Analysis of Furazolidone residues in shrimps using High Performance Liquid Chromatograph

BY

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Declaration

I carried out the work described in this thesis at the SGS Lanka (Pvt) Ltd. And Faculty of Applied Sciences under the supervision of Mr.Jagath Wansapala and Mrs.Sumathi Rajasingham. A report on this has not been submitted to any other University for another degree.

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AFECTIONALY DEDICATED
TO MY LOVING FAMILY MEMBERS, TEACHERS

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Abstract

Furazolidone is a banned synthetic nitrofuran derivative. This is used both therapeutically and prophylactically as an antimicrobial agent, which is effective in treating especially bacterial and fungal infections in fish, shrimp, poultry, pigs and rabbit. It is widely used through out Asia. High doses of Furazolidone may cause mutagenic action, carcinogenic action and development of drug resistant bacteria, shrimp, and human. Furazolidone is added to shrimp with feeds. Due to use of over doses and due to incorrect withdrawal time from ponds, shrimps are containing antibiotic residues.

This research was aimed the estimation of the Furazolidone residue level and the development of extraction method. Not only that but also the effect of heat and salt on Furazolidone was tested.

Extraction method was developed. Several steps are involved to analyze Furazolidone residues in shrimps. They are sampling, extraction, extract clean up, concentration, separation of extract components, detection, determination and confirmation of identity.

All the farm samples were free from Furazolidone. Six of the seven fresh market samples were positive for Furazolidone test. But after cooking four market samples of seven were positive. Results were statistically analyzed with T-test of the mean using MINITAB package. Market samples were exceeded the residue limit, which has laid down by Federal Office of Public Health. Furazolidone may be added to shrimps to preservation purpose. Salt and cooking are effecting to residue level. It lower the residue level.

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Chapter 01

1. Introduction

1.1 Antibiotics in shrimp

Antibiotics are usually administered in feed, either compounded during manufacture or surface-coated on to feed pellets. They are also used in the hatchery as bath medication in the shrimp industry. For medication of juvenile shrimp, the antibiotic is applied to feed as oil based coating as necessary. At semi-intensive to intensive scales of production, farmers use 5-10g antibiotics per kg feed at least once per day at weekly intervals. Thus antibiotic would be used in about 10% of feed. (David, 1999).

Rampant use of antibiotic in shrimp hatcheries and incorrect withdrawal time of shrimp from ponds will cause undesirable effects. Furazolidone (Nitrofuran parent compound degrades rapidly in the treated animal and metabolites are stored in the tissue and products where they can remain long after treatment. Studies have found that these metabolite residues are very stable, as they have a long half-life. (Holmstrom et al., 2003).

1.2 Furazolidone as an antibiotic

Furazolidone is a synthetic nitrofuran derivative used both therapeutically and prophylactically as an antimicrobial agent, which is effective in treating especially bacterial and fungal infections in fish, shrimp, poultry, pigs and rabbit (Vermeulen, 2003). But the Sri Lankan fisheries and aquatic resources act (No.2 of 1996), U.S. food and drug administration, EU regulations recently withdrew approval for the use of this drug in food producing animals because of it's carcinogenicity. And Furazolidone is never approved for use in aquatic food animal in the United State. Furazolidone is an unauthorized substance mentioned as in schedule 1(group A) in Fisheries and aquatic resources act. Furazolidone residues present in shrimp are a threat to human health. (Stehly et al., 1994). But it is widely used throughout Asia in large quantities in aquaculture industry.

1.3 Furazolidone as a disease-causing agent

These antibiotics used in aquaculture may be toxic not only to targeted pathogens or pest but also to non-targeted population such as culture species, wild flora and fauna and human consumers. High doses of Furazolidone may cause mutagenic action, carcinogenic action and development of drug resistant bacteria. The Federal Office of Public Health has laid down a residue limit of 1ug/kg for each active substance including its metabolites (Federal Office of Public Health, 2002). Furazolidone are available as several types of preparations such as Furazolidone mixture, Furazolidone premix, Furazolidone aerosol powder (Dunnavan, 2002)

1.4 Other uses of Furazolidone

1.4.1 Medicines in human health care

It is used in human medicine as a treatment of bacterial diarrhoea and Gastro-enteritis. It is also active against *Giardia lamblia.* But it is rarely used in Human medicine; the usual dosage is 100mg 4times a day for 2 to 5 days and for children 5mg per kilogram body-weight daily in divided doses. (The pharmaceutical CODEX, 1979)

1.4.2 Veterinary uses of Furazolidone

Furazolidone is used in animals as an antimicrobial agent and for the prevention and treatment of histomoniasis. The usual dose for the treatment of salmonella infections in large animals is 10 to 12 milligrams per kilogram body-weight for 5 to 7 days. For *Escherichia coli* and salmonella infections in weaned pigs the usual prophylactic dosage is 100ppm in the feeds, and the usual therapeutic dosage is 300ppm in the feed for 7 days; these doses may be doubled in unweaned pigs. For histomoniasis in poultry, the usual prophylactic dosage is 100ppm in the feed and the usual therapeutic dosage is 400ppm in the feed for 10 days. (The pharmaceutical CODEX, 1979)

1.5 Objectives

(a) Development of extraction method for Furazolidone residues in shrimps.

(b) Determination of the level of Furazolidone residues presents in shrimps at the farms & markets.

(c) Assessment of the effect of cooking & addition of salt make on Furazolidone residues in shrimp.

(d) Make recommendations for analysis of Furazolidone residues in shrimps.

CHAPTER 02

2 Literature review

2.1 Shrimps

2.1.1 Importance and Background of shrimp.

As highly prized seafood delicacy, shrimps and prawns are a cash crop grown mainly for the affluent export (and urban) markets. From farms in Southeast Asia, East Asia, South Asia, and South America shrimp are exported to the major market in Japan, the USA and Europe. Domestic consumption accounts for only 5-20% while foreign markets absorb 80-95% of total farmed production. Often, products rejected for the export market due to small size, bacterial load, or chemical residue levels are shunted to local markets.

Long standing, sustainable tradition is being transformed by modern, commercially oriented, high-output, intensive culture process that is causing severe environmental damage. As the extensive style has given way to intensification and industrialization, the degree and extent of environmental damage intensifies too. (Hagler, 1997)

2.1.2 Biological and Nutritional information of shrimp

Shrimps belongs to,

Kingdom: Animalia

Phylum: Artropoda

Class: Crustacea

Genus: Peneaus

Species: Monodon (James,1993).

There are several species. Among 300 species of Penaeid shrimp known worldwide, only a few dozen are commercially important in capture fisheries. Among the leading cultured species, the giant or black tiger shrimp *Peneaus monodon* has increased its share in global production from 33% to 56% and the Eucador white shrimp *P.vannamei* from 14% to19%, whereas the Chinese white shrimp *P.shinensis* has declined from 28% to 6% in the last few years.

(Hagler, 1997)

Nutritional value of shrimp

The nutritional values of shrimp are numerous and eating shrimp reduces the risk of heart disease. Seafood may prolong life after a heart attack. Seafood lowers blood triglycerides (fats). Shrimp may improve heart function and reduce damage from heart disease. It can lower blood pressure and may improve symptoms of inflammatory diseases, arthritis and psoriasis.

Seafood is a rich source of protein, vitamins and minerals. Seafood contains about twenty percent of the high quality proteins of red meat and poultry. It is also low in fat and most of the fat it has is polyunsaturated. Shrimp is also a good source of "B" Vitamins (B-6, B-12, Biotin and Niacin). Frozen shrimp is a good source of several minerals especially Iron, Phosphorus, Potassium and Zinc. (Siam Canadian foods Co., Ltd., 2003)

Total calories	80 cal.
Protein	18g
Carbon	0
Total fat	1g
Saturated fat	0
Omega-3	0.3g
Cholesterol	165mg

(Charleston Sea food, 2003)

Life cycle

Shrimp are swimming crustaceans that inhabit the warm marine waters of the tropics and sub tropics. Belonging to the Family penaeidae, their complex life history starts in near shore water where ripe females spawn eggs, which hatch in to free-, swimming larvae. These metamorphose through a series of stages in to postlarvae, which move toward nursery habitats along the coastline and estuaries. Here they develop further in to juveniles and subadults until they undertake the reverse migration to offshore water where they spawn and remain until they die. This life cycle is typical for most penaeids although a few species are able to complete the cycle in purely marine or estuarine water.

Breeding time of *P.monodon*- November, December, January

Fig.2.1 life cycle

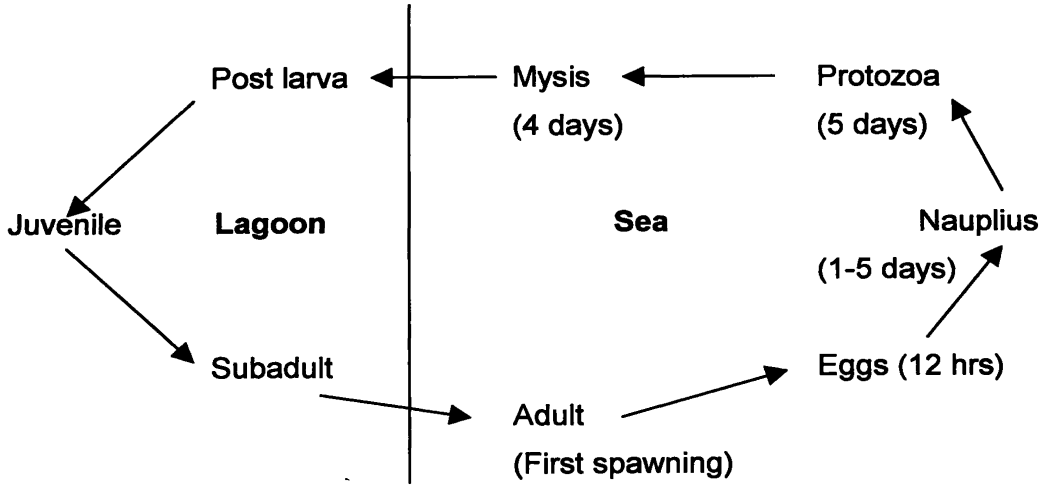
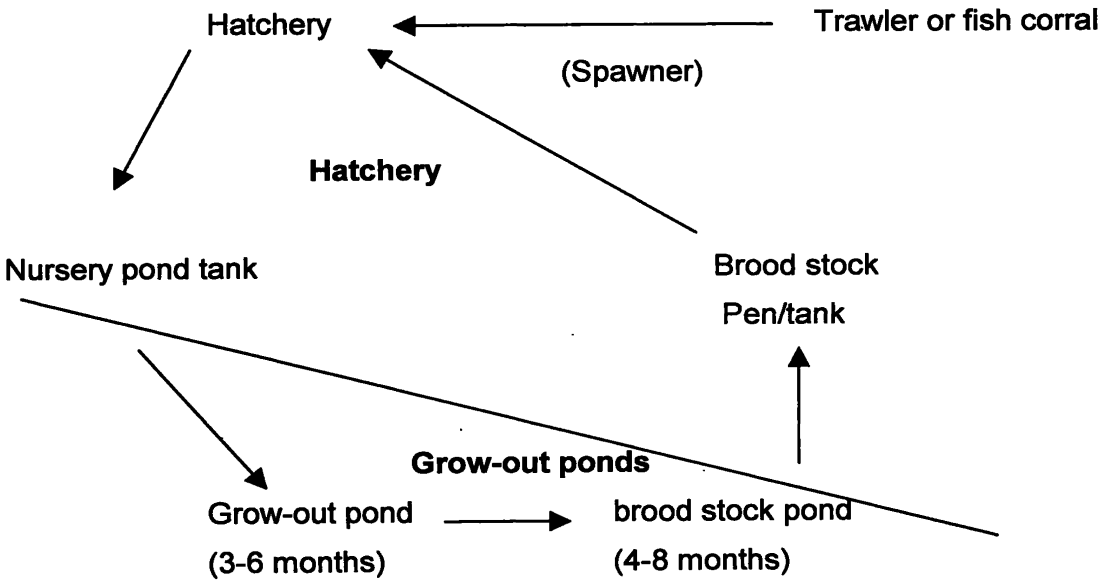


Fig. 2.2 Brood production cycle



Grow-out or farming systems for shrimps is classified into four categories

(a) Traditional
(b) Extensive
(c) Semi-intensive
(d) Intensive

2.2 Disease associated with shrimp

Vibriosis and Related Bacterial Diseases

For nearly as long as penaeid shrimp have been cultured, reports of infections and disease caused by *Vibrio* spp. have been by far the most numerous of the reported bacterial agents of penaeid shrimp. Gram-negative Bacteria are predominant bacteria in marine environments and usually constitute the majority of bacteria present in the normal microflora of cultured and wild penaeid shrimp. *Vibrio* spp. was found to constitute the majority of culturable bacteria associated with the gut, gills or cuticle of wild or cultured penaeid shrimp by a number of researchers. Virtually all if not all of the reported species of bacterial pathogens of penaeid shrimp (especially the vibrios) have also been reported to be part of their normal microflora (James, 1993)

Because of the relatively large numbers of *Vibrio* spp. normally present in the shrimp's microflora, it is not surprising that many investigators have found species of *Vibrio* to be frequent and apparently opportunistic pathogens of penaeid shrimp. This opportunistic pathogenic *Vibrio* spp. apparently establish lethal infections as a result of other primary conditions that might include other infectious disease, nutritional disease, extreme environmental stress, wound, etc. Many attempts to complete Koch's postulates with *Vibrio* spp. isolates from diseased shrimp have been unsuccessful, or successful only after challenge with a relatively massive inoculum, further supporting the argument that many bacterial infections (due most often to *Vibrio* spp.) are due to infections by opportunistic pathogens that are part of their host's normal microflora. Despite the apparent historically opportunistic nature of most *Vibrio* pathogens of penaeid shrimp, some more recently occurring disease syndromes of penaeid shrimp have been caused by *Vibrio* spp., which behave more like true pathogens than opportunistic invaders.

Most bacterial isolates from diseased penaeid shrimp have been *Vibrio* species, usually

V. alginolyticus	*V. splendidus*
V. parahaemolyticus	*V.vulnificus*
V.harveyi	*V.damsela*

Other Gram negative rods include including,

Flavobacterium spp.

Pseudomonas spp.

Aeromonas spp.

As well as Gram positive bacteria,

Mycobacterium marinum

And occasional *micrococcus* may be involved in bacterial disease syndromes in penaeid shrimp. Most of these organisms are part of the normal microflora of these animals.

Bacterial infections in shrimp may take three forms:

(1) Localized pits in the cuticle, which constitute the condition called bacterial shell disease

(2) Localized infections of the gut or hepatopancreas or localized infections from puncture wounds, limb loss, etc.

(3) Generalized septicemias.

All life stages of the penaeid shrimp may be affected (James, 1993).

Fig. 2.3 Shrimp with bacterial shell disease

Among pathogen *Vibrio spp.* Especially the luminous *V.harveyi* have been implicated as the main bacterial pathogens of shrimps. Antibiotics have been used in attempts to control these bacteria, but their efficiency is now, in general, very poor. In the Philippines, luminous Vibrio disease caused a major loss in shrimp production in 1996, and many farms have ceased to produce shrimp because survival was so poor. The vibrio spp. were resistant to every antibiotic used, including Chloremphenicol, Furazolidone, Oxytetracycline, and streptomycin and were more virulent tan in previous years.

Chlorine is widely used in hatcheries and ponds to kill zooplankton before stocking shrimp. But its use stimulates the development of multiple antibiotic resistance genes in bacteria. There is a rapid increase in *Vibrio harveyi* numbers after the chlorine is removed. Chlorine treatment will lower the numbers of competitors for nutrients and kill algae, thus increasing food resources. It is likely,

therefore, that the vibrios surviving after chlorine treatment are not only more resistance to antibiotic, but also pathogenic. (David, 1999).

2.3 Uses of antibiotics in shrimp culture

Antibiotics are used to treat bacterial infections and not for other reason. It cannot be used in treatment of viral diseases such as white spot or yellow head diseases. Antibiotics are used prophylactically, therapeutically or as a preservative.

The ability of broad-spectrum antibiotics to retard bacterial food spoilage was first observed with fish. This discovery was the culmination of a long search by Tarr and his associates for chemical agents to improve the keeping qualities of fish. Many of the bacteria, which cause fish spoilage, are psychrophillic or cold loving and therefore proliferate at refrigerated temperatures. Consequently, icing or refrigerated storage is only moderately effective in preventing spoilage.

Antibiotic may be applied to fish by spraying or dipping. In relation to food preservation the antibiotic can be considered as a special group in the category of chemical preservatives. The activity of the antibiotics is of a new order, 100-1000 times greater than that of the conventional chemical preservatives. But the results obtained with shrimp have been disappointing. (Wrenshall, 1959)

Also antibiotic is used as an antimicrobial agent with feeds for shrimps, pigs, poultry and for rabbit.

When use prophylactically and therapeutically antibiotics introduce to shrimps with feeds in intensive and semi intensive farming e.g. Oxytetracycline, Oxolinic acid, Chloremphenicol, Furazolidone (Vermeulen, 2003).

2.4 Furazolidone

2.4 .1 Physical characteristics of Furazolidone

It is a yellow crystalline odourless powder. Furazolidone is very slightly soluble in water, in alcohol and in chloroform; practically insoluble in Ether. It is a light sensitive compound. (The pharmaceutical CODEX, 1979)

Molecular weight :- 225.16 g/mole

Melting point :- 259^{0}C

Storage temperature :- room temperature

2.4.2 Chemistry of Furazolidone

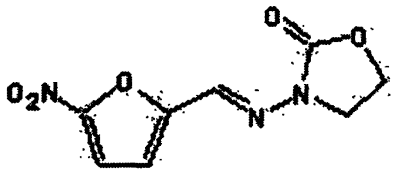
Furazolidone is a synthetic Nitrofuran.

(a)IUPAC nomenclature.

3-(5-Nitrofurfurylideneamino)oxazolidine-2-one

(b)Structural formula

Fig.2.4 Structural formula



Furazolidon

(c)Formula

$C_8H_7N_3O_5$

(d)Other names

Coryzium; Furoxone, Neftin, Furaphen (with chloremphenicol); Bifuran (with nitrofurazone)

2.4.3 Identification of Furazolidone

Test – Dissolve about 1mg in 1ml of Dimethylformamide and add 1 drop of 1N alcoholic potassium hydroxide; a deep blue color is produced (The pharmaceutical CODEX, 1979)

2.4.4 Metabolism of Furazolidone

In treated animals the drug is rapidly metabolized and residues of the parent compound cannot be detected in edible products normally. However, part of the metabolites of Furazolidone bind to tissue proteins and as a result has a very long half-life. In the case of Furazolidone, it was hypothesized that 3-amino-2-oxazolidinone (AOZ) the side chain of the drug, might be released from such protein bound residues under the acidic conditions in stomach (Hoogenboom, 2002).

The distribution, excretion and biotransformation of radiolabelled Furazolidone were studied in rats, pigs and humans. According to results of these studies, there were several observations.

(a) Liver metabolizes high amount of Furazolidone and highest levels being found in liver, kidney, fat and muscle.

(b) Furazolidone was rapidly metabolized and excreted predominantly in urine. In chicken and human urine only trace amounts of unchanged Furazolidone could be detected.

(c) On the basis of the positive effects of Furazolidone in genotoxicity test in vitro and the increased incidence of malignant tumours in mice and rats and concluded that Furazolidone was a genotoxic carcinogen.

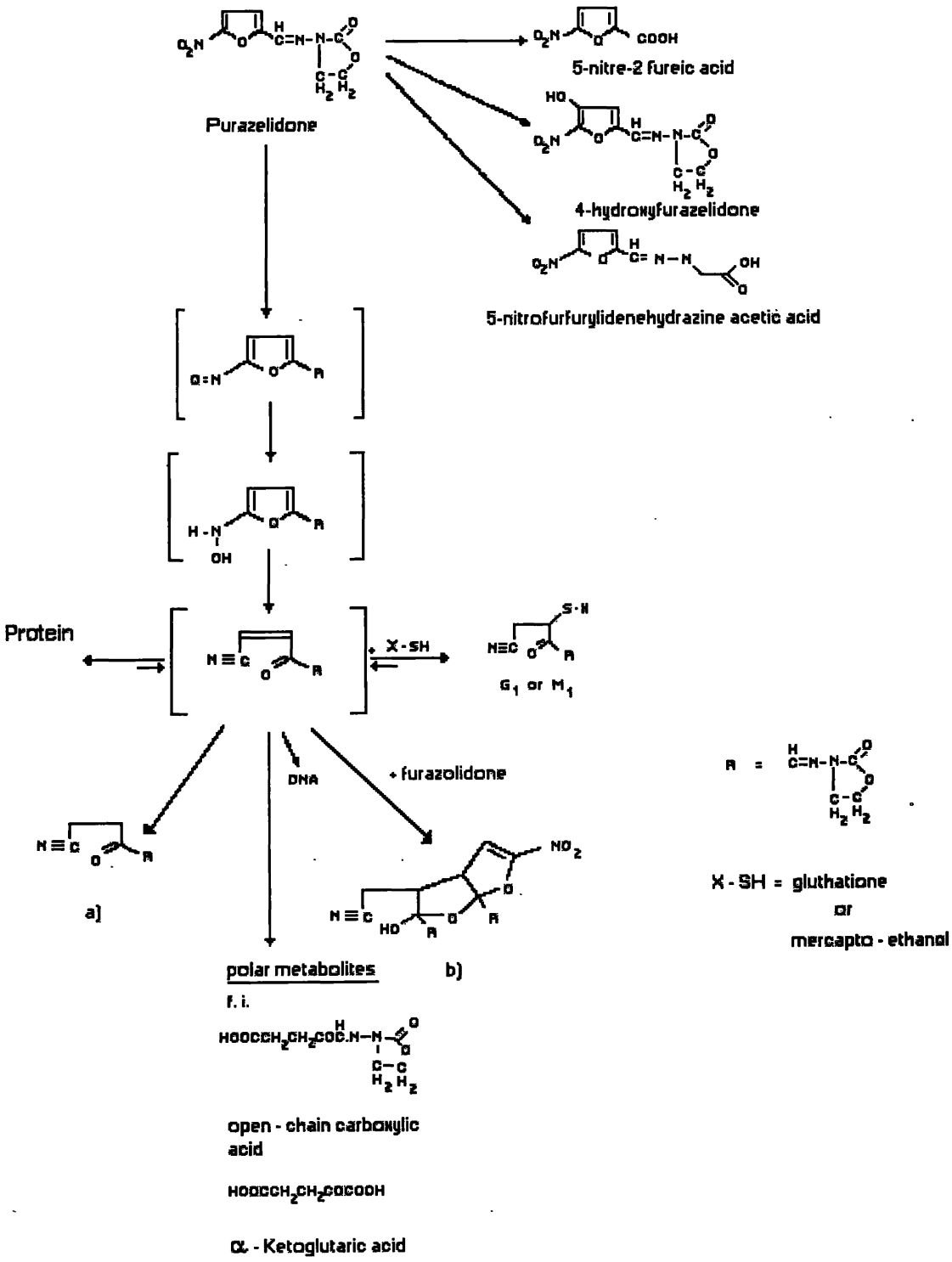
(d) Biotransformation

One of the major metabolites of the incubation mixture was identified as 2,3-dihydro-3-cyanomethyl-2-hydroxy-5-nitro-1alpha,2-di(2-oxo-oxazolidin-3-yl)iminomethyl-furo [2,3-ß]furan. In addition N-(5-amino-2-furfuryliden)-3-amino- 2-oxazolidone was identified as a minor metabolite.

Metabolism was studied with ^{14}C-furazolidone (methylene-labelled) in swine liver microsomes under aerobic and anaerobic conditions (Fig.2.5).

3-(4-cyano-2-oxobutyli-deneamino)-2-oxazolidone and 2,3-dihydro-3-cyanomethyl-2-hydroxyl-5-nitro-1alpha,2-di(2-oxo-oxazolidin-3-yl)iminomethyl furo[2,3ß]furan were the major ethylacetate extractable metabolites, formed via the open-chain acrylonitril-derivative of furazolidone. Another metabolite formed by microsomes in the presence of mercaptoethanol was identified as a mercaptoethanol conjugate M_1-(3-(4-cyano-3-ß-hydroxyethyl-mercapto-2-oxobutylideneamino)-2-oxazolidone). (Vermeulen, 2003).

Figure 1. Proposed metabolic pathway of furazolidone by swine liver microsomes (in vitro, in vivo)



a = 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone.

b = 2,3 - dihydro-3cyanomethyl-2-hydroxy-5-nitro-1 α ,2-di(2-oxo-oxazolidin-3-yl) iminomethyl-furo[2,3-β]furan

2.4.5 Antibiotic activity of Furazolidone

2.4.5.1 Functions.

It is well known that antibiotics are commonly used in shrimp farming to prevent or treat disease outbreaks. (Holmstrom et al., 2003) Other than that it is used as an antimicrobial agents in poultry, pigs, rabbit and fish. Nitrofuran drugs are widely used as inexpensive and effective medicines. Furazolidone is a bactericide, which is absorbed only slightly from intestinal mucosa. As well as it has a fungicidal effect. Furazolidone is used as a therapeutic drug in human diseases. (The pharmaceutical CODEX, 1979).

2.4.5.2 Disease causing activity

Toxicity of Furazolidone when use as a therapeutic agent in human medicine.

Mild toxic symptoms, including headache, nausea and vomiting may occur; vesicular or morbilliform rashes occur with high dosage but usually subside on reduction of the dose. High doses of Furazolidone may cause haemolysis in genetic groups with glucose-6-phosphate-dehydrogenase deficiency conditions, which is prevalent among dark-skinned people (The pharmaceutical CODEX, 1979)

The use of antimicrobial agents in the aquatic environment would cause concern, in terms of both potentials environmental impacts and potential human health implications (Graslund, 2002).

Resistivity of humans and pathogens to furazolidone

Antibiotics in aquaculture can cause the risk of development of resistant bacteria ("Stubborn bacteria"). This can occur among bacteria infecting shrimp, bacteria infecting humans, bacteria. When bacteria have acquired resistance, it is no longer possible to get rid of them with the antibiotic that caused the resistance. Furthermore, some antibiotics can cause bacteria to develop resistance not only to that specific antibiotic, but also to other, different antibiotics. Additionally, resistance-encoding elements can be transferred from one bacteria species to another. These way bacteria can indirectly become resistant to an antibiotic without being directly exposed to it (Graslund, 2002).

Carcinogenicity & Mutagenicity

The metabolites of the nitrofurans exhibit mutagenic potential. High doses of Furazolidone may cause haemolysis in genetic groups with glucose-6-phosphate-dehydrogenase deficiency, a condition that is prevalent among dark-skinned people. (The pharmaceutical CODEX, 1979).

A carcinogenicity study was conducted in Swiss MBR/ICR mice, which received a diet containing concentration of Furazolidone equal to average daily doses of 12, 24, 47mg/kg bw/day for 13 months, followed by a controlled diet for 10 months. In the mid-and high-dose groups, a significant increase in the incidence of bronchial adenocarcinomas was observed in both sexes, and the incidence of lymphosarcomas was significantly increased in male mice (Vermeulen, 2003).

Environmental impact

Many of the antibiotics that are used in shrimp farming are quite persistent in the environment and can spread to surrounding water ways with the outlet water or sludge flushing or removal. In the surrounding environment they can change the ecosystems by changing the normal composition of bacteria, and also have acute toxic effects on aquatic animals and plant. They can also be taken up by organisms, for example mussels, which are collected and eaten by locals. Therefore, antibiotic residues in food is not only a threat to shrimp consumers in importing countries, but also a threat to people living in shrimp farming areas (Graslund, 2002).

2.5 Analysis of Furazolidone

Background

Furazolidone is a Nitrofuran. Not only Furazolidone but also Furaltadone, nitrofurazone and Nitrofurantoin are the Nitrofuran. Furazolidone and occasionally also Furaltadone and Nitrofurazone have been detected in levels ranging between 0.5 and 88 μg/kg in shrimps from Thailand, Vietnam, China, Indonesia, India and Bangladesh.

Legal principles

Parameter assessment

Furaltadone	1μg/kg (including metabolite)
Furazolidone	1μg/kg (including metabolite AOZ)
Nitrofurantoin	1μg/kg (including metabolite)
Nitrofurazone	1μg/kg (including metabolite)

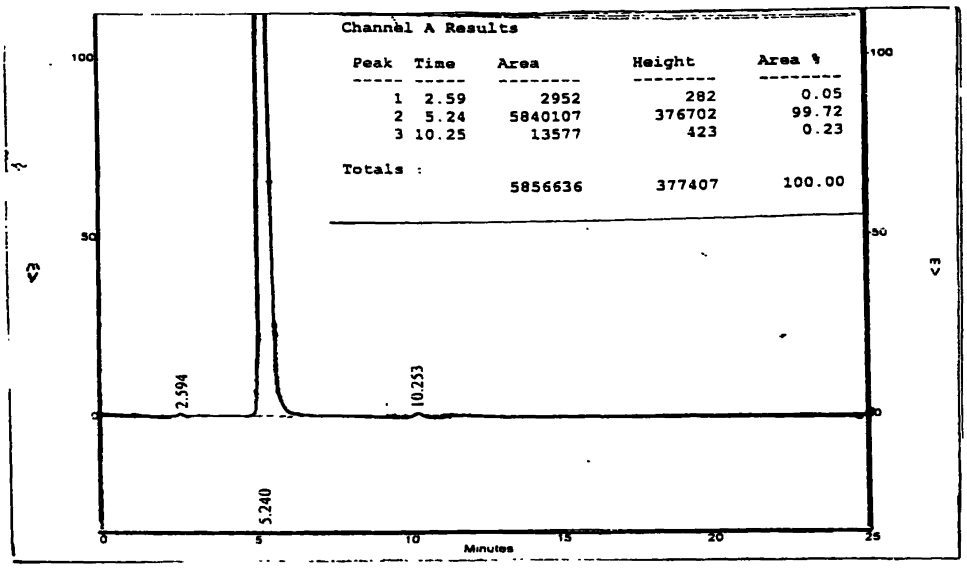
Metabolites are bound or unbound forms; calculated as active substance.

(Federal Office of Public Health, 2002)

2.5.1 Analysis of Furazolidone using HPLC

High Performance Liquid Chromatography is a technique that a solvent (mobile phase) is passed through a sorbent (stationary phase) to effect the separation of the Furazolidone from other component. It differs in that the high efficiency sorbent is packed in to a column and the solvent is pushed through with a high-pressure pump. In normal phase HPLC columns are packed with silica gel phase are composed primarily of low polarity solvents. In reverse phase HPLC, columns are packed with silica to which has been bound a particular functionality (eg., C8, C18, phenyl, cyano), which produces a relatively non-polar stationary phase. Mobile phase are quite polar, normally consisting of combination water, methanol, Acetonitrile, etc. After Furazolidone elute from the column, they immediately pass in to a detector, which usually measures the absorbance (UV detection) of the Furazolidone. This can be compared with similar measurement of known concentrations of standards to produce accurate quantitation (Udayakumara , 2000).

Fig.2.6 Chromatogram of Furazolidone Standard



Separation is controlled by several factors.

Among these characteristics, characters of solvent are important factors.

These are Polarity index (k') and separation factor (α) of solvent.

For rapid analysis low k' is desired, where as for good separation, a high k' is needed. The compromise is a k' value of 2-6.

The separation factor is a ratio of the net retention time (equilibrium distribution coefficient) for any two components. If α is 1, then the separation between two component is zero.

K' and α are controlled by the solvent.

In Furazolidone analysis Acetonitrile is used as a solvent.

Acetonotrile,

K' = 6.2

α = 6

Dipole moment = 0.41

Viscosity = 0.37

(Pomeranz et al., 1996)

Three basic steps of Analysis of Furazolidone; Extraction, Filtration and cleanup, Analysis

(a) Extraction

Typical extraction solvent is Acetonitrile.

Formula: - C_2H_3N (CH_3CN)

Physical properties of Acetonitrile.

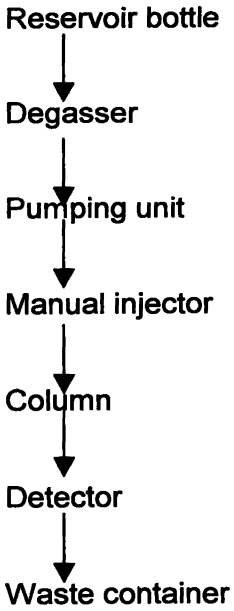
Acetonitrile is an organic solvent
Melting point: - 82^{0}C
Molecular weight: - 41g
Density (at 20^{0}C): - 0.78 g/ml
Solubility in water: - a (g/100g)

(b) Filtration and cleanup

Filter through two filter papers to get a filtrate and cleanup procedure is done through Cep-pak cartridges purify the filtrate. Purity is a critical factor for the HPLC determination of any samples. Ensure that there are no suspended or undissolved particles in the samples before it is injected on to the column.

(c) Analysis

Parts of HPLC and solvent flow of HPLC



Reservoir bottle

Mobile phase is drawn out of the reservoir bottle and pumped through the tubing by the pumping unit.

Degasser

Removes dissolved air from the mobile phase preventing air bubbles and consequent rise-drift or other baseline irregularity caused by dissolved air.

Pumping unit.

Pumps the mobile phase through the manual injector, column and detector, in that order, and finally in to the waste container.

Manual injector

Samples are injected in to the system via manual injector, with a micro syringe (25ul).

Column

It is a reverse phase column. In the column the components are separated by means of the mutual interactions of the mobile phase and the packing of the column.

Detector

The detector detects the components eluted from the column and output the signal data to a chromatopac or personal computer.

Waste container

Mobile phase from the detector drains in to the waste container.

2.5.2 Detection limit of HPLC.

Detection limit indicates the performance of an instrument at low analyte concentration. This indication may be used as a guide to instrument optimization, as a gauge of the suitability of an instrument for a particular application or a criterion for the interpretation of low concentration measurement (Lloyd, 1988).

2.6 Other methods for analysis of Furazolidone

-Enzyme-linked Immunosorbent Assay (ELISA).

-HPLC/MS(High Performance Liquid Chromatography / Mass Spectrometry) and LC/MS methods.

ELISA test

This is a technique widely used for the detection of an antigen (eg. A drug) by the use of a bio specific antibody raised against the drug and an enzyme chemically linked to the antibody, the antigen or a secondary antibody. ELISA method is low cost, rapid and can screen large number of samples. Positive samples are then tested by more sophisticated technique such as LC-MS to confirm the presence of a drug.

When tissue-bound residues of the nitrofuran are treated with mild acid, specific side chains are released (for examples, Furazolidone release AOZ). Two approaches will be taken in different laboratories to produce antibodies against the nitrofuran side chains. Chemical derivative of side chains (AOZ) will be produce and conjugated to a protein, which will elicit an immune response, yielding antibodies to AOZ. By varying the site of attachment of the protein to AOZ or by introducing a spacer molecule, antibodies may be produced which are specific for each nitrofuran or generic for 2 or more nitrofurans. The most suitable antibody or antibodies will be incorporated in one or more ELISA tests and sample extraction and clean-up procedures will be developed. (Hoogenboom et al., 2003)

HPLC/MS (High Performance Liquid Chromatography / Mass spectroscopy) and LC/MS

Analysis of nitrofuran metabolites is the only way to ensure detection long after treatment of the animal has occurred. Analysis of the metabolite is a very specialized procedure requiring analysis using a LC/MS. (Hoogenboom et al., 2002).

The metabolites of the four nitrofuran active substances analyzed were extracted from the samples with solvent and converted with 2-nitrobenzaldehyde for the purpose of increasing the sensitivity of the detection process. After a clean-up stage via a solid phase cartridge, the derivatives were identified by means of HPLC/MS with the aid of their full-scan mass spectra and quantified with the aid of their characteristic mass traces (Federal Office of Public Health, 2002).

Chapter 03

3 Materials and Method

3.1 Sample collection

At farm level

Seven shrimp samples were collected from seven ponds among 3 farms. These farms were located Chilaw and Bangadeniya areas.

At market level

Seven shrimp samples were collected. These are three different fresh market products. Three samples were collected from local market. One sample from export market level. Three samples from two hotels and one sample among these three is a Sea shrimp.

All of these fourteen samples were Head-on shrimps. Other than sea shrimp sample other samples were Tiger shrimps (*Peneaus monodon*). Each sample was 150g in weight.

3.2 Furazolidone estimation

3.2.1 Materials

3.2.1.1 Equipment

(a) Blender :- Waring 2 speed commercial blender
(b) Flasks :- Pyrex 50ml , 100ml
(c) Flask shaker :- Gallenkamp flask shaker (manufacturer)
(d) Filter papers :- Whatman No:42
(e) Funnels
(f) Cep-pak catridge :- ALLTECH silica
(g) Cyringer :- 25ml (TERUMO cor. TORKYO,JAPAN)
(h) Measuring cylinders :- SILBER Brand 50ml , 1000ml
(i) Aluminium foil
(j) Vials
(k) Spatula
(l) Analytical microbalance

Instruments

(a) High Performance Liquid Chromatograph (HPLC) :- SCL-10A vp system controller , SPD-10A vp UV-visible detector (wave length 365nm) HPLC column- LC18 Octadecane silicone (25cm x 4.6mm, 5um)

(b) Instrument software :- Class vp SCL-10A vp ver.5.30

(c) Microfilter :- Nylon 66 membrane , 0.45umx47mm (Supelco 5-8063)

(d) Vaccume pump :- Model 13156 Pressure/vaccume pump(Gelman little Giant -Gelman sciences)

3.2.1.2 Reagents

(a) Solvents :- Acetonitrile

(b) Mobile phase :- 0.2% NaH_2PO_4 : Acetonitrile; 70:30

(c) Sodium sulfate :- Anhydrous powder

Standard

(a) Standard Furazolidone (Sigma chemical cor.)

3.2.2 Method

Furazolidone was analyzed by developing a extraction method, following procedure as described by, Stehly G.R., Plakas S.M., El saidK.R. (Fig.3.1)

3.2.2.1 Sample preparation

To remove possible contaminations all apparatus use in extraction were rinsed with Acetonotrile and all glassware use in extraction was covered with Aluminium foil.

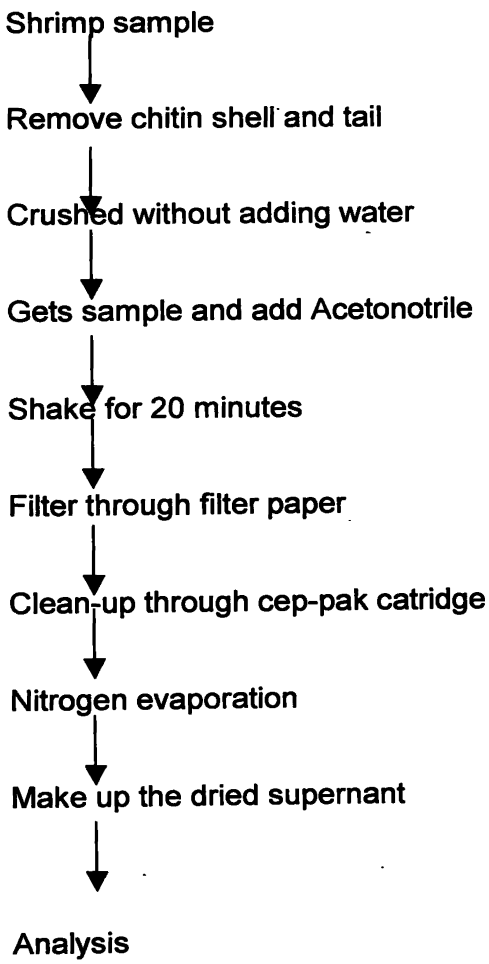
Tail and chitin shell was removed from shrimps and homogenized meat in Waring blender with no additional liquids.

3.2.2.2 Extraction, Clean up & Separation

8.0 g test portion of shrimps was weighed in to 100ml flasks. About 4.0 g of anhydrous Na_2SO_4 and 40ml Acetonitrile were added to flask. Then mixture was standed for about 10 minutes and shaked for 15 minutes with 1 speed in a flask shaker. The mixture was filtered through filter paper. Remainder in a flask was washed with Acetonitrile & filtered. Filtrate was filtered trough cep-pak cartridge to 50ml flask. Then filtrate was evaporated under Nitrogen gas. Precipitate was washed with small amount of Acetonitrile in to vials and again aliquot was evaporated under Nitrogen gas. Remainder was dissolved in Acetonitrile. Acetonitrile was added on a

weight basis until get 1.56 g. (Density of Acetonitrile x volume; 0.78 g/ml x 2ml). Same procedure was followed after cooking the shrimps with about 3g of table salt.

Fig. 3.1 Flow chart for method



Before analysis with HPLC, using following order of solvents column washing was done to remove impurities.

(a) 5% Methanol
(b) Methanol (HPLC grade)
(c) Acetonitrile
(d) Mobile phase

3.2.2.3 Preparation of mobile phase

Mobile phase was prepared according to SIGMA-ALDRICH leaflet supplied with Furazolidone standard. It is the mixture of Acetonotrile and 0.2% NaH_2PO_4 With the 30:70 ratio. 2 g of NaH_2PO_4 powder was dissolved in a 1000ml of distilled water to prepare the 0.2% Buffer solution. 700ml of 0.2% NaH_2PO_4 and 300ml of Acetonotrile were mixed and made mobile phase. Then mobile phase was filtered through Microfilter.

3.2.2.4 Preparation of Standard solution

0.01g of Standard Furazolidone was weighed and dissolved in a 10ml of Acetonitrile to prepare 1000ppm Standard Furazolidone solution.
Then make up 5ppm, 1ppm, 0.1ppm, and 0.05ppm solutions. The solutions are stable for 1 week if protected from direct sunlight and refrigerated when not in use.

3.2.2.5 Recovery test

250g of Shrimp sample was ground and got 4 test portions with 5g weights. 5ppm Standard Fuarzolidone (50µl) was added to three samples and other was a control. Then extraction cleanup and separation were done in a same procedure used in furazolidone residue extraction of samples and analyzed. Analyzed results (detected concentration) were plotted in a graph against real concentration. 50µl of 5ppm.standard was measured and dried with Nitrogen evaporation. Analyzed with HPLC.

3.2.2.6 Analysis

Mobile phase was run through column of HPLC. Extract solution was injected at the volume of 20ul to HPLC through injection port. HPLC was run with mobile phase at the rate of 1.5 ml/min. Furazolidone was monitored at 365nm wavelength by UV visible detector. Standard method was used for quantification on the basis of peak area measurements.

3.2.3 Calculation

Calculations were done on the basis of peak area measurement.
(Appendix 01).

Weights of Furazolidone residues were calculated according to formula mentioned below.

$$\text{Weight of Furazolidone} = \frac{\text{Y ppm x 2ml x wt. of made up solution x1000g}}{\text{Wt. of shrimp sample x 1.56g}}$$

Density of Acetonitrile = 0.78 g/ml

1.56g = wt. of 2ml of Acetonitrile.

Y ppm. = Concentration of Furazolidone in made up solution,

Furazolidone concentrations were calculated using standard curve of Furazolidone. (Appendix 01)

Calculations of Recovery test.

The percentage of loss of Furazolidone

$$\text{Loss percentage of sample} = \frac{\text{Concentration of loss x 100\%}}{\text{Concentration of standard solution}}$$

3.2.4 Statistical analysis.

Test of significance for the analytical study was done using T- test of mean using MINITAB package. (Appendix 03 and Appendix 04)

3.2.5 Find out the detection limit

First machine was washed to remove impurities. Then acetonitrile was injected and noted the peak area, which was shown at the retention time of Standard Furazolidone. This was repeated 7 times and got the lowest peak area and find out its concentration. It was the detection limit.

Chapter 04

4 Results and Discussion

The major objective of the study is to develop the extraction method for Furazolidone residues.

According to that, Furazolidone residues were extracted in to Acetonitrile after following several steps. Due to light sensitivity of Furazolidone, have to careful during extraction. Otherwise loss of drug residue may be high. So it is needed to find out the loss during extraction. Shrimps were spiked with Furazolidone and recovery test was proceeded and results were got. (Table 4.4)

The concentration of Standard Furazolidone solutions, after the HPLC determination is 0.12 ppm.

Add 50μl of 5 ppm = concentration was 0.12 ppm

Table 4.1 Results of recovery test

Sample	**A**	**B**	**C**	**D**
Amount of std. Added (μl)	00	50	50	50
Concentration 0f 2ml(after extraction)(ppm)	0.0	0.095	0.083	0.0964
Loss of Furazolidone (ppm)	00	0.025	0.037	0.0236

According to results (Appendix 02), there is 23.77% loss of furazolidone during extraction method, which was developed. Losses of samples were 20.83%, 30.83% and 19.67%. The concentration of sample, which was not added Furazolidone standard, was zero. Recovery percentage is 76.23%.

The second aim of this study was the determination of the Furazolidone residue level in the shrimps at the market and at the farm.

In the sampling, samples were collected from 3 farms. Because at that days, due to White spot disease, most of farms were closed. That lower number of farms selecting might be affected for the negative results of sample testing at farm level. At the farm level all the shrimp samples were free from Furazolidone residues (Table 4.1). The shrimp feed from one farm was tested for Furazolidone, but it was also free from that antibiotic.

At the market level 6 of the seven samples were contained residues of the Furazolidone in excess of the limit of 1μg/kg, without the metabolite. Those were local market products (Table 4.2). The sample, which was taken from export market level, was free from Furazolidone residues. The two samples got from hotels were exceeded the residue limit. But it is too low amount than other local market products. Then tested, if there were any different in residue level after cooking with table salts. After cooking Furazolidone residues were presented only in four local market products and these also exceeded the residue limit. In other samples were free from residues after cooking.

These results were got according to detection limit of Shimadzu 10A vp HPLC instrument. Detection limit of Shimadzu 10A vp HPLC is 1 ppb (1μg/kg) that is a residue limit, which has laid down by Federal Office of Public Health. Area related to this detection limit is near 50. Residue amount may be lower than that detection limit. But not observe with this instrument.

Table 4.2 Calculated results of sample testing at the farm level (weight of Furazolidone)

Sample	Before cooking (μg/kg)	After cooking (μg/kg)
A	Not detected	Not detected
B	-Do-	-Do-
C	-Do-	-Do-
D	-Do-	-Do-
E	-Do-	-Do-
F	-Do-	-Do-
G	-Do-	-Do-

Table 4.3 Results of sample testing at the market level (weight of Furazolidone)

Sample	Before cooking (μg/kg)	After cooking (μg/kg)
A	Not detected	Not detected
B	1117.27	97.86
C	222.09	73.66
D	155.84	141.28
E	15.37	5.73
F	1.65	Not detected
G	2.38	-Do-

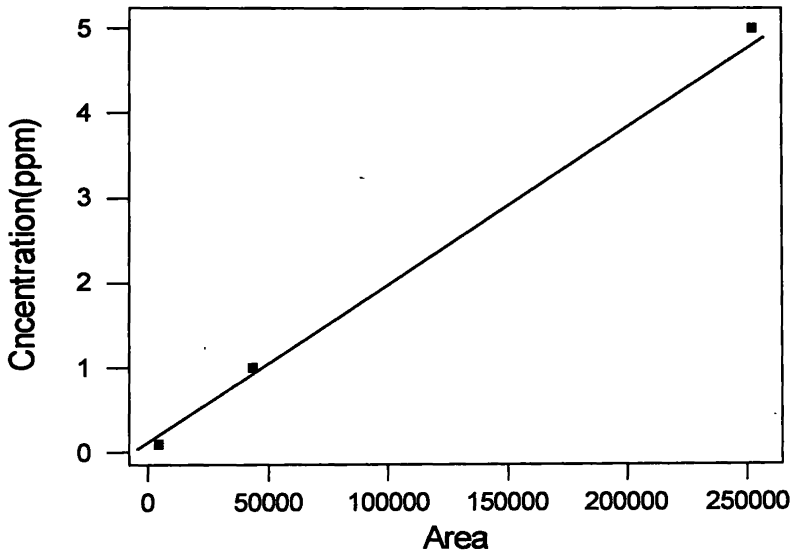
The results were analyzed statistically. According to results statistical analysis the residue level of fresh market samples was exceeded the residue limit (Appendix 03). And the residue level of cooked market samples was also exceeded the residue limit, which has laid down by the Federal Office of Public Health. (Appendix 04) Concentrations of Furazolidone residues in samples were got automatically with Class Vp software in the HPLC.

Concentration of Furazolidone was calculated by using standard curve (Calibration curve) of Furazolidone. Calibration curve was everyday tested with standard Furazolidone before analyze the sample. Standard curve was plotted using three different Furazolidone standard solutions; 0.1 ppm, 1 ppm, 5 ppm (Appendix 03). All of these tests were repeated and results were plotted. (Fig.4.1)

Table 4.4 Resulted peak areas for furazolidone standard

Concentration of Furazolidne standard (ppm)	0.1	1	5
Result (peak area)	4290	43478	252632
Repeated result (peak area)	4596	43538	252680

Fig.4.1 Standard curve for Furazolidone



Chapter 05 Conclusion

5.1 Conclusion

Extraction method was developed according to facilities, which SGS laboratory have. But the recovery is nearly 76.23%. Loss is 23.77%. Losses may be occurred due to use of filter papers, due to light (Furazolidone is light sensitive), during Nitrogen evaporation. So carefulness is more important during extraction.

According to results of statistical analysis in the market samples (both fresh and cooked samples), the level of Furazolidone is exceeded the residue limit (1μg/kg), (Appendix 03 and 04) which The Federal Office of Public Health has laid down. At the farm level do not present the Furazolidone residues.

Considering the results can be concluded that the Furazolidone antibiotic is added during preservation. It is added in the place between the farm and the market. Therefore it is present as a parent drug (Furazolidone). The metabolite also may be present. But the metabolite is not detected with HPLC.

The Furazolidone level was considerably lower in cooked shrimp sample than fresh samples. So can be concluded that the heat and table salt can change the structure of Furazolidone.

The detection limit of HPLC machine is 1ppb. The residue level may be lower than that. But not be detected with this instrument. It is a drawback of this HPLC.

5.2 Recommendations

Should be developed the extraction method to reduce the losses during extraction.

Should be developed the method for extracting and analyzing the Furazolidone metabolite.

Study the effects of other spices on Furazolidone residues and the relationship between the amount of table salt and lowering amount of Furazolidone.

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Appendix 01

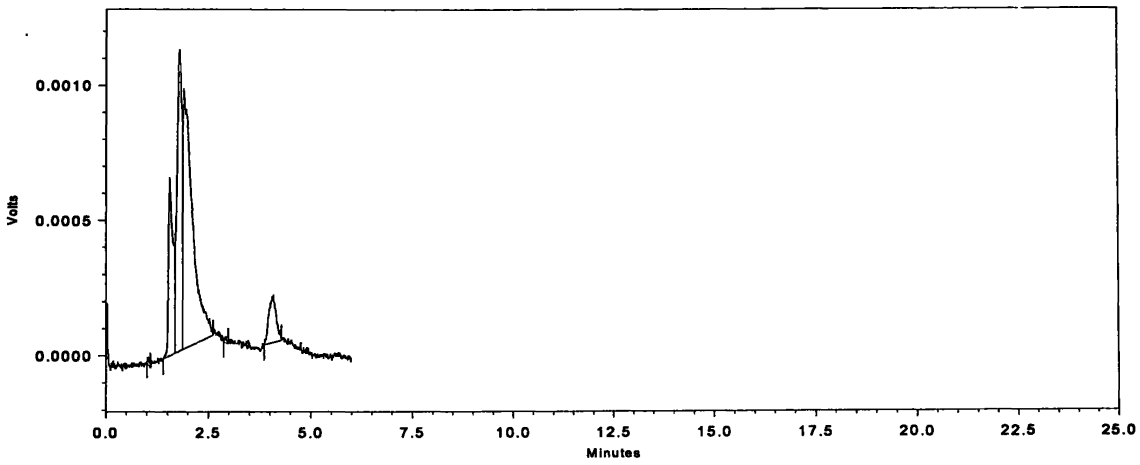
Results of Market sample testing

Sample	Area		Concentration (ppm)	
	Fresh	Cooked	Fresh	Cooked
A	Not detected	Not detected	00	00
B	160433	13340	3.5349	0.2939
C	32060	10655	0.7064	0.23477
D	23129	19814	0.5096	0.4366
E	2148	Not detected	0.04733	0.02315
F	276	-Do-	0.00069	00
G	352	-Do-	0.00776	00

Chromatogram and calculation of Sample testing (Market E-fresh)

UV detector
(365 nm)

Pk #	Retention Time	Area	Units	Start Time	Stop Time
6	4.092	2148	ppm	3.86	4.28
Totals		2148			



A – Peak for Acetonitrile

B – Peak for Furazolidone

Weight of Furazolidone = $\frac{\text{Y ppm x 2ml x wt. of made up solution}}{\text{Wt. of shrimp sample x 1.56g}} \times 1000\text{g}$

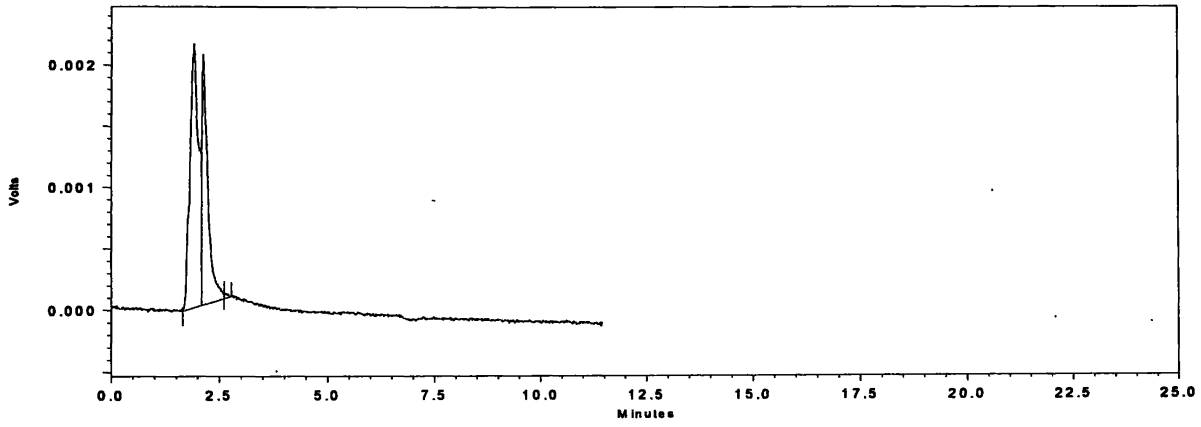
$= \frac{0.04733 \text{ ppm x 2ml x 1.561g}}{8.08\text{g x 1.56g}} \times 1000\text{g}$

= 0.0117228 mg/kg

= 11.72 μg/kg

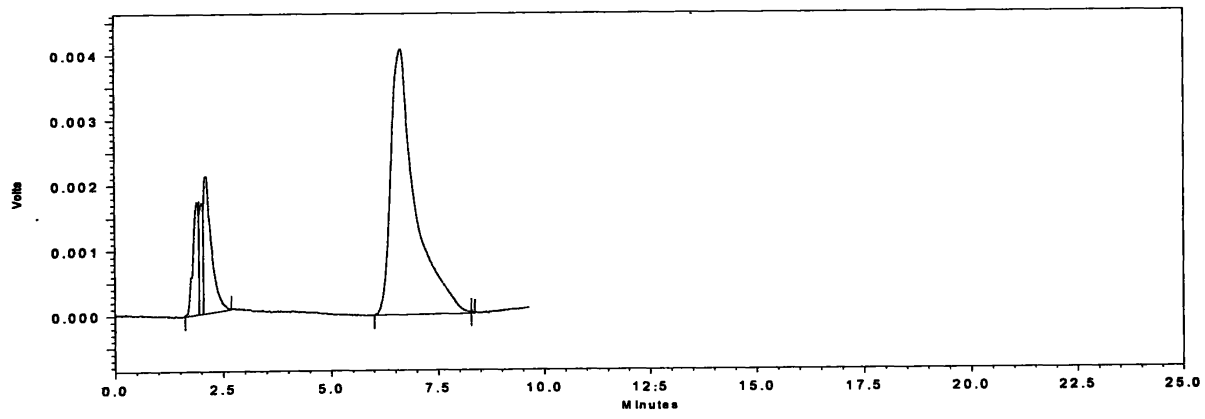
Market-fresh-A

Detector A	(365nm)				
Pk #	Retention Time	Area	Units (ppm)	Start Time	Stop Time
Totals					



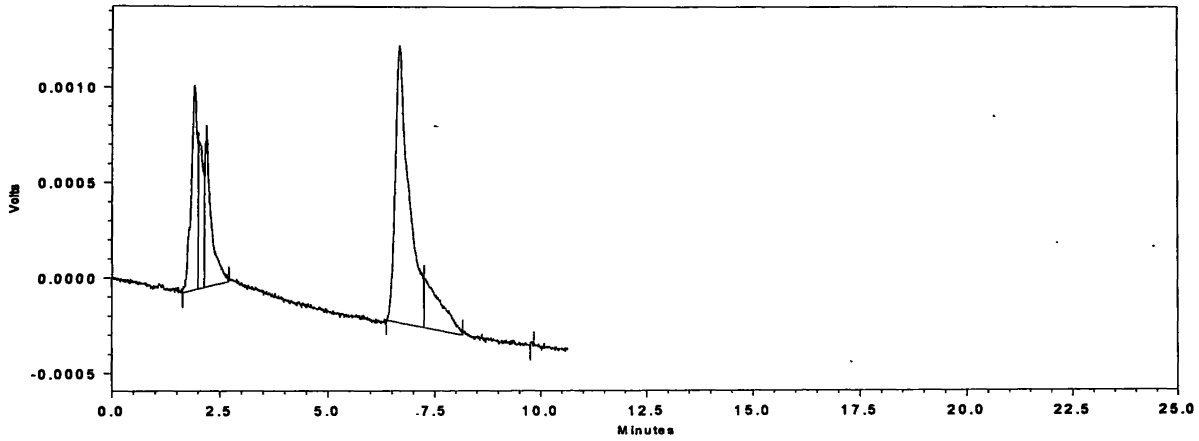
Market-fresh-B

Detector A	(365nm)				
Pk #	Retention Time	Area	Units ppm)	Start Time	Stop Time
4	6.642	160433		6.01	8.28
Totals		160433			



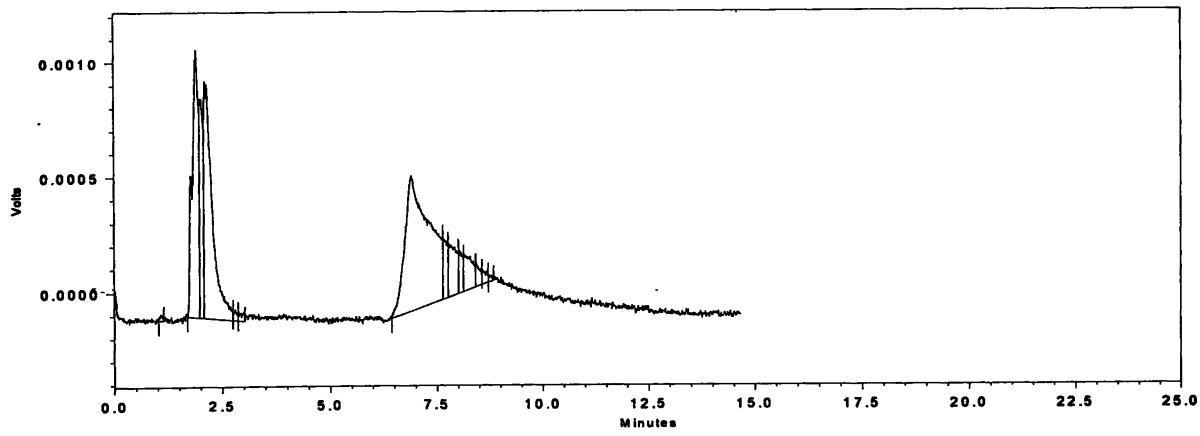
Market-fresh-C

Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
4	6.675	32060	ppm	6.37	7.25
Totals		32060			



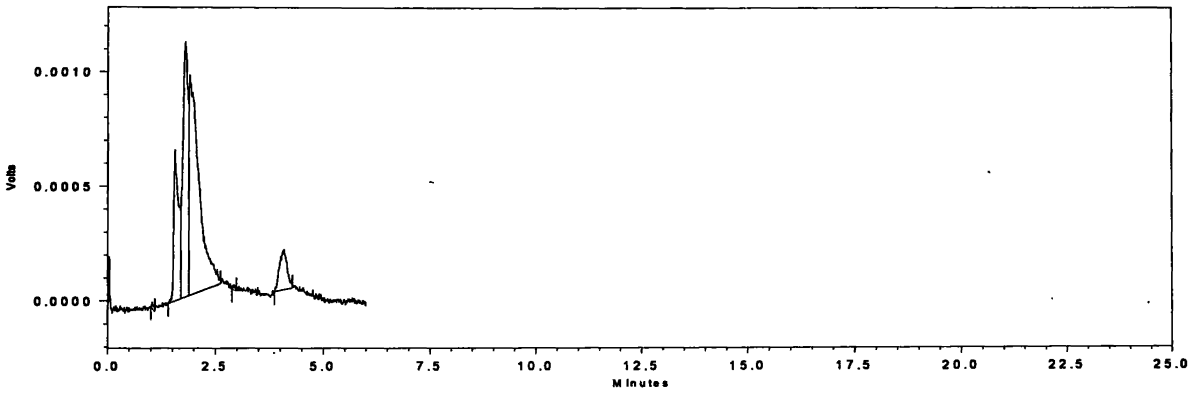
Market-D-fresh

UV-Detector Pk #	(365nm) Retention Time	Area	Units (ppm)	Start Time	Stop Time
7	6.900	23129		6.44	7.64
Totals		23129			



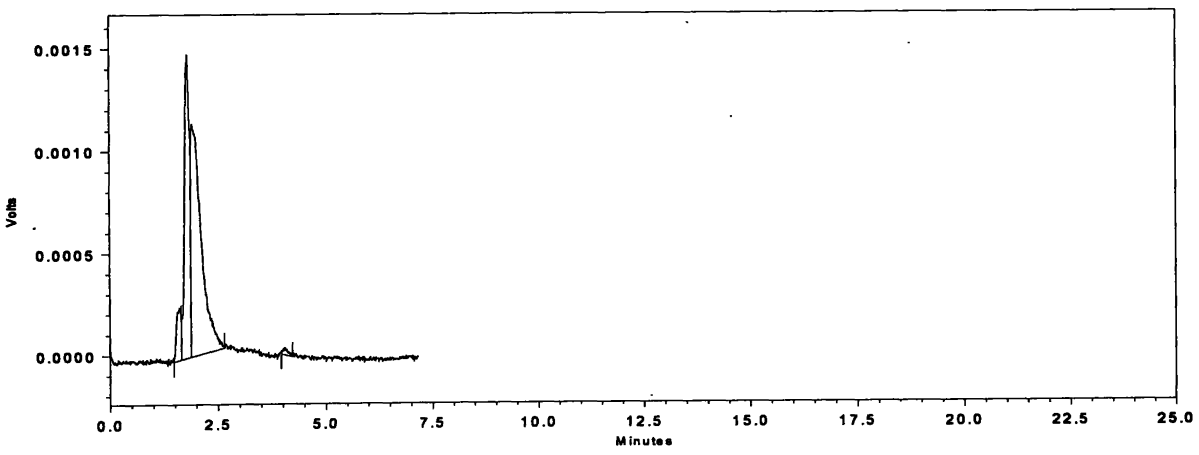
Market-E-fresh

Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
6	4.092	2148	ppm	3.86	4.28
Totals		2148			



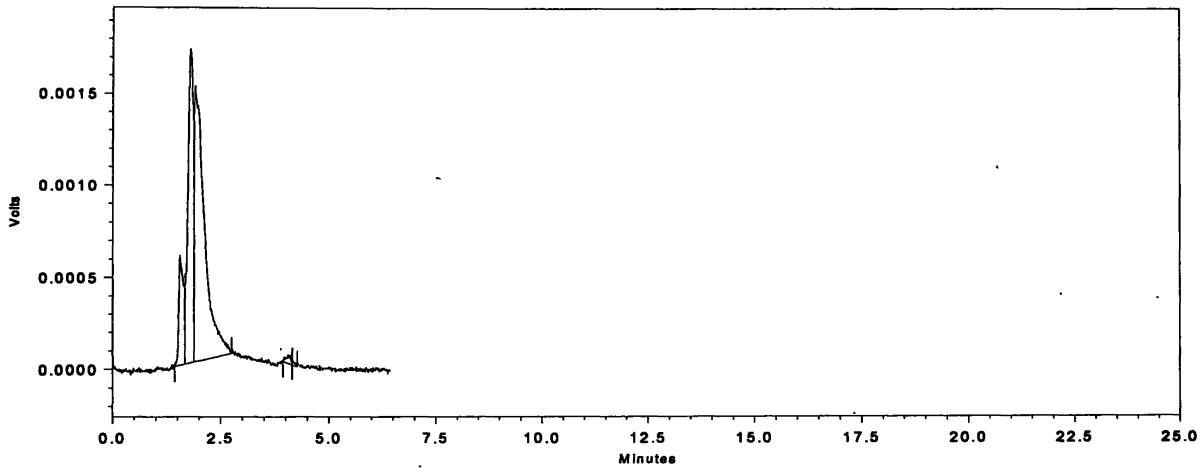
Market-F-fresh

Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
4	4.058	276	ppm	3.96	4.22
Totals		276			



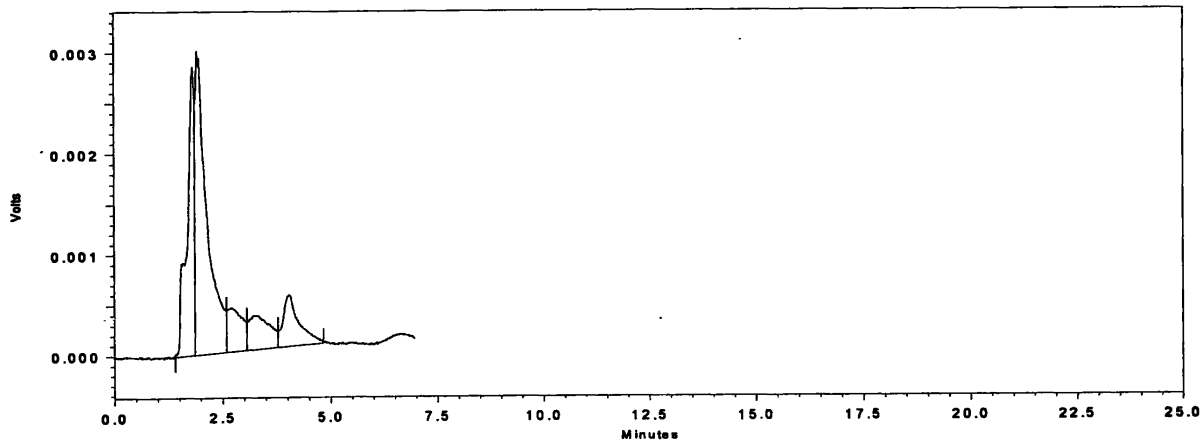
Market-G-fresh

Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
4	4.058	352	ppm	3.93	4.15
Totals		352			



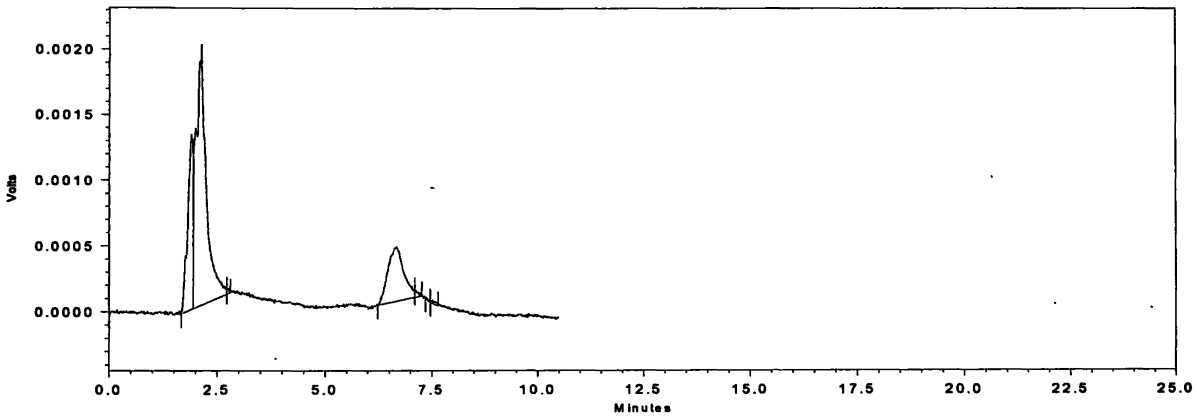
Market-B-cooked

Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
5	4.042	13340	ppm	3.77	4.83
Totals		13340			



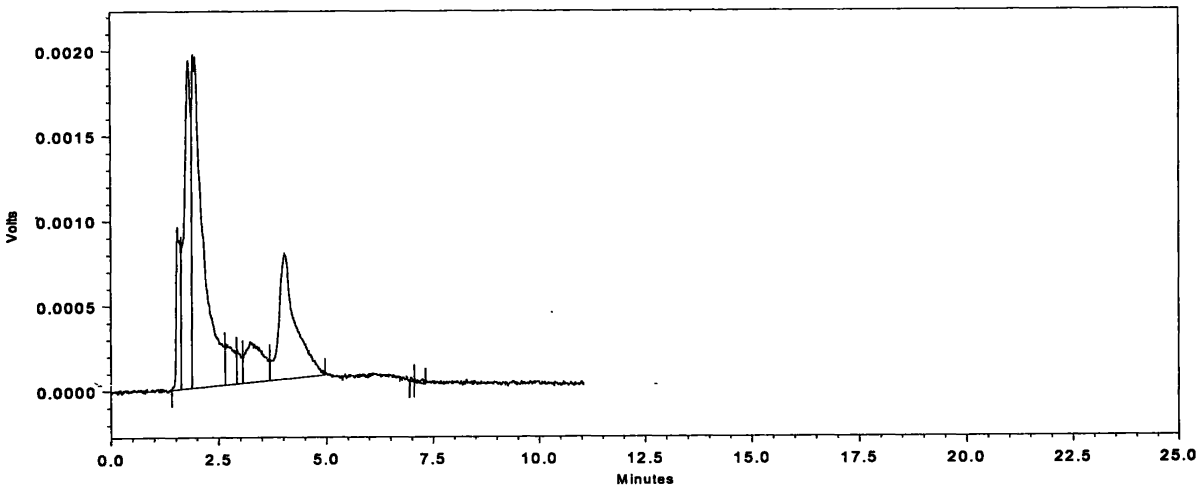
Market-C-cooked

Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
4	6.667	10655		6.23	7.10
Totals		10655			



Market D-cooked

Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
7	4.025	19814	ppm	3.68	4.97
Totals		19814			



Appendix 02

Recovery test and standard curve

Details of Recovery test

Sample	A	B	C	D
Sample weight (g)	5.445	5.417	5.755	5.65
Amount of std. Added (µl)	00	50	50	50
Weight after made up (g)	1.56	1.56	1.56	1.56

The percentage of loss of Furazolidone

Loss percentage of sample B $= \frac{0.025 \text{ ppm}}{0.12 \text{ ppm}} \times 100\%$

$= 20.83\%$

Loss percentage of sample C $= \frac{0.037 \text{ ppm}}{0.12 \text{ ppm}} \times 100\%$

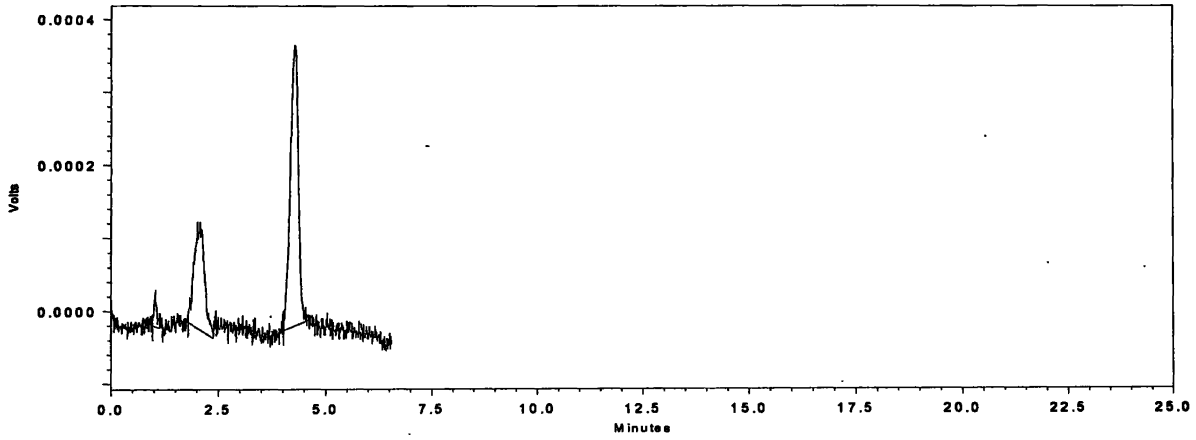
$= 30.83\%$

Loss percentage of sample D $= \frac{0.0236 \text{ ppm}}{0.12 \text{ ppm}} \times 100\%$

$= 19.67\%$

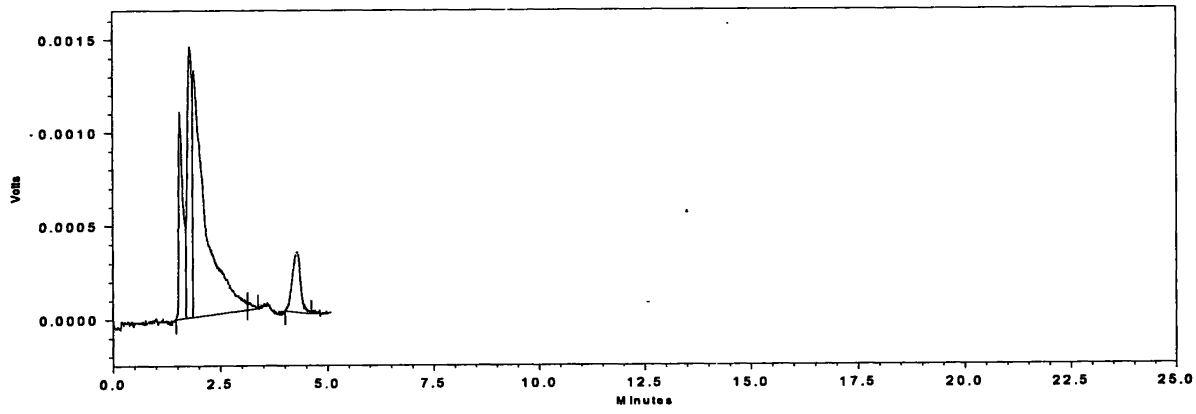
Standard-recovery-5ppm

Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
3	4.275	5007	ppm	4.02	4.53
Totals		5007			



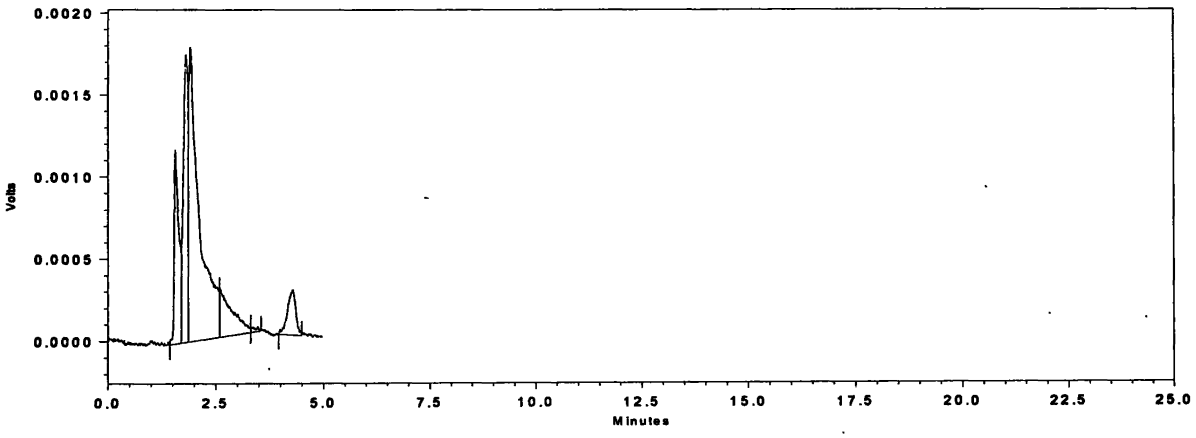
Recovery-B

Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
5	4.283	4302	ppm	4.01	4.62
Totals		4302			



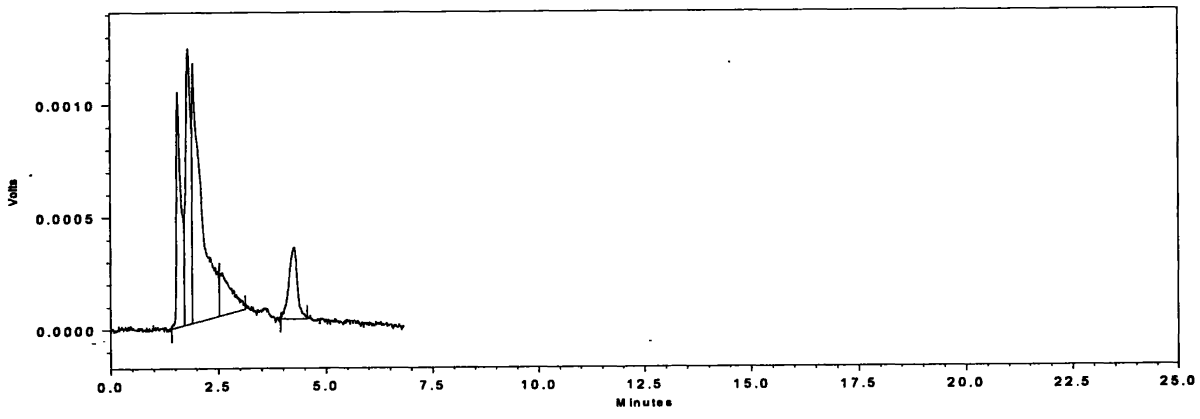
Recovery-C

Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
6	4.292	3683	ppm	3.96	4.49
Totals		3683			



Recovery-D

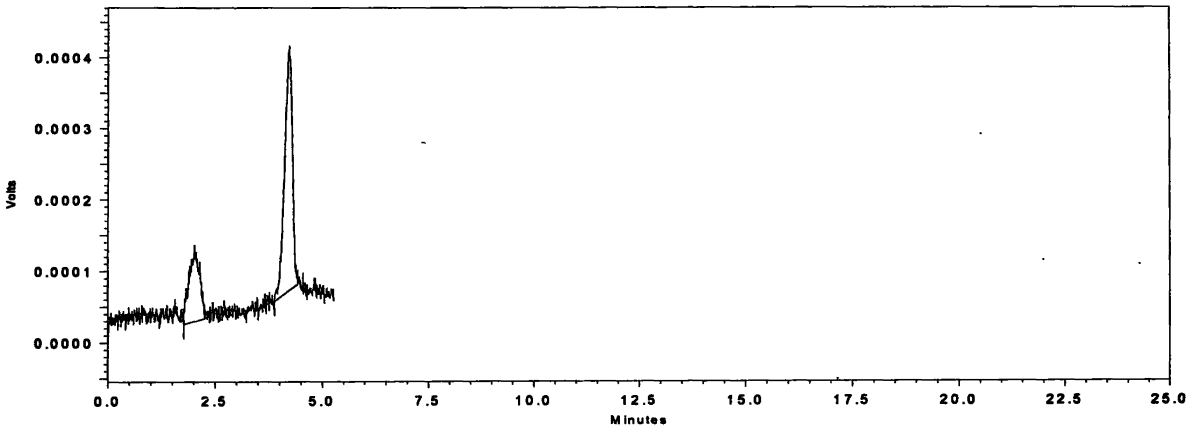
Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
5	4.267	4376	ppm	3.94	4.56
Totals		4376			



Results for standard curve

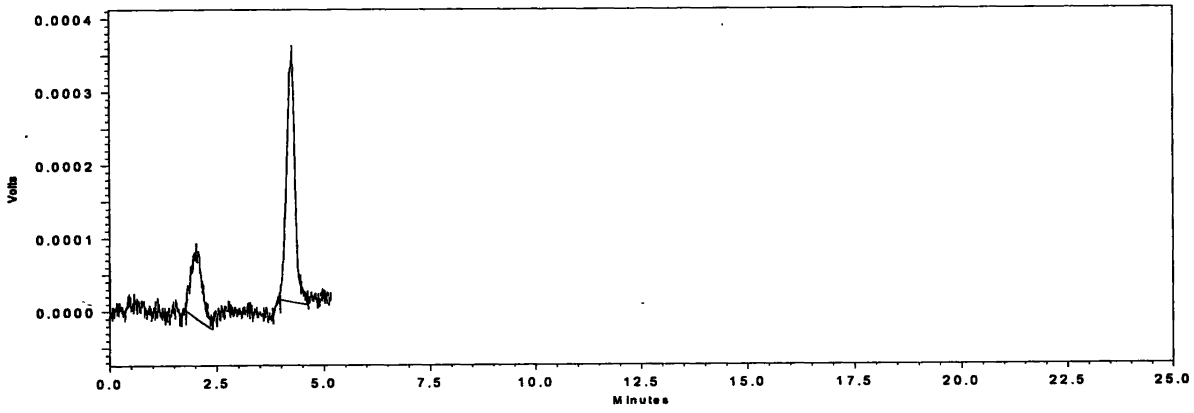
Standard 0.1 ppm

Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
2	4.233	4290	ppm	3.88	4.42
Totals		4290			



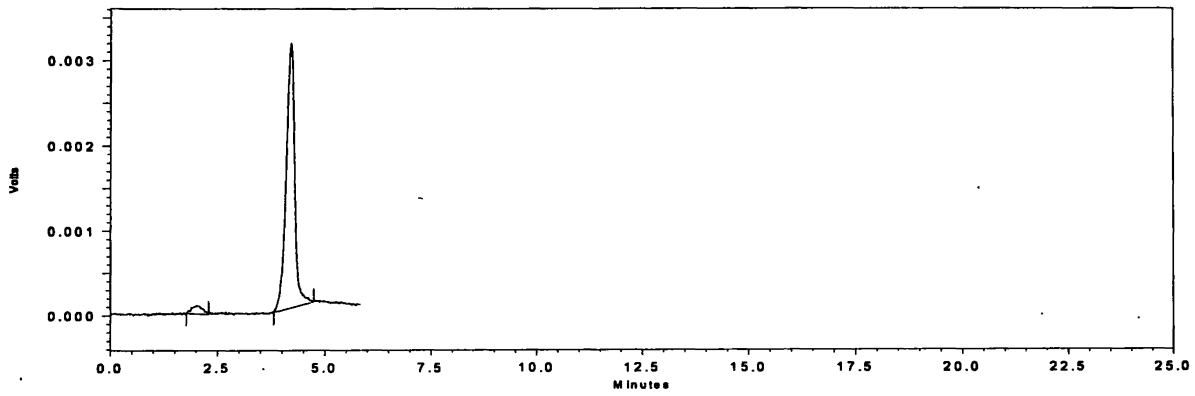
Standard 0.1 ppm

Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
2	4.258	4596	ppm	3.98	4.61
Totals		4596			



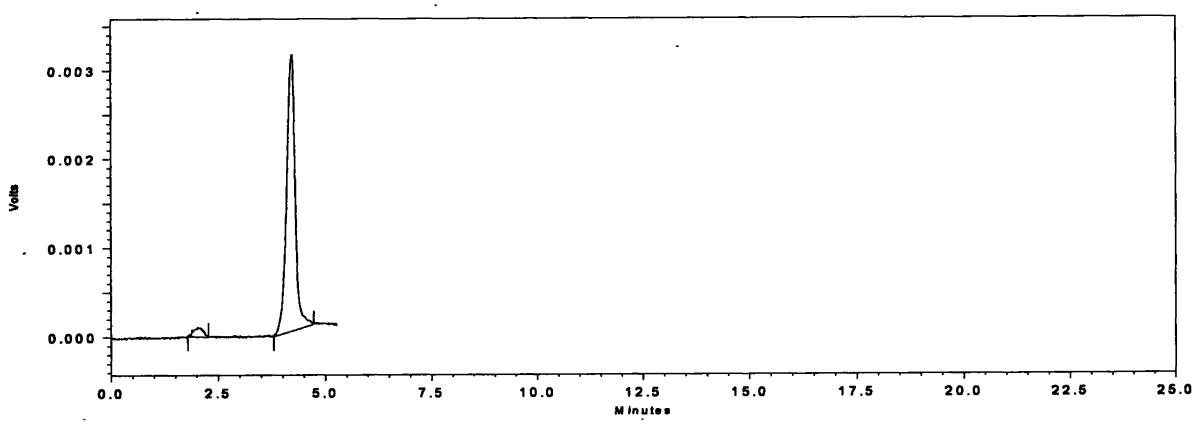
Standard 1ppm

Detector A (365nm) Pk #	Retention Time	Area	Units	Start Time	Stop Time
2	4.217	43478	ppm	3.81	4.74
Totals		43478			



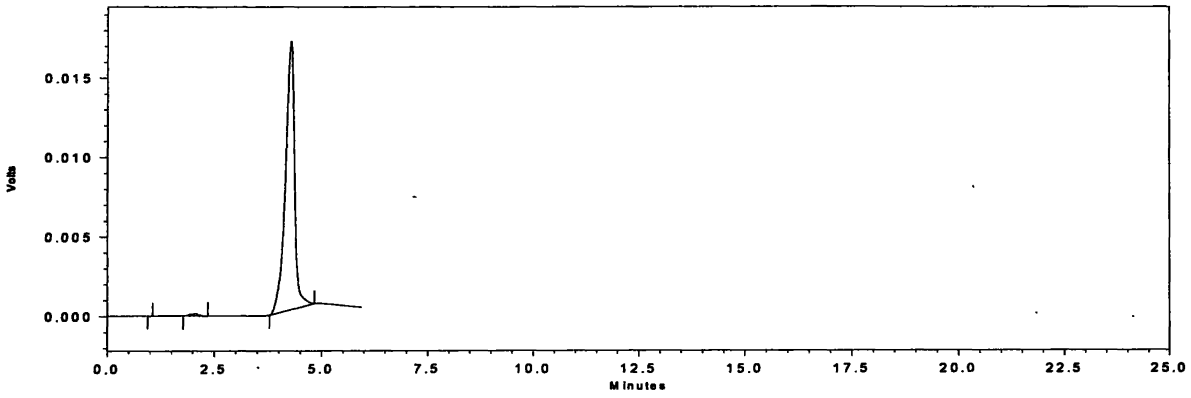
Standard 1ppm

Detector A (365nm) Pk #	Retention Time	Area	Units	Start Time	Stop Time
2	4.217	43538	ppm	3.79	4.72
Totals		43538			



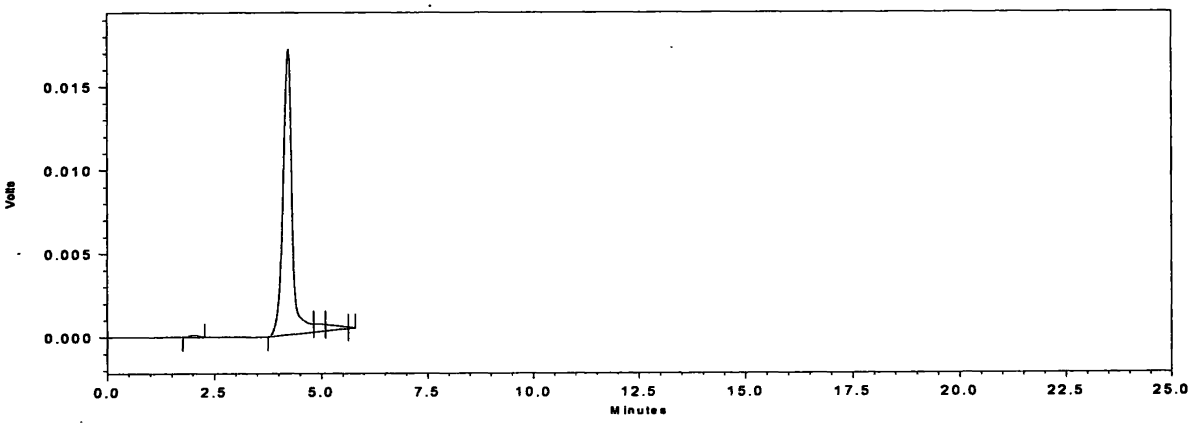
Standard 5ppm

Detector A Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
3	4.283	252632	ppm	3.78	4.83
Totals		252632			



Standard 5ppm

UV Detector Pk #	(365nm) Retention Time	Area	Units	Start Time	Stop Time
2	4.233	252680	ppm	3.75	4.82
Totals		252680			



Appendix 03

One-Sample T-Test of the Mean of fresh samples

Test of mu = 1 vs mu < 1

Variable	N	Mean	StDev	SE Mean
C4	7	216	407	154

Variable	95.0% Upper Bound	T	P
C4	515	1.40	0.894

H_0 : μ = 1 μg/kg

H_1 : μ < 1 μg/kg

Appendix 04

One-Sample T- test of the Mean of cooked samples

Test of mu = 1 vs mu < 1

Variable	N	Mean	StDev	SE Mean
After cooking	7	45.5	58.5	22.1

Variable	95.0% Upper Bound	T	P
After cooking	88.4	2.01	0.955

H_0 : μ = 1 μg/kg

H_1 : μ < 1 μg/kg

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