molar ammonium Acetate plus 0.2 % Acetic acid in Water 2. Mobile Phase B-5 milli molar ammonium acetate plus 0.2 % Acetic acid in Acetonitrile.
Flow rate	0.300 ml/min
Column Temp.	30.0 ^{o}C

6. **Mass Spectrometer Conditions:**

Scan type	Multiple reaction monitoring
Mode	ESI positive mode
Run time	27 min
Gas temperature	350 °C
Capillary	4500 V
Nebulizer	35 psi
Nozzle voltage	500 V
Sheath gas Flow	12 (L/min)
Sheath gas temperature	400 °C
Polarity	Positive
Data type	Centroid
MS Acquire time	27.00 min
Capillary voltage	4 500 V
Delta EMV	2 50
H igh Pressure Limit	1 200.00 bar
G as flow	1 2 (L/min)

ANALYTICAL VALIDATION

LINEARITY

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration (amount) of analyte. Linearity should be evaluated visually to plot signals as a function of analyte concentration. For the establishment of linearity a minimum of 5 different concentrations are recommended. Linearity is determined by analyzing reference material of at least 5 different concentrations within the linear range and

calculates the regression coefficient, y-intercept, slope of the regression line. Acceptance Criteria for linear quantification, regression coefficient (R2) for analytical standard solution should be ≥ 0.99

RECOVERY

Recovery studies were performed to examine the efficacy of extraction and clean up. Untreated oils samples were spiked with known concentration of the pure phenthoate and phorate standard solutions. The concentration of each pesticide in the final extracts was calculated

$$\boldsymbol{Recovery}\ \% = \boldsymbol{Spiked\ Value} / \boldsymbol{S□汳iked\ Concentration} \times 100$$

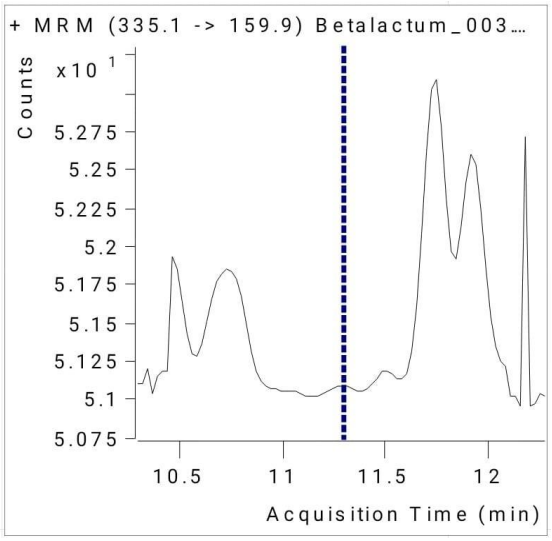
According to sante guidelines, recovery should be in range between 70% - 120%. And from recovery quantitative analysis of both beta lactam is done by:

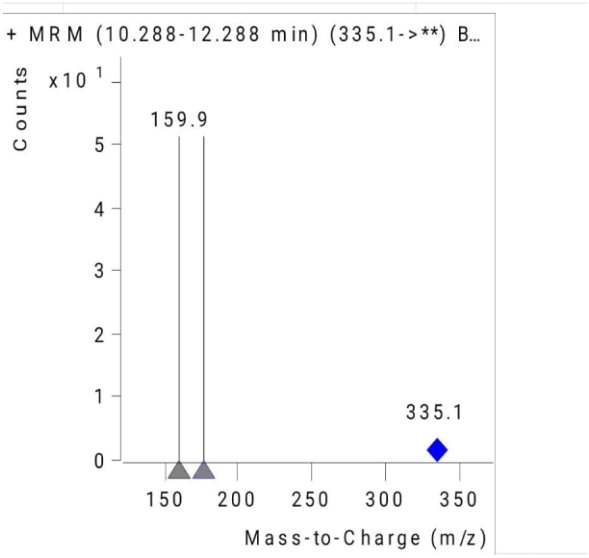
$$\mathbf{Result\ (ppb)} = \mathbf{Sample\ Respon□档e/\ Recovery} \times \mathbf{100}$$

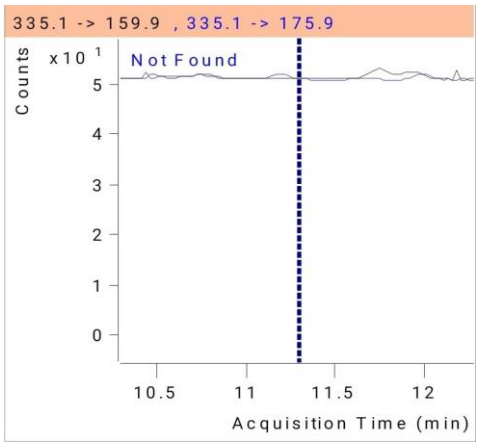
RESULT AND DISSCUSSION

Results

Beta lactam (penicillin-G) not detected.







penicillin G

CONCLUSION

Conclusion

The use of antibiotics in modern agriculture and dairy industry has significantly increased the productivity, but it has also increased antibiotic residue in food and in our environment resulting negative effect on human health. Determination of antibiotics residue in milk is very challenging due to its complex matrix. Thus in this study a novel extraction & cleanup method was adopted for analysis of different oils sample. The extraction and clean based on QuEChERS method. Analysis was done with sample, one skimmed milk powder named as sample A and sample B. The study concluded that sample A and B does not have Beta Lactam.

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