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Determination of Antibiotic Residues in Ice Creams by Using LC/MS/MS Technique

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Abstract

Two different brands of ice creamhave been procured from the market and used for the present study.Five commonly used antibiotics such as Nitrofuran metabolites,Sulfonamides, quinolines &Fluoroquinolone residue,Nitrofuran Parent compounds, Nitroimidazole, Chloramphenical and tetracycline.0.1 grams of standard were dissolved in 2 milliliters of methanol to create stock solutions. To create a standard working solution with varying concentrations, this solution was further diluted using the same solvent.Samples were analyzed for antibiotic residues using HPLC coupled with triple quadrupole mass spectroscopy (LC/MS/MS). The separation was carried out using isocratic mode on an Agilent ODS C18 column with a suitable mobile phase.The technique's linearity, recovery, accuracy, and specticity were all confirmed.

Keywords: Antibiotics, Ice creams, LC/MS/MS

1. Introduction

A major worldwide health concern, antibiotic resistance is thought to be the cause of about 0.7 million deaths a year, with estimates indicating that figure could increase to 10 million by 2050. Antibiotic therapy may fail, and human lives may be in danger if milk products with antibiotic residues exceeding the Maximum Residue Limit (MRL) are consumed. Other dangers include harm to humans and the emergence of superbugs.Additionally, prolonged exposure to these residues may disrupt the gut microbial, potentially exacerbating various diseases [1]. Therefore, our study uses High-Performance LiquidChromatography (HPLC) to detect five distinct veterinary medicines in order to ascertain the residue levels of selected antibiotics in ice cream.

Antibiotic Resistance

The antibiotic resistance dilemma has been attributed to the overuse and misuse of antibiotics as well as the pharmaceutical industry's inability to develop new medications due to onerous regulatory constraints and a lack of financial incentives. Around the world, antibiotics have had comparable positive benefits. Antibiotics reduce morbidity and mortality from food-borne illnesses and other infections linked to poverty in developing nations with inadequate sanitation. Globally, antibiotics have proven to be effective in a similar way. Antibiotics reduce morbidity and mortality from food-borne infections as well as other infections connected to poverty in developing nations with continued inadequate sanitation.[2]

Use of antibiotics in ice creams

Antibiotics are used in animal husbandry for both preventative and therapeutic purposes. It has been found that the use of antibiotics in animals is double that of their utilization in humans. Human health benefits greatly from milk, a nutrient that is widely consumed worldwide. Milk is a foodstuff that is extensively consumed worldwide and has significant health advantages for humans. Due to their negligent use in treating animal diseases, antibiotic residues are primarily found in milk. A further source of antibiotic residues in milk and the final cause of any possible public health concern is the use of certain antibiotics as feed additives.

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Additionally, milk is a basic ingredient in ice cream manufacturing. Antibiotic residues are the parent antibiotics or their metabolites that are administered and end up deposited in animal tissues and animal-derived materials meant for human consumption when the concentration is higher than what is allowed for a specific amount of time. The use of antibiotics in the treatment of mastitis and dry cow therapy are two major contributing factors to the development of antibiotic residues in milk. Poor detection facilities and an inadequate food residue monitoring system that takes maximum residue limits (MRLs) into account could be considered major contributions. These residues contribute to antibiotic resistance, allowing microorganisms to become resistant and potentially spread resistance genes. Additionally, antibiotic resistance can cause allergic reactions, such as serum sickness and anaphylaxis [3,4].

Harmful effects of Antibiotics Resistance on consumers

For the dairy industry, even minimal concentrations of antibiotic resistance are highly concerned. The presence of low levels of antibiotic residues in milk and dairy products can lead to significant public health and industry issues.These residues contribute to antibiotic resistance, allowing microorganisms to become resistant and potentially spread resistance genes. Additionally, antibiotic resistance can cause allergic reactions, such as serum sickness and anaphylaxis, particularly with penicillins. They have carcinogenic and mutagenic effects, potentially causing DNA mutations and chromosome damage, leading to conditions like infertility and congenital anomalies. Furthermore, antibiotic resistance can disrupt the normal intestinal flora, making pathogenic microbes more prominent and disturbing the gut environment. [5,6]

Detection of Antibiotics in Ice Creams

LC-MS/MS Technique: In clinical testing facilities, methods like HPLC and LC–MS/MS are typically used to measure medication concentration for different antimicrobials. The LC- MS/MS method has been proven to overcome limitations of lack of specificity, time consumption, and a long waiting time for the procurement of results. A wide variety of chemicals were evaluated using simultaneous methods for measuring several analytes. [7,8]A literature survey reveals that antibiotic residues have not been estimated in ice cream so far. Hence, we performed the quantitative estimation of antibiotics in the ice cream samples for the first time.

2. Materials and Methods:

2.1 Equipment's:

Agilent: LC:(G1211B)1260 Quat pump. MS MS: G6430A 6430 Triple Quad LCMS,

Agilent: LC:(G1312B) HPLC -1260 Binary pump MS MS: G6430A Triple Quad MS

Agilent: LC:(G1311B) 1260 Binary pump MS MS: G6430A 6430 Triple Quad LCMS

3. Standard Preparation:

A stock solution of 1.0 mg/ml is obtained by dissolving the approximate quantity of each antibiotic. From this 1 µ/ ml standard was prepared using methanol. Two commercial samples of ice cream were procured from an ice cream shop located inChennai and used for the present study.

3.1 Nitrofuran Metabolites

Sample Extraction:

Hydrolysis and Derivatization: Weighed out 2.0 gm of sample in a centrifuge tube. To this add 0.2 M hydrochloric acid. Added 50µl of 2-NBA-solution in methanol. Closed the screw cap and shook by hand to disperse the sample. Incubated the sample overnight at 37 ± 2°C at 200 RPM

Neutralization:

After cooling, added 500 µl 0.3 M Na3PO4 solutions to the sample. Shook the sample by hand. Adjusted the pH to 7.

LC/MS/MS Technique

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Extraction:

A total of 5 mL of ethyl acetate was added to the pH-adjusted sample.Vortexing thoroughly mixes and centrifuges at 3500 rpm for 10 minutes. The resulting organic layer was carefully transferred to a clean evaporation tube. A second extraction was performed by adding another 5 mL of ethyl acetate to the remaining aqueous phase, followed by vortexing and centrifugation under the same conditions. The organic layer from this second extraction was collected and combined with the first. The combined organic extracts were evaporated under a gentle stream of nitrogen at 35 °C until dry. The residue is dissolved with 2ml of water & the lipid content in the sample was removed with 2ml of hexane and aqueous layer filtered through 0.22µm filter & injected into LC/MS/MS.

Instrumental Settings:

1. HPLC Conditions:

The column used is eclipse plus which is of 4.6 x 50 mm (3.5 µm particle size) C18 was used with a 5M ammonium formate in 0.1 % FA.

The analysis was performed using a methanol (MeOH) gradient under a flow rate of 0.400 mL/min. A total of 40 µL of each sample was injected into the system, with a total run time of 15 minutes. Solvent composition for LC-MS/MS is given in (Table 1)

Source Parameter for mass spectrometry is given in (Table 2).

Timetable:

Table 1: The Liquid Chromatography Gradient elution method

LC /MS/ MS Conditions:

Selected reaction monitoring MS/MS was performed on the protonated molecule ion for antibiotics using the following general parameters

Polarity: Positive
Ion Source: Electro spray

Table 1: Gradient Table: Solvent Composition

	Channel	Solvent 1	Name 1	Used	Percent
1	A	H_20	5mM Ammonium formate in 0.1 % FA	Yes	85.0 %
2	B	MeOH		Yes	15.0 %

Table 2:Source Parameter for mass spectrometry

Parameter	Value (+)	Value (-)
Gas temp (c degree)	300	300
Gas flow (l/min)	12	12
Nebulizer (psi)	45	45
Capillary (V)	4000	2000

3.2 Sulfonamides, Quinolones & Fluoroquinolone Residue

Sample Extraction:

Extraction of antibiotics:

Weighted out 2.0g of sample &2 ml of water was added and then 10 ml of CAN was added.

Vortexed& 1g of MgSO4 is added, 0.5g Na3 citrate dihydrate ,0.5 g Na2H citrate sesiquehydrate,1 g sodium chloride is added. Solvent Composition A (H2O 75%), and B (MeOH 25.0%).

Shook immediately & centrifuged at 5000 rpm 5 min 20˚C.

6 ml of clear ACN layer for cleanup with 150 mg PSA was taken, 900 mg MgSO4 & 45 mg GCB.

Vortexed & centrifuged at 5000 rpm 5 min 20˚C. took out 1 ml clear solution to GC MS MS.

Pipetted out 1ml of Extract & it was evaporated using Turbo evaporator at 45±2°C under Nitrogen.

Reconstituted the residue with 2 ml of water and injected it to LC-MS/MS.

Instrumental Conditions for LC/MS/MS:

HPLC Conditions:

Agilent: LC: (G1311B) 1260 Quat pump. MS MS: G6430A 6430 Triple Quad LCMS C18 column 150 x 4.6 x 3.5µm.

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The Liquid Chromatography Gradient elution method for sulfonamides, quinolones& fluroquinolone residue (Time gradient : 2min (A:75%, B:25%,6 min (A:5% B:95%),,6.10min (A:5%, B:95%,,10 min (A:75%, B:25%, and 15 min (75%, B:25%))

3.4 Nitrofuran Parent

Compounds, Nitroimidazole, Chloramphenical

Sample Extraction: Weighed 2.0 gm of cleaned ground Sample into a Centrifuge tube.

Quantification of antibiotics for the following commercial samples of ice creams

Added 10mlof the supernatant layer. Repeated the extraction procedure two times. The above combined extract was evaporated to dryness under nitrogen atmosphere at 35◦C, the dried extracts are reconstituted into 2 ml of water were filtered and injected into the LC/MS/MS.

3.5 Tetracycline

Solutions:

McIlvaine buffer/EDTA solution: 2.841 grams of Na2HPO4 were dissolved in 100 milliliters of purified water. Mix after diluting to volume. 2.101 grams of citric acid monohydrate were dissolved in 100 milliliters of distilled water. Mix after diluting to volume. Combine 62.5 ml of the Na2HPO4 solution with 100 ml of the citric acid solution. This mixture's pH was checked. The result was found to be 4.0 + 0.05.

Prepare the McIlvaine buffer to contain 0.1 M EDTA: Added 3.7224 gm of EDTA in 162.5 ml of pH solution (100 ml of citric acid and 62 ml of Na2HPO4 solution).

Sample Extraction:

Accurately weighed 2.0 gm of cleaned ground Sample into a Centrifuge tube.10 ml of 0.1 M Na2EDTAMcIlvaine buffer. Vortexed for 1 min at 5000 rpm. Centrifuged for 10 min at 3500 rpm, collecting the supernatant layer.

Standards:

Repeated the extraction two more times, combining the extracts. The combined extract was again centrifuged and filtered. Conditioned each SPE C18 column with 3.0 ml of MeOH and followed by 3.0 ml of water. Transferred the extract to a conditioned C18 column. A vacuum manifold allows flow through at about 1 drop/sec. The tube was rinsed with 10 ml of water and passed through the cartridge. Dry the cartridge for 5 min by air. TCs were eluted with 10 ml of MeOH.

Instrumental Conditions for LC/MS/MS:

HPLC Conditions:

Agilent: LC: (G1311B) 1260 Binary pump. MS MS: G6430A 6430 Triple Quad LCMS, C18 column 50 x 4.6 x 2.7µm.

LC-MS-MS Conditions:

The liquid Chromatography gradient elution method.Mobile phase composition solvent.Selected reaction monitoring MS/MS was performed on the protonated molecular ion for pesticides using the following general parameters.

Results and Discussion

Concurrently, it is highly difficult to quantify antibiotics in single method analysis when considering ionization, sample extraction, compatibility with potential antibiotics, and chromatography. The process was linear from 5 to 125 ppb, µg/kg, and the LC/MS/MS is a more sophisticated and sensitive approach.

Preparation of Calibration Curve Standard

Chromatograms of the standards metronidazole, levofloxacin, escitalopram, sulfamethoxazole, trimethoprim, amoxy-1, and tetracyclinewere given in the (Figs. 1.1-1.5).

Calibration Curve Ranges From 5 to 125 ppb, microgram /

Quantification of antibiotics for the following commercial samples of ice creams was performed by LC/MS/MS*:*

Sample 1: 36 antibiotics
Sample 2- 41 antibiotics

These antibiotics' MRM transition was administered in (Tables 3 to 6).

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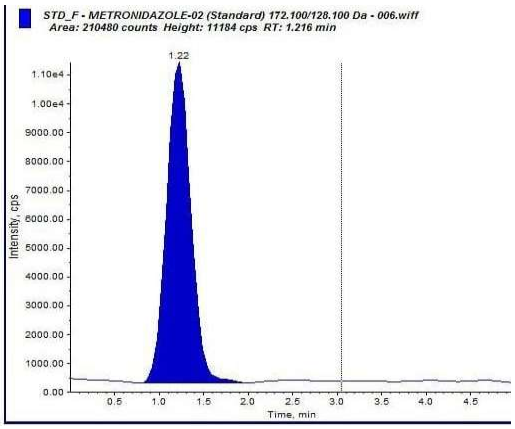

Fig. 1.1Metronidazole standard

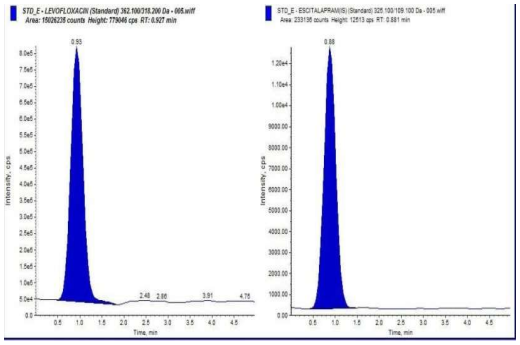

Fig.1.2: Levofloxacin and Escitalapram (Standards)

Nitrofuran Metabolites

Sulfonamides, Quinolines & Fluoroquinolone Residues
Nitrofuran Parent Compounds, Nitroimidazole, Chloramphenicol:

Tetracyclin41 Antibiotics were detected in samples 1 & 36. Antibioticsare detected in sample 2.

The concentration of antibiotics in the sample was found to be below the limit of Quantification (BLQ). Antibioticresidues pose a major threat to the long-term viability of public health. To check the residual antibiotics in ice-cream samples, the LC-MS/MS technique is used. The concentration of 41 antibiotics in sample 1 & 36 Antibiotic in sample 2 were

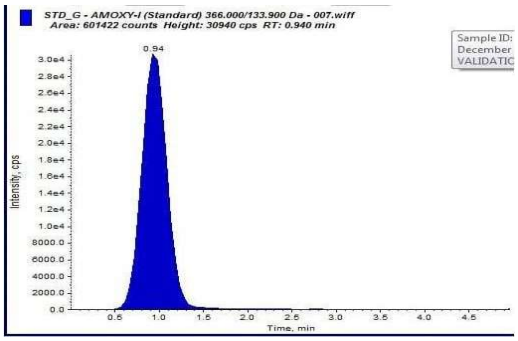

Fig. 1.3: Amoxy-1 (Standard)

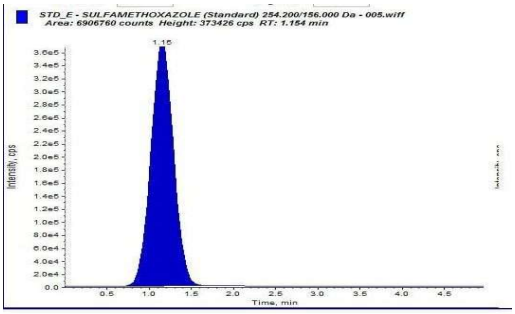

Fig. 1.4: Sulfamethoxazole

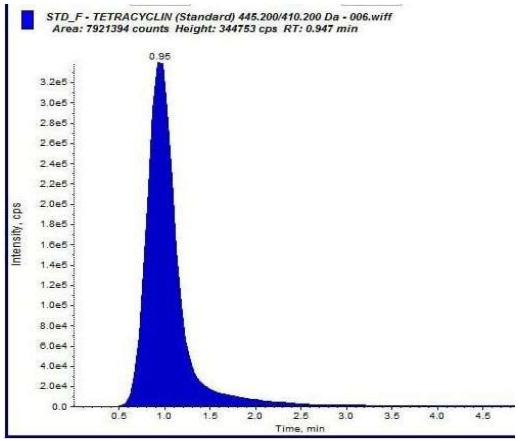

Fig. 1.5: Tetracycline

determined by LC/MS/MS Technique and found to be below the LOQ.The absence of antibiotic residues in ice cream sample suggests that the procured ice cream sample could be consumed by humans and it is safe to consume. To guarantee food safety and shield consumers

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Table 3: The MRM conditions for the antibiotic by LC/MS/MS

Compound Name	Quantifier Ion(Prec Ion)	Qualifier Ion (Prod Ion)	Polarity
AMOZ-d5	340.3 ->299.3	340.3 ->296.2	Positive
3-amino-5morpholinomethyl- 1,3oxazolidin [AMOZ]	335.2 ->291.1	335.2 ->262.1	positive
1-aminohydantoin [AHD]	249.1 ->134	249.1 ->104.1	Positive
AOZ-d4	240.2 ->134.1	240.2 ->104.2	Positive
3-aminooxazolidinone [AOZ]	236.2 ->134	236.2 ->104	Positive
SCA- 13C,15N2-	212 ->194.9	212 ->167.9	Positive
Semicarbazide [SEM]	209.1 ->166.1	209.1 ->134	Positive

Table 4: The MRM conditions for the antibiotic by LC/MS/MS

Compound name	Quantifier Ion (Prec Ion)	Qualifier Ion (Pro Ion)	Polarity
Difloxacin	400.21 ->382.2	400.21 ->356.2	Positive
Sarafloxacin	386 ->368	386 ->342	Positive
Enrofloxacin	360.2 ->342.2	360.2 ->316.2	Positive
Ciprofloxacin	332.35 ->314.2	332.35 ->231.1	Positive
Norfloxacin	320.34 ->302.2	320.34 ->276.2	Positive
Sulfadimethoxine	311.3 ->156.1	311.3 ->108.1	Positive
Sulfadoxin	311.2 ->156.1	311.2 ->92.2	Positive
Trimethoprim	291.3 ->229.9	291.3 ->122.9	Positive
Sulfachloropyridazine	285 ->156.1	285 ->92.2	Positive
sulfamethoxypuridazine	281.3 ->156	281.3 ->92.2	Positive
Sulfamethacin	279.3 ->186.1	279.3 ->124.1	Positive
Sulfamerazin	271.3 ->156.1	271.3 ->92.2	Positive
Sulfamethizole	271.3 ->156.1	271.3 ->92.2	Positive
Flumequine	262.26 ->244.1	262.26 ->126.1	Positive
Oxolinic acid	262.07 ->244.1	262.07 ->244.1	Positive
Sulfathiazole	256.3 ->156.1	256.3 ->92.1	Positive
Sulfamethoxazole	254.1 ->156.1	254.1 ->92.1	Positive
Sulfadiazine	251.3 ->156.1	251.3 ->108.1	Positive
Sulfapyridine acid	250.3 ->156.1	250.3 ->92.2	Positive
Nalidixic	233.25 ->215.1	233.25 ->187.1	Positive
Sulfaguanidine	215.2 ->156.1	215.2 ->108.1	Positive
Sulfacetamide	215.2 ->92.1	215.2 ->65.2	Positive

LC/MS/MS Technique

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Sulfapyridine acid	250.3 ->156.1	250.3 ->92.2	Positive
Nalidixic	233.25 ->215.1	233.25 ->187.1	Positive
Sulfaguanidine	215.2 ->156.1	215.2 ->108.1	Positive
Sulfacetamide	215.2 ->92.1	215.2 ->65.2	Positive

Table 5: The MRM conditions for the antibiotic by LC/MS/MS

Compound name	Quantifier Ion (Prec Ion)	Qualifier Ion (Pro Ion)	Polarity
Furaltadone	325.1	281.2	Positive
Furaltadone	325.1	100.1	Positive
Furazolidone	226	95.1	Positive
Furazolidone	226	67.1	Positive
Nitrofurantoin	237	152.1	Negative
Nitrofurantoin	237	151.8	Negative
Nitrofurantoin	237	77	Negative
Nitrofurazone	197	150.1	Negative
Nitrofurazone	197	124	Negative
Ronidazole	201.1	140.1	Positive
Ronidazole	201.1	55.2	Positive
Metronidazole	172.1	128.1	Positive
Metronidazole	172.1	82.1	Positive
Ipronidazole	170.1	124.1	Positive
Ipronidazole	170.1	109.1	Positive
Dimetridazole	142.1	96.1	Positive
Dimetridazole	142.1	95.1	Positive
Dimetridazole	142.1	54.2	Positive
Chloramphenicol-D5	326.1	157.1	Negative
Chloramphenicol	321	257.1	Negative
Chloramphenicol	321	152	Negative

Table 6: The MRM conditions for the antibiotic by LC/MS/MS

Compound name	Quantifier Ion (Prec Ion)	Qualifier Ion (Prod Ion)	Polarity
Chlorotetracycline	479.1-> 462.1	479.1-> 98.1	Positive
Oxy tetracycline	461.2-> 444.1	461.2 -> 426.1	Positive
Doxycycline	445.5 -> 410.1	445.5 -> 154	Positive
Tetracycline	445.2 -> 428.2	445.2-> 427.2	Positive
4-epi-tetracycline	445.2-> 427.2	445.2 -> 410.2	Positive
4-epi Chlortetracycline	479.28-> 444.20	479.28-> 468.20	Positive
4-epi Oxytetracycline	461.28-> 426.28	461.28-> 443.40	Positive

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from exposure, it is essential to keep an eye out for antibiotic residues in any food sample.

Outcomes

It is frequently overlooked that food exposure to antibiotic residues poses possible harm to public health. Antibiotic residues in ice cream are hence the causeof caution. The purpose of this project is to offer baseline data that can guide policy decisions and further inquiries, particularly risk assessment in order to safeguard public health.To guarantee food safety and safeguard the public's health, this study might be expanded, and a database of different antibiotic residue concentrations and related danger thresholds could be created.

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