# MONITORING THE EFFECTIVENESS IN ELIMINATING THE TRACE PRESENCE OF AOZ (FURAZOLIDONE DERIVATIVE) RESIDUE IN *PENAEUS MONODON* AND ITS PRODUCTS UNDERGOING DIFFERENT PROCESSING REGIMES

**TAN THUAN CHEW**

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**by**

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| Abbreviations / Symbols | Caption |
|---|---|
| 2-NPAOZ | Nitrophenyl derivatised AOZ |
| 2-NPAOZ-$d_4$ | Deteurated nitrophenyl derivatised AOZ |
| ADI | Acceptance daily intake |
| AHD | 1-aminohydantoin |
| AMOZ | 5-morpholino-3-amino-2-oxazolidone |
| AOZ | 3-amino-2-oxazolidinone |
| AOZ-$d_4$ | Deteurated 3-amino-2-oxazolidinone |
| APCI | Atmospheric pressure chemical ionisation |
| API | Atmospheric pressure ionisation |
| C2 | Carbon number 2 |
| C5 | Carbon number 5 |
| CC$\alpha$ | Decision limit |
| CC$\beta$ | Detection capability |
| CD | Commission Decision |
| CV | Coefficient of variation |
| DMSO | Dimethyl sulfoxide |
| EC | European Council |
| ESI | Electrospray ionisation |
| EU | European Union |
| FOZ | N-(5-amine-2-furfuryliden)-3-amine-2-oxazolidinone |
| ISD | Standard deviation |
| JECFA | Joint Expert Committee on Food Additives |
| LC-MS | Liquid chromatography mass spectrometry |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| LLE | Liquid-liquid extraction |

| | |
|---|---|
| LOD | Limits of detection |
| LOQ | Limits of quantification |
| MAO | Monoamine oxidase |
| MRL | Maximum residue limit |
| MRPL | Minimum required performance reporting limit |
| NBA | 2-nitrobenzaldehyde |
| ODS | Octadecylsilyl |
| r | Repeatability |
| R | Within-laboratory reproducibility |
| RIC | Reconstructed ion chromatogram |
| RPC | Reversed-phase chromatography |
| RRT | Relative retention time |
| RSD | Relative standard deviation |
| RT | Retention time |
| SA | Analytical limit standard |
| SC | Matrix-match calibrating standard |
| SD | Standard deviation |
| SEM | Semicarbazide |
| S/N | Sound-to-noise ratio |
| SPE | Solid-phase extraction |
| SR | Recovery standard |
| SRM | Selected reaction monitoring |
| SV | Validating standard |
| TIC | Total ion current chromatogram |
| $Tr_1$ | Less intense transition ion |
| $Tr_2$ | More intense transition ion |
| UHQ | Ultra high quality |

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# PEMANTAUAN KEBERKESANAN DALAM PENYINGKIRAN KEHADIRAN SURIH AOZ (DERIVATIF FURAZOLIDON) YANG TERDAPAT DALAM *PENAEUS MONODON* DAN PRODUKNYA MELALUI PROSES YANG BERBEZA-BEZA

## ABSTRAK

Pihak berkuasa Malaysia telah mengharamkan penggunaan furazolidon (salah satu daripada nitrofuran utama yang ada) dalam haiwan ternakan. Hal ini adalah berdasarkan risiko dari segi kesihatan yang boleh memudaratkan orang umum. Penekanan diberi dalam mengesan kehadiran metabolit utama furazolidon, iaitu 3-amino-2-oxazolidinona (AOZ). Metabolit ini adalah lebih stabil dan kekal lebih lama di dalam tisu haiwan berbanding dengan furazolidon. Oleh itu, pengesanan metabolit merupakan kaedah paling sesuai dalam penilaian tentang penyalahgunaan furazolidon. Kaedah pengesahan melibatkan penggunaan pengionan elektrosembur positif kromatografi cecair-spektrometri jisim tandem (ESI LC-MS/MS) untuk menentu paras AOZ dalam udang harimau (*Penaeus monodon*). Sebelum analisis LC-MS/MS dijalankan, sampel akan melalui pelbagai peringkat penyediaan, termasuk penghomogenan, hidrolisis asid metabolit yang terangkai pada protein, dan derivatasasi dengan 2-nitrobenzaldehid (2-NBA). Pembersihan sampel dan penulenan analit dijalankan dengan melibatkan pengekstrakan berganda dengan etil asetat. Pemisahan molekul dijalankan dengan menggunakan kromatografi cecair dengan kolum C18 (Inertsil® ODS-3 50.0 mm x 2.1 mm, 3 µm) pada suhu bilik. Kuantifikasi analit dijalankan berdasarkan garis panduan daripada EU dengan melibatkan pemantauan tindakbalas selektif (SRM) yang melibatkan satu ion prekursor dan dua ion produk sebagai penentu. Kuantifikasi yang lebih jitu dan sensitif diperoleh melalui penggunaan standard terdeturasi (AOZ-$d_4$) sebagai standard dalaman (IS). Kaedah yang digunakan divalidasi berdasarkan garis panduan daripada Commission Decision 2002/657/EC dengan melibatkan penggunaan sampel blank dan standard matrik (ditambah dengan AOZ ataupun 2-NPAOZ, bersama-sama dengan AOZ-$d_4$). Validasi

dijalankan terhadap spesifisiti analit, identifikasi molekular (termasuk RRT, titik pengesahan, nisbah S/N, dan nisbah ion transisi), kelinearan, pengekstrakan perolehan, persisi (termasuk kebolehulangan, dan kebolehulangan intra-makmal), dan had analitikal (termasuk LOD, LOQ, CC$\alpha$, and CC$\beta$) untuk menentukan kesuaian keadah ini. Had analitikal untuk semua matrik udang berada di bawah paras MRPL untuk nitrofuran (1 ng/g). Kurva piawai berada dalam bentuk linear untuk julat kepekatan AOZ dari 0 hingga 2,000 ng/g untuk semua matrik udang. Udang harimau dirawat dengan furazolidon melalui kaedah rawatan mandi dengan kepekatan 30 mg furazolidon/L air garam untuk 10 hari berturut-turut. Residu AOZ dalam otot, kepala dan eksoskeleton udang mencapai paras maksimum, iaitu pada kepekatan 8,671.08 ± 628.80 ng/g, 9820.14 ± 4,15.47 ng/g, and 11,025.12 ± 730.76 ng/g, masing-masing sebaik sahaja selepas rawatan furazolidone. AOZ adalah sangat susah untuk disingkirkan. Oleh demikian, AOZ masih boleh dikesan selepas 2 bulan dari rawatan terakhir. Tiga jenis produk udang dipilih, iaitu udang beku tanpa kulit, urat and kepala, udang masak beku tanpa kulit, urat and kepala, dan udang jeruk. Proses yang terlibat dalam penghasilan produk-produk yang terpilih tidak berjaya dalam menyingkirkan kesemua residu AOZ dalam udang yang dirawat dengan furazolidon. Penyingkiran AOZ yang terbanyak boleh diperoleh melalui proses yang terlibat dalam penyediaan udang masak beku tanpa kulit, urat dan kepala yang melibatkan penggunaan 1 % asid laktik, iaitu sedikit kurang daripada 50 % (termasuk peratusan penyingkiran daripada pra-pemprosesan).

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## ABSTRACT

Due to its risk showered upon public health, Malaysia authorities has prohibited the use of furazolidone (one of the main nitrofurans available), in food-producing animals. Monitoring compliance with the ban has focused on the detection of its main metabolite, 3-amino-2-oxazolidinone (AOZ), which is more stable and persists in the animal tissues with comparison to the parent compound. Thus it's the best approach to evaluate their utilization. A confirmatory method based on positive electrospray ionization liquid chromatography-tandem mass spectrometry (ESI LC-MS/MS) has been utilised for the trace level determination of AOZ in tiger prawns (*Penaeus monodon*). Prior to LC-MS/MS analysis, samples undergo various preparation, which entails homogenisation, acid hydrolysis of the protein-bound metabolites and derivatisation with 2-nitrobenzaldehyde (2-NBA). Sample clean-up and analyte enrichment was performed by a double liquid-liquid extraction with ethyl acetate. Separation of the molecules was performed by liquid chromatography in a C18 column (Inertsil® ODS-3 50.0 mm x 2.1 mm, 3 µm) at room temperature. Analyte quantification was performed according to EU guidelines, using selective reaction monitoring (SRM) with one precursor ion and two product ions as identifiers. A reliable and more sensitive quantitation is obtained by using deuterated standard (AOZ-$d_4$) as internal standard (IS). The "in-house" validation of this method has been performed taking into account the Commission Decision 2002/657/EC. The performance characteristics of the method were established by in-house validation procedures employing assays with sample blanks, matrix-match standards (spiked with AOZ or 2-NPAOZ, with AOZ-$d_4$). Analyte specificity, molecular identification (including RRT, identification points, S/N ratios, and transition ion ratios), linearity, extraction recovery, precision (including repeatability,

and within-laboratory reproducibility), and analytical limits (including LOD, LOQ, CC$\alpha$, and CC$\beta$) were validated. The fitness for purpose of this method was assessed based on its performance characteristics. The analytical limits obtained for all the three type of tiger prawn matrices were below the MRPL for nitrofuran, which is 1 ng/g. Linear standard curves were obtained in the ranges 0–2,000 ng/g for all types of tiger prawn matrices. Results showed that after furazolidone treatment, via bath treatment, of 10 mg furazolidone/L salt water for 10 consecutive days, AOZ residue in tail muscle, head, and exoskeleton reached its maximum, 8,671.08 ± 628.80 ng/g, 9820.14 ± 4,15.47 ng/g, and 11,025.12 ± 730.76 ng/g, respectively, right after stopping treatment. AOZ was very difficult to eliminate in vivo, thus AOZ is still detectable even after 2 month from the last furazolidone treatment. Three types of prawn product were selected, which are frozen peeled, deveined, headless prawn, frozen peeled, deveined, cooked, headless prawn, and marinated prawn. None of the processing methods used were able to eliminate all the AOZ residues in the incurred tiger prawns, with the most reduction, slightly less than 50 %, was observed in the incurred tiger prawns undergoing pre-processing coupled with frozen peeled, deveined headless cooked tiger prawn processing (utilising 1 % lactic acid dipping).

# CHAPTER ONE
# INTRODUCTION and OBJECTIVES

## 1.1 Introduction

Drugs such as antibiotics, antimicrobials, and growth hormones have been developed and used to help veterinarian and the food animal producer to prevent and also cure diseases. It has been administered in relatively large dosages to treat sick animals and in lower dosages to prevent diseases in exposed animals. Bacterial disease of penaeid prawns can be caused by agents such as *Vibrio spp*., *Beneckea sp*., *Flavobacterium spp*., *Aeromonas spp*., Filamentous bacteria (*Leucothrix sp.*). All life stages of penaeid prawn are susceptible to bacterial infection and this is stimulated through the exposure of penaeid prawns to severe stress.

The nitrofurans are synthetic antimicrobial compounds, containing in their molecules a characteristic 5-nitrofuran ring. Examples of nitrofurans that have been widely used are furazolidone, nitrofurazone, furaltadone, and nitrofurantoin (Leitner *et al*., 2001). These compounds are antibacterial drugs and are often added to feed to stimulate growth and to prevent and control several bacterial and protozoan infections, such as fowl cholera and coccidiosis black-heads (Pereira *et al*., 2004). These antibiotics are very effective drugs and do not appreciably contribute to the development of resistance.

In contrast to the short half-life of the parent compounds, their metabolites persist for longer periods linked to proteins. Leitner *et al*., (2001) reported that furazolidone (parent compound) residues disappear from tissues after 12 h of withdrawal while it is metabolite, 3-amino-2-oxazolidinone has a half-life time between 4 and 9 days. Thus, illegal use of nitrofuran can be monitored most effectively by determining the presence of bound residues (Pereira *et al*., 2004).

Controversy regarding the use of nitrofurans in food-producing animals such as swine, cattle, poultry, prawn, and fish has arisen during the last two decades because indications have appeared that residues of the drugs exhibited carcinogenic and genotoxic properties (McCracken and Kennedy, 1997). As a result, systemic use of all nitrofurans, as a chemical class, in food-producing animals was prohibited with effect from 1 January 1997 in the United States and EU (Leitner *et al*., 2001).

Even though the use of nitrofurans has been prohibited in food producing animals, farmers are still using it illegally. Farmers from countries like Vietnam, Bangladesh, China, Indonesia, and Thailand were believed to be still using this antibiotic (Johnston and Santillo, 2002). Although farmers have been practicing the withdrawal period during harvesting of tiger prawns (*Penaeus monodon*), however, these have not been so successful and this antibiotic is still traceable in the end product. That is the reason why tiger prawns imported from these countries were inspected thoroughly for the trace presence of nitrofuran. For our country, Malaysia, the EU authorities did random testing on tiger prawns imported into EU countries.

Malaysia Health Ministry is still finding residues of nitrofuran during samplings of food-producing animals. From the routine monitoring of nitrofuran carried out between 1999 and 2001, the percentage of samples found positive was 3.5 % in 1999, 1.5 % in 2000, and 0.09 % in 2001 (Masindian, 2006). In March 2002, health authorities in Europe found nitrofuran residues in chicken meat imported from Thailand (Delforge, 2004). People's Daily Online (2006) reported the presence of nitrofuran in turbot. The Daily Star (2006) also reported incidence of illegal usage of nitrofuran in shrimps imported to EU from Bangladesh.

By looking back at statistics and reports from government agencies and websites, the usage of this type of veterinary drug is still going on even though it is a

banned veterinary drug in food-producing animal ever since June 1995 due to concerns regarding to their carcinogenicity and mutagenicity. Since the law could not eliminate the usage of these types of drugs, alternative methods are needed to assure that the health of consumers is not at stake.

Even though chemical usage is widespread in the aquaculture industry, and the generic chemicals in use are known, accurate statistics on usage are hard to come by. While the microbiological quality of tiger prawn is closely monitored, chemical residues have received little attention (Johnston and Santillo, 2002). Chemical residues must be treated as important as microbiological quality of tiger prawn traded worldwide. That is why monitoring is important and need to be implemented so that all our export products must be free from nitrofurans or any other banned veterinary medicines. The economy of our country might be affected if monitoring is being ignored.

Recent studies by Reed *et al*., (2004) on the dosing and sampling technique will be used as the guideline in the determination of true disposition characteristics of 3-amino-2-oxazolidinone in tiger prawns. Numerous studies on the determination of the metabolites of nitrofuran antibiotics in animal tissues by high-performance liquid chromatography-tandem mass spectrometry (Conneely *et al.*, 2003; Leitner *et al.*, 2001; McCracken and Kennedy, 1997) results in development of analytical methods for identification of nitrofuran metabolites. This shows the importance and need of monitoring the trace presence of nitrofuran metabolite derivatives in food producing animals.

Greater and more understanding on the behaviour and the disposition of AOZ residue in tiger prawn can be learnt and this will benefit the next generations. This research can be the stepping-stone for those who have the interest in this research area, as there are still quite a number of drugs that can be investigated. Besides the

existing drugs, with the ever-growing science and technology, new drugs are emerging every now and then. These new drugs certainly need to undergo similar if not exactly the same pathway to ensure the degree of safeness when used in food-producing animals.

## 1.2 Objectives

The method used to analyse AOZ residue using LC-MS/MS was previously developed by USM Doping Control Centre (Leitner *et al*., 2001; Conneely *et al*., 2003; O'Keeffe *et al*., 2004), but with some modifications are aimed at shortening the analytical time. Validation on the method was performed based on the guidelines set in CD 2002/657/EC (2002). Determination of disposition of AOZ residue in tiger prawn was performed using tiger prawns that were treated with furazolidone. The results obtained from this finding will be used on the next objective of this study, which is to study the effectiveness of prawn processing in eliminating AOZ residue. In general, the processing regimes involve peeling, deveining, beheading, washing, lactic acid dipping treatment, cooking, marination, and freezing. The trace presence of AOZ residue will be monitored in the tiger prawns while undergoing these processing regimes. Thus, this study will reveal any leaching or draining or breakdown of AOZ residue from the process that will help in reducing this residue from tiger prawns before consumption.

In summary, the objectives of this study are:

1. To validate the method used to measure the trace presence of AOZ residue using LC-MS/MS based on the guidelines set in CD 2002/657/EC (2002).
2. To determine the disposition of AOZ residue in tiger prawn *(Penaeus monodon)*.
3. To monitor the effectiveness of different processing regimes in eliminating the trace presence of AOZ residue in tiger prawn and its products.

# CHAPTER TWO
# LITERATURE REVIEWS

## 2.1 *Penaeus monodon*

Named for its huge size and banded tail, *Penaeus monodon* (common name include giant tiger prawn, black tiger prawn, leader prawn, sugpo, and grass prawn) still accounts for most of the farmed prawn coming out of Asia. Native to the Indian Ocean and the southwestern Pacific Ocean from Japan to Australia, tiger prawn is the world largest (maximum length approximately 360 mm and weigh up to 650 g) and fastest growing of the farmed prawn. Tiger prawn is very susceptible to two of the most lethal prawn viruses: yellowhead and whitespot (Kontara, 1988).

The scientific classification for tiger prawn is as below (De Bruin, 1995):

Kingdom = Animalia

Phylum = Anthropoda

Subphylum = Crustacea

Class = Malacostraca

Order = Decapoda

Suborder = Dendrobranchiata

Family = Penaeidae

Genus = Penaeus

Species = monodon

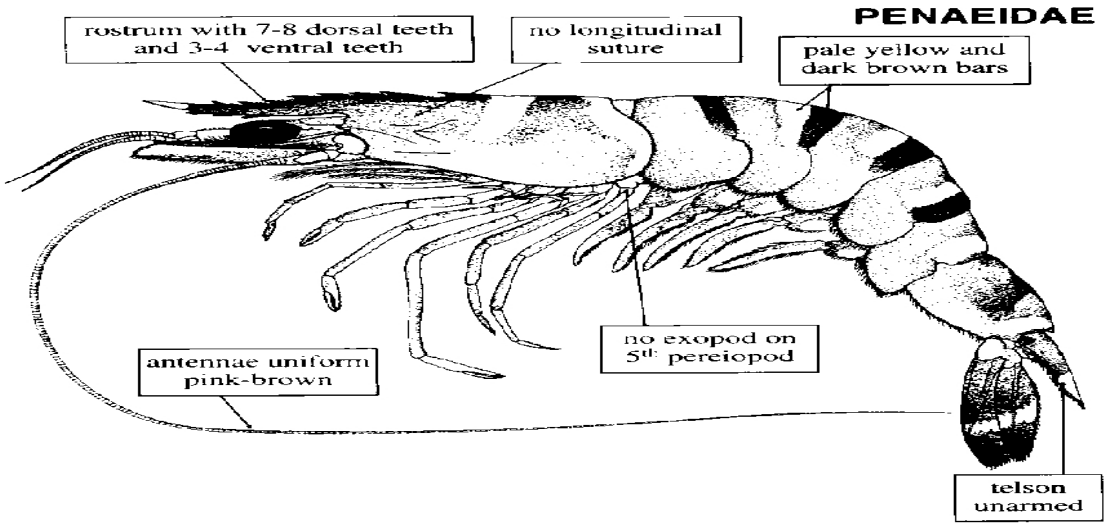


Figure 2.1: External anatomy of a tiger prawn.
*Source: De Bruin (1995)*

Tiger prawn is the most widely cultured prawn species in the world, although it is gradually losing ground to the *Litopenaeus vannamei* (common name: western white shrimp). Farmers throughout Asia are switching to *vannamei*, and it has now become the dominant species around the world. In 2006, tiger prawn and western white shrimp probably accounted for approximately 85 % of the shrimp produced on farms around the world (Rosenberry, 2006).

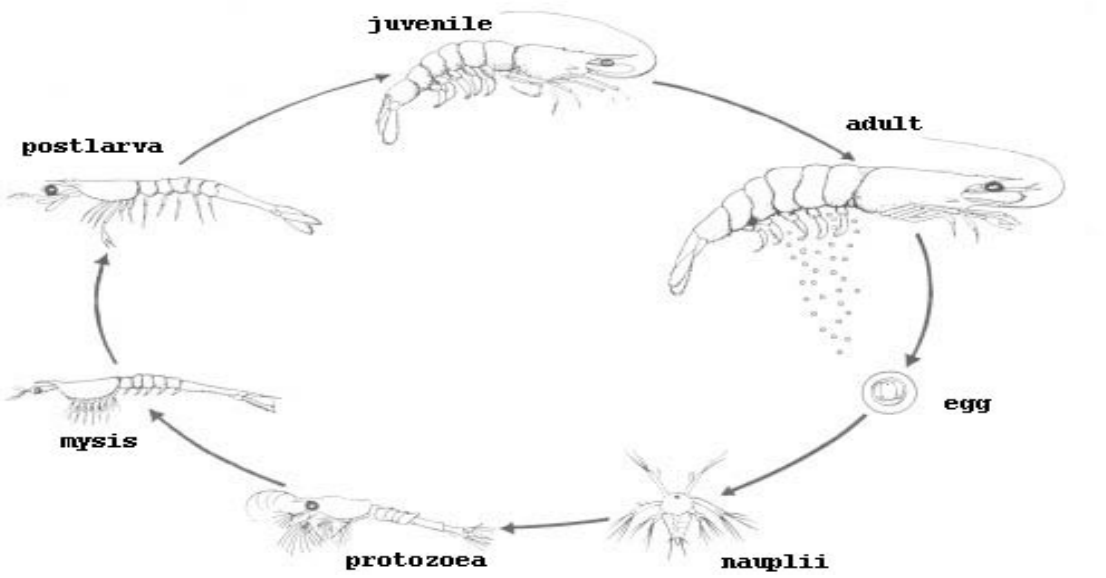


Figure 2.2: The life cycle of Panaeid prawn
*Source: Lankester et al. (2003)*

### 2.1.1 Prawn Culture

The most important consideration in prawn cultivation is the water quality. Water quality should be conducive to good growth of the prawn. Some of the essential qualities of pond water includes temperature, salinity, dissolved oxygen, pH, nitrogen, phosphorous and potassium which enhance the growth of algae which serve as food. Hydrogen sulfide also affects the growth of prawns. Parameters for all the qualities are described as follows (Kontara, 1988):

1) **Temperature**: The rate of growth increases with temperature, however, higher temperature causes mortality. Temperatures between 26°C to 30°C are considered best in terms of maximum production. Temperature above 32°C should be cause of concern.
2) **Salinity**: Young prawns can tolerate wide range of salinity. However, very little is known of the salinity tolerance of sub-adult and adult prawn. Growth was good within the range from 20 to 30 ppt.
3) **Dissolved Oxygen**: Growth is best at dissolved oxygen level above 3 ppm. Oxygenation of pond water is provided by the use of aeration devices and frequent water exchange.
4) **Water pH**: Desirable pH is above 7 to 8.5. In water with pH of 6.4 and less, growth was reduced. When pH fell below 5.0, heavy mortalities occurred.
5) **Nitrogen compounds**: There are three forms of nitrogen namely; nitrate, nitrite and ammonia. The concentration range of these nitrogen compounds which can be tolerated by *P. monodon* are:

   Un-ionized ammonia ($NH_3$) - 0.0 – 1.0 mg/L

   Ionized ammonia ($NH_4^+$) - 0.0 – 0.5 mg/L

   Nitrite ($NO_2$) - 0.0 – 6.0 mg/L

   Nitrate ($NO_3$) - 0.0 – 200 mg/L

6) **Hydrogen sulfide ($H_2S$)**: Hydrogen sulfide ($H_2S$) in the pond is the result of the chemical reduction of organic matter, which accumulates on or in the pond bottom. The accumulation of $H_2S$ could be avoided by periodic drying of the pond bottom and cultivation of the pond soil to expose the same under the sun until it hardens and cracks to dryness.

Water temperature and salinity are measured daily at 0800 and 1700 while other parameters are measured every seventh day.

Based on type of management, the stocking rates for tiger prawn are as follows: traditional method, less than 2.0/$m^2$; semi-intensive, 2–4/$m^2$; and for intensive, more than 10/$m^2$. Tiger prawns are fed either twice, thrice or four times a day. The rate of feeding ranges from 5 to 10 % of the biomass (Kontara, 1988).

## 2.2 Processing

### 2.2.1 Beheading

The head (cephalothorax) of fresh prawn contains organs rich in various digestive enzymes, which could lead to rapid deterioration of the flesh in the tail segment. Manual beheading is done by squeezing the prawn ahead of the tail section between the thumb and the fingers. Manual beheading contributes to cost saving, higher efficiency, and higher yields (Kanduri and Eckhardt, 2002).

### 2.2.2 Peeling and Deveining

The vein is the prawn intestine that runs down the dorsal side near the surface. It is usually filled with food and sand; its removal improves product quality (Kanduri and Eckhardt, 2002).

### 2.2.3 Additive Dipping

Okolocha and Ellerbroek (2005) mentioned that with the right modification and acceptable concentrations of acids and/or alkalis, additive dipping can give a good reduction on the populations of bacteria and increase the shelf life of meat products. Several researches have focused on additive treatment on ground beef (Jimenez-Villarreal *et al*., 2003), chilled beef carcasses (Gill and Badoni, 2004) and poultry meat (Okolocha and Ellerbroek, 2005). Jimenez-Villarreal *et al*. (2003) listed some of the common additives used, which are chlorine dioxide, cetylpyridinium chloride, organic acids, and trisodium phosphate.

Cassens (1994) mentioned that the usage of 1 – 2% of lactic acid in carcass rinses, as a means to control microbiological contamination, is effective without causing colour or flavour problems.

### 2.2.4 Cooking

Erdoğdu *et al*. (2001) listed two changes during thermal processing of prawns, which are denaturation of proteins and also reduction on water holding ability. These two changes lead to yield loss and dimensional changes. Reduction in microbial load is an important factor in processing with proper cooking schedules (Erdoğdu *et al*., 2003). Due to its higher resistance compared to other microorganisms, *Vibrio cholera* ($D_{65}$ = 93s, z = 7.7°C) was chosen as the target organism in safety hazards in seafood products (Rippen and Hackney, 1992).

Erdoğdu *et al*. (2003) reported that long cooking times and/or high cooking temperatures contribute to better product safety and speed but with the price of higher yield loss and also lower sensory quality. As a result, they produce two separate

cooking charts, one for large tiger prawn (35 to 44 prawns/kg) (Figure 2.3) and another one for medium tiger prawn (90-110 prawns/kg) (Figure 2.4).

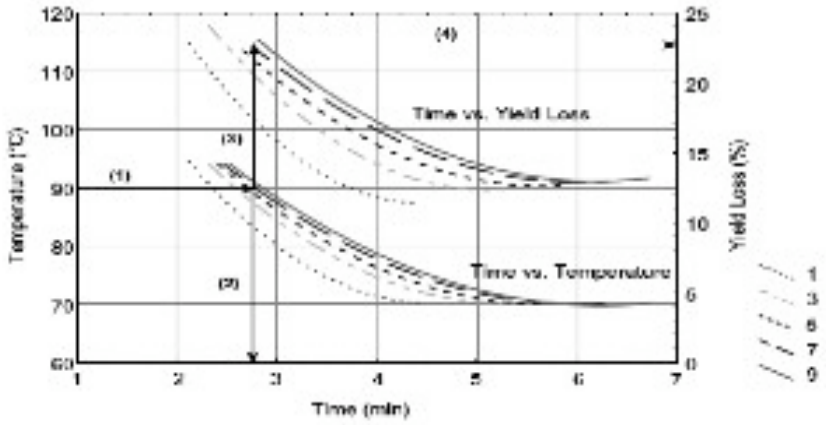


Figure 2.3: The constructed cooking chart for large tiger prawn (35 - 44 prawns/kg). *Source: Erdoğdu et al. (2003)*

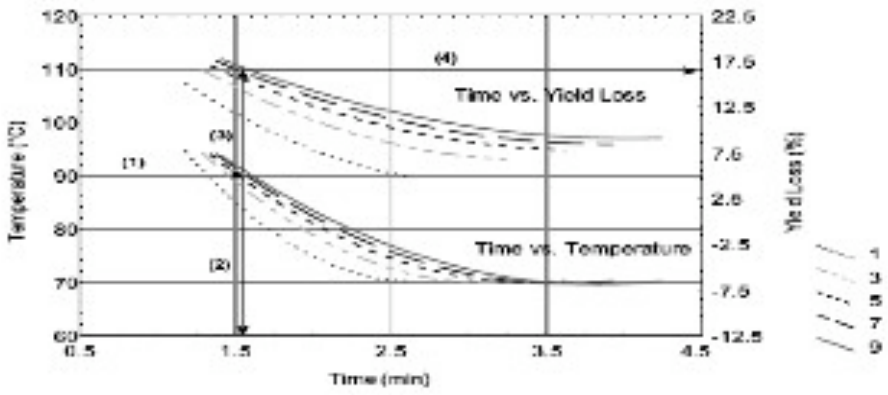


Figure 2.4: The constructed cooking chart for medium tiger prawn (90 - 110 prawns/kg).
*Source: Erdoğdu et al. (2003)*

### 2.2.5 Marinating

The word “marinate” originated from the Latin word “marine” to Italian, Spanish, and French languages with the meaning of soaking/pickling in salt brine. According to Cadun *et al*. (2005), marinated prawns are widely consumed in European countries and America. Formulations for marinades include sugar, spices, oil, acids (such as fruit juice, vinegar, and wine) (Zheng *et al*., 1998), rheology-improving additives (such as guar gum, and xanthan), antimicrobial agents (such as benzoate, and sorbate), and aroma enhancers (Björkroth, 2005).

Besides improving taste (tenderness, juiciness, flavour, and aroma), marinating also acts as a preservation method by preventing the growth of pathogenic bacteria and most spoilage bacteria. The ability to suppress microbial growth is due to the high acidity (low pH value, not higher than 4.8) since most of the food poisoning and spoilage bacteria are inhibited at pH lower than 4.8. Thus, increasing the shelf life and safety of the marinated products (Kilinc and Cakli, 2004; Björkroth, 2005; Cadun *et al*., 2005).

Acceptability of marinated prawns must also be based on sensory analysis besides low bacterial counts. Cadun *et al*. (2005) reported that pink prawns (*Parapenaeus longirostris*) marinated for 40 days have low bacterial count, but due to high rancidity or fat oxidation, the sensory score shows low acceptability by sensory panelists.

### 2.2.6 Freezing

Jaacobsen and Fossan (2001) mentioned that the quality of prawn is altered during frozen storage particularly by oxidation, proteins denaturation, sublimation, and recrystallisation of ice crystals. Both the oxidation and sublimation processes during frozen storage are the main culprits behind the formation of bad aroma, yield loss, rancidity, and toughening of the meat in frozen prawns.

### 2.2.7 Glazing

For ages, ice-glazing has been a well-known method to prevent dehydration and oxidation in frozen products during long-term storage, in which both deteriorations will lead to changes in colour, odour, and texture of the frozen products (Jaacobsen and Fossan, 2001). Mathen *et al*. (1970) listed 2 glazing methods, which were dipping and spraying the frozen products with glazing water at temperature below 1°C

(Mohamed Hatha *et al*., 1970) before rapidly refrozen. At times, salt-sugar solutions are also used to substitute glazing water. Rapid refreezing is an important step as this will preserve the overall consumer acceptability, in terms of taste, smell, and texture as well as to minimize thaw drip loss (Jose and Sherief, 1993).

## 2.3 Veterinary Medicines

Veterinary medicine is defined as any substance or combination thereof presented for its ability to treat or prevent disease in animals or which may be administrated to animals with the aim of medical diagnosis or restoring, correcting or modifying physiological functions in animals (McEvoy, 2002).

It is commercially available in two form, either in its original form or mixed with animal feed, which is known as medicated feeding stuffs. McEvoy (2002) termed medicated feeding stuffs as any mixture of a veterinary medical product(s) and feed(s), which is ready prepared for marketing and readily used to feed animals without any further alteration to it, because of its curative or preventive properties or other properties as a medicinal product.

### 2.3.1 Antibiotic

The term ‘antibiotic’ is normally reserved for a very diverse range of both natural and semi-synthetic low molecular weight compound, which possess antibacterial activity. Strictly speaking, penicillins, tetracyclines, macrolides, aminoglycosides, and amphenoicols are antibiotics while sulfonamides, nitroimidazoles, nitrofurans, and quinolones are not. Rather, they are antibacterials because of being synthetic (Kennedy *et al*., 1998; Di Corcia and Nazzari, 2002). Gentili *et al*. (2005) also stated that natural substances at high molecular weight, such as polyether antibiotics are also considered as antibacterials.

### 2.3.2 Antimicrobial

Antimicrobial drugs are synthetic compound, which possess antibacterial activity (Kennedy *et al*., 1998; Di Corcia and Nazzari, 2002). These types of veterinary drug are used in food producing animals for a wide range of purpose. Besides having the therapeutic effects against disease agents, it is responsible for changes in the structure or function of systems (or maybe both) within the target animal (Yan and Gilbert, 2004).

## 2.4 Methods of Drug Delivery

Dosage and time treatment are some of the important parameters to achieve desirable result. Dosage level or treatment time that exceeds the normal threshold might lead to danger of toxicity to the animals. On the other hand, if the dosage level or treatment time is too low, the bacteria will survive and will contribute a great portion to the possibility of the bacteria developing resistance to the veterinary drug (Yanong, 2003).

Yan and Gilbert (2004) listed the three main methods of veterinary drug delivery in food producing animals, which are parenteral administration, oral administration and intrauterine infusion. In addition, Yanong (2003) listed another major route of administration, which is bath treatment. The criteria for choosing which method to be used are based on the animal species, the infectious disease target, the drug formulation or purpose for which the antimicrobials are employed.

### 2.4.1 Oral Administration

Oral administration is a convenient method due to the easiness to administer the drugs to the animals (Yan and Gilbert, 2004) and is commonly used in aquaculture production due to the cost effectiveness (Yanong, 2003). Normally, antimicrobials

(including nitrofuran) are administered with this method either mixed with animal feeds (using fish oil or canola oil as a binding agent) or drinking water (Díaz *et al*. 1997; McEvoy, 2002; Yanong, 2003).

The bioavailability of particular drug is dependent on the degree of absorption of the drug itself, and the conditions of the gastrointestinal luminal environment (e.g., pH, ionic tension, food material interference, etc) (Yan and Gilbert, 2004). Plakas *et al*. (1994) reported that the oral bioavailability of furazolidone administered in solution was 58%, compared with 28% in a feed mixture. Yan and Gilbert (2004) reported that drinking water medication is better compared to feed medication because diseased animals generally continue to consume fluids even after they have stopped eating.

### 2.4.2 Parental Administration

Parenteral administration is normally the choice after failure of oral administration to deliver an adequate dose of the veterinary drug presented in feed or water. The failure of oral administration is normally due to the failure of sick animal to intake any veterinary rug orally (Yan and Gilbert, 2004). The two most common parenteral routes of administration are intramuscular or subcutaneous administrations.

Reed *et al*. (2004) used intrasinus dosing on the white prawn, *Litopenaeus setiferus* to study the pharmacokinetics of oxytetracycline. Oxytetracycline doses were delivered through the arthrodial membrane at the junction of the carapace and abdomen on the ventral side into the ventral intrasinus space of the prawn.

### 2.4.3 Intrauterine Infusion

Intrauterine infusion is a type of veterinary drug administration that is organ-specific drug delivery method. This type of delivery method allows a relatively high

concentration of drug at the infected site compared to other part of the animal. Generally, antimicrobial drugs delivered via these routes have less impact on the gut microflora due to the low concentration of drug in the gastrointestinal. (Yan and Gilbert, 2004).

### 2.4.4 Bath Treatment

Yanong (2003) mentioned that bath treatment is a popular route of antibiotics administration even though it required much more drug when compared to other routes to achieve the desired results. In order to achieve the most effective result when using furazolidone, using cover or finding a suitable treatment location is important due the susceptibility of furazolidone to deterioration by light.

## 2.5 Withdrawal Period

Withdrawal period refers to the appropriate time interval starting from the day of the last administration of the veterinary drug under normal conditions of use until the day when the treated animals is considered safe to be slaughtered for the production of safe food products. Withdrawal period is important because it should archive the ultimate goal of having the concentration of residues in food derived from these treated animals not exceeding the maximum residue limits (MRLs) with high degree of assurance (Díaz *et al*. 1997; Anadón and Martínez-Larrañaga, 1999; Vranic *et al*., 2002).

Vranic *et al*. (2002) listed the three main methods for estimation of the withdrawal period for veterinary drugs used in food producing animals, which are Decision Rule, Linear Regression, and Non-parametric Approach. All these three methods have the same ultimate goal. But, there is a slight difference between these three methods, and that is the length of the interval period. Vranic *et al*. (2002) reported

that the estimated withdrawal period by the method of Linear Regression is the shortest, while the longest is by the method of Non-parametric Approach.

Hoogenboom *et al*. (1991) and (1992) reported that the withdrawal period for medicated animals with furazolidone is long. AOZ was still detectable in liver, kidney and muscles of furazolidone treated pigs (300mg furazolidone per kg of feed for a period of 7 days) at a concentration of 41, 7 and 10ng/g respectively even after 4 weeks from the day of the last medication. Conneely *et al*. (2002) reported that the metabolite can still be detected in the tissues of furazolidone treated animal for up to seven weeks after withdrawal of furazolidone.

## 2.6 Furazolidone

In 1944, Dood and Stillman discovered for the very first time that by adding a single nitro group in C5 of the furan ring, it gave rise to a molecule with bacteriostatic properties. Thus, nitrofurans were developed. With the correct modification on C2 of the furan rings, different types of nitrofurans were generated. The inclusion of a conjugated carbon – carbon or carbon – nitrogen double bond between the nitrofuril group and the terminal portion of the lateral chain in C2 will enhance the antibacterial activity. (Monasterios *et al*., 2005). Among them there are four of the most prominent nitrofurans, which are furazolidone, nitrofurantoin, nitrofurazone, and furaltadone (Mottier *et al*., 2005).

N-(5-nitro-2-furfurylidene-3-amino)-2-oxazolidinone or commonly known as furazolidone (*fyoor-a-ZOE-li-done*) is a synthetic chemotherapeutic agent that belongs to the class of 5-nitrofurans (Draisci *et al*., 1997; McCracken and Kennedy, 1997a; Kennedy *et al*., 1998; Cooper *et al*., 2004). Furazolidone occurs as a yellow odorless crystalline powder with a bitter aftertaste. It is practically insoluble in water and alcohol. The presence of alkali will decompose furazolidone. Exposure to daylight for 6 h will

cause nearly total (around 93%) decomposition of the furazolidone but was unchanged when exposed to fluorescent light in the same time period (Muth *et al*., 1996). Thus it should be stored in light resistant containers.

The presence of a 5-nitro group enables furazolidone to have a broad spectrum of activity and for this reason it is widely used as veterinary drugs (Draisci *et al*., 1997; Kennedy *et al*., 1998; Balizs and Hewitt, 2003; Auro *et al*., 2004). Monasterios *et al*. (2005) mentioned that furazolidone is useful in the treatment of invasive bacterial diarrhea and parasitic condition due to its low absorption through the intestine. Furazolidone is predominantly bacteriostatic. But at high doses, it acts as bactericidal (Kennedy *et al*., 1998). The ability to act as bactericidal is due to its ability to interfere with several bacterial enzyme systems, possibly including prevention of acetylation of coenzyme A and also acts as a monoamine oxidase (MAO) inhibitor (Hoogenboom, 1991; Di Corcia and Nazzari, 2002; Balizs and Hewitt, 2003; Cash and Johnston, 2004).

### 2.6.1 Furazolidone as Veterinary Medicine

Since 1953 (before furazolidone was banned), it was used for treatment in turkeys and chickens of fowl typhoid, paratyphoid, and pullorum; blackhead (histomoniasis); nonspecific enteritis (blue comb, mud fever), ulcerative enteritis (quail disease), and synovitis (arthritis due to filterable virus); and paracolon infection (*Paracolobactrum*). In chicken its use for infectious hepatitis and coccidiosis. While in turkeys its use for hexamitiasis, and in swine for bacterial enteritis (nercotic enteritis, necro, black scours) or vibrionic (bloody) dysentery (Bryan, 1978).

Normally, antibacterial drugs including furazolidone at therapeutic dose are administrated orally or mixed with animal feed as food additive (Draisci *et al*., 1997). Furazolidone is widely used as food additives for the treatment of gastrointestinal

infections caused by *Escherichia coli* and *Salmonella* spp in cattle, swine, fish, prawns, and poultry (Hartig *et al*., 2003; Gao *et al*., 2003; Cooper *et al*., 2004; Finzi *et al*., 2005; Szilagyi and de la Calle, 2006). Furazolidone is also given to prevent and control several bacterial and protozoan infections. For example, fowl cholera, coccidiosis blackheads and swine enteritis in poultry and pigs and mastitis in dairy cattle (Draisci *et al*., 1997; Auro *et al*., 2004).

Doses lower than the usual therapeutic level are administrated to promote weight gain, for feed conversion (McCracken and Kennedy, 1997a; Cooper *et al*., 2004), to prevent disease, and to increase feed efficiency (Bryan, 1978), thus increasing the meat yield per pound of feed used as well as for maintaining adequate growth rate, feed conversion (McCracken and Kennedy, 1997a; Cooper *et al*., 2004) and enhanced feed efficiency (Bryan, 1978).

### 2.6.2 Furazolidone as Human Medicine

Furazolidone is also used to treat human as a medicine. Furazolidone has been used for the treatment of bacillary dysentery, typhoid and paratyphoid fevers, giardiasis (caused by protozoan *Giardia lamblia*), brucellosis, and intestinal infections of undetermined etiology (Bryan, 1978; Díaz *et al*., 1997). It is also used in the treatment of trichomoniasis, giardiasis (for adult, dosage of furazolidone is 100mg, four times daily for seven to ten days), cholera (for adult, dosage of furazolidone is 100mg, four times daily for four to seven days), and other vibrio infections. Besides that, furazolidone has been reported to possess anti-Helicobacter activity and to have some ulcer-healing properties (Monasterios *et al*., 2005).

Backer (2000) mentioned that furazolidone is used commonly in treating children who suffered from giardiasis because it is available in a liquid suspension and

is well tolerated. Cash and Johnston (2004) mentioned that furazolidone is the antibiotic of choice in pregnant individuals in treating giardiasis.

Furazolidone also holds a particular advantage in treating cholera over other antibiotics, including tetracycline, in that several antibiotic-resistant strains are susceptible to it in vitro (Glass *et al*., 1980). Rabbani *et al*. (1991) added that furazolidone, given as either a single dose (7mg/kg/day) or a dose of 7mg/kg/day divided in four equally daily doses for 3 days, is effective treatment for childhood cholera. Furazolidone also seems to be more effective compared to ampicillin in therapy for acute traveler's diarrhea. The mean duration of diarrhea was shortened on the average from 72 to 57 h for all cases irrespective of entiology (DuPont *et al*., 1984)

### 2.6.3 Furazolidone Metabolism

Furazolidone has an in-vivo half-life of only a few hours (Hartig *et al*., 2003), whilst Cooper *et al*. (2004) confirmed that furazolidone disappeared from tissue within 12 h of withdrawal. Elimination of furazolidone in channel catfish was also extremely rapid, with a terminal half-life of 0.27 h and total body clearance of 1901 mL/h/kg (Plakas *et al*., 1994). McCracken and Kennedy (1997a) reported that the concentration of furazolidone found in kidney and liver of furazolidone treated porcine, fell below 5 ng/g within 4 and 6 h, respectively. Due to this very instability or rapid metabolism property of furazolidone and its incapability to persist in edible tissue, analysis of intact furazolidone in tissues is both difficult and of limited value. For this reason, it has in the past prevented the detection of this veterinary drug in food producing animals (McCracken and Kennedy, 1997a; Conneely, *et al*., 2002; Hartig *et al*., 2003; Xu *et al*., 2006). However Tatsumi *et al*., (1984) successfully isolated N-(4-carboxy-2-oxobutylideneamino)-2-oxazolidone, α-ketoglutaric acid, 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone, and N-(5-acetamido-2-furfurylidene)-3-amino-2-oxazolidone from the urine of rats treated orally with furazolidone.

Metabolism of furazolidone gives rise to different forms of extractable and protein-bound residues (O'Keeffe *et al*., 1999). The major metabolite from the use of furazolidone in mammals, birds and fish is 3-amino-2-oxazolidinone (AOZ) while the minor metabolite is N-(5-amine-2-furfuryliden)-3-amine-2-oxazolidinone (FOZ) (Auro *et al*., 2004). These metabolites are covalently bound to cellular protein in vitro (Draisci *et al*., 1997; Di Corcia and Nazzari, 2002). AOZ has a higher stability and longer residence time (between 4 and 9 days half life time and are detectable for up to 7 weeks after administration) when compared to the parent drug (Leitner *et al*., 2001; Conneely *et al*., 2002; Yang, 2003).

### 2.6.4 Furazolidone Toxicity

Concerning the toxicology properties of nitrofurans, mutagenic activity has been observed and reported in research using yeasts, fungi, bacteria and sub-mammalian systems. In addition, researches using rats and mice have shown that nitrofurans are tumorigenic. Besides that, nitrofurans have been shown to be cyctoxic to mammalian cells in culture. The tumorigenic and cyctoxic effects seem to be related to the nitrofurans compound itself and also to the metabolite formation (Draisci *et al*., 1997). For furazolidone, it has been suggested that its metabolite, AOZ can be metabolized into β-hydroxyethylhydrazine, which is a mutagenic and carcinogenic compound (O'Keeffe *et al*., 1999).

Díaz *et al*. (1997) mentioned that even though the toxic effects (also mutagenic and carcinogenic) of most of the nitrofuran are well documented, their precise mode of action is not fully clarified (and neither is their fate). Therefore, further and advance research on the mode of action must be carried out in order to affirm the toxicity of furazolidone and its metabolites.

### 2.6.5 Contradiction for Furazolidone Toxicology

Cancer induction was established using laboratory animals that were treated directly with large quantities of nitrofuran. Human as we know, show no danger sign when nitrofuran is consumed. That is why it is still being used in treating giardiasis and cholera. Rather, humans are in danger of consuming the residues of the main nitrofuran metabolites, AOZ and a minor metabolite, FOZ. But, up to this date, evidence that AOZ/FOZ can induce genotoxic or carcinogenic effect remains inconclusive (Auro *et al*., 2004).

Auro *et al*. (2004) reported that both tilapias (*Oreochromis niloticus*) fed with tilapia-meal (feed made from tilapia) contaminated with AOZ/FOZ and mollies (*Poecilia Formosa*) fed with mollie-meal (feed made from mollie) meal contaminated with AOZ/FOZ, both at concentration of 500g of fishmeal/kg of furazolidone-free commercial feed, show no sign of developed tumours. These results do not support the established viewpoint that furazolidone must be banned from tropic chains based on its potential carcinogenic properties.

Klee *et al*. (1999) reported that no acute toxicity of AOZ or digested food residues in gut segments at Caco-2 cells at concentration that were substantially above the maximum residue levels to be expected in the food of animal origin after administration of therapeutic doses. The inability to find acute toxicity in gut at Caco-2 cells is due to the extensive metabolism and detoxification of furazolidone by glutathione (De Angelis *et al*., 1999). The results demonstrate that the chemical nature of drug residues can be altered by digestive process. In addition, this process may also yield degradation products that may be bioavailable for the consumer. Thus, the covalently bound xenobiotics at macromolecular tissue cannot necessarily be regarded as an irreversible endpoint of residue bioavailability and toxicity (Klee *et al*., 1999).

### 2.6.6 Source of Furazolidone Contamination

Trace amount of nitrofuran contamination has been detected in warm-water prawns, prawn, and chicken (Yang, 2003). It has been reported that there are numerous ways for the contamination to occur. Contamination may occur from deliberate, direct misuse of the drugs, as well as from contaminated feed, or environment sources (Leitner *et al*., 2001; Gao, 2003; Yang, 2003; Pereira *et al*., 2004). Furthermore, contamination may also occur from transfer between animals via ingestion of faeces and/or urine (Kennedy *et al*., 2000; Leitner *et al*., 2001). Kennedy *et al*. (2000) and McEvoy (2002) reported that contamination of feeds is linked to human error, improper cleaning, carried over of contamination from medicated feeds to subsequent batch (unmedicated feeds), production practices and handling procedures in the feed mill, during transport and on farm.

McCracken *et al*. (2000) successfully setup a criterion that makes the differentiation between furazolidone abuse and contamination possible. Porcine treated with furazolidone illegally will have a concentration ratio of AOZ in bile:kidney of less than 0.3; while unmedicated porcine contaminated with AOZ will have concentration ratio of AOZ in bile:kidney of greater than 3.0.

### 2.6.7 Current Issues on Furazolidone Contamination

The presence of nitrofurans in food producing animals are undeniable even after the implementation of regulation against the use of nitrofurans in the mid 90's. A survey was done to investigate the occurrence of furazolidone residues in pigs from Northern Ireland between February 1995 and July 1995. Out of one hundred samples, seventeen percent of the samples were found positive (McCracken and Kennedy, 1997a). In 2002 nitrofurans and their metabolites were frequently detected in imported poultry from Brazil and Asia, and prawns from Asia (Johnston and Santillo, 2002;

McEvoy, 2002; Hartig *et al*., 2003). The detected concentrations varied as high as 320ppb and as low as 0.6ppb (Hartig *et al*., 2003). Conneely *et al*., (2003) have also reported that residues of nitrofuran drugs have been detected in poultry and shellfish imported into the EU.

## 2.7 AOZ (Furazolidone Metabolite)

Covalently bound residues are derived from the covalent binding of the parent drugs or its metabolites to endogenous macromolecules. These covalently bound residues are not readily extractable from the tissues by mild aqueous or organic solvent extraction, denaturation, and solubilisation techniques (Burgat-Sacaze and Rico, 1990). Mostly, to breakdown this type of binding, more extensive and rigorous chemical treatments are required, such as treatment with acid, base, or enzymes (O'Keeffe *et al*., 1999).

Covalently bound residues play an important role in food safety since it possess high stability to remain intact within the macromolecules for a long period of time after the withdrawal of the drug treatment. In addition, the ability of the binding to breakdown during digestion to release a compound that is in biologically active forms also contributes to the importance in food safety (O'Keeffe *et al*., 1999).

Each nitrofurans have its own metabolites. The main metabolites that are of concerns in detection of furazolidone, furaltadone, nitrofurantoin, and nitrofurazone are AOZ, 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ), 1-aminohydantoin (AH), and semicarbazide (SC), respectively (Leitner *et al*., 2001; Edder *et al*., 2003; Pereira *et al*., 2003; Yang, 2003). Samuelsen *et al*. (1991) reported that AOZ had no detectable antibacterial activity.

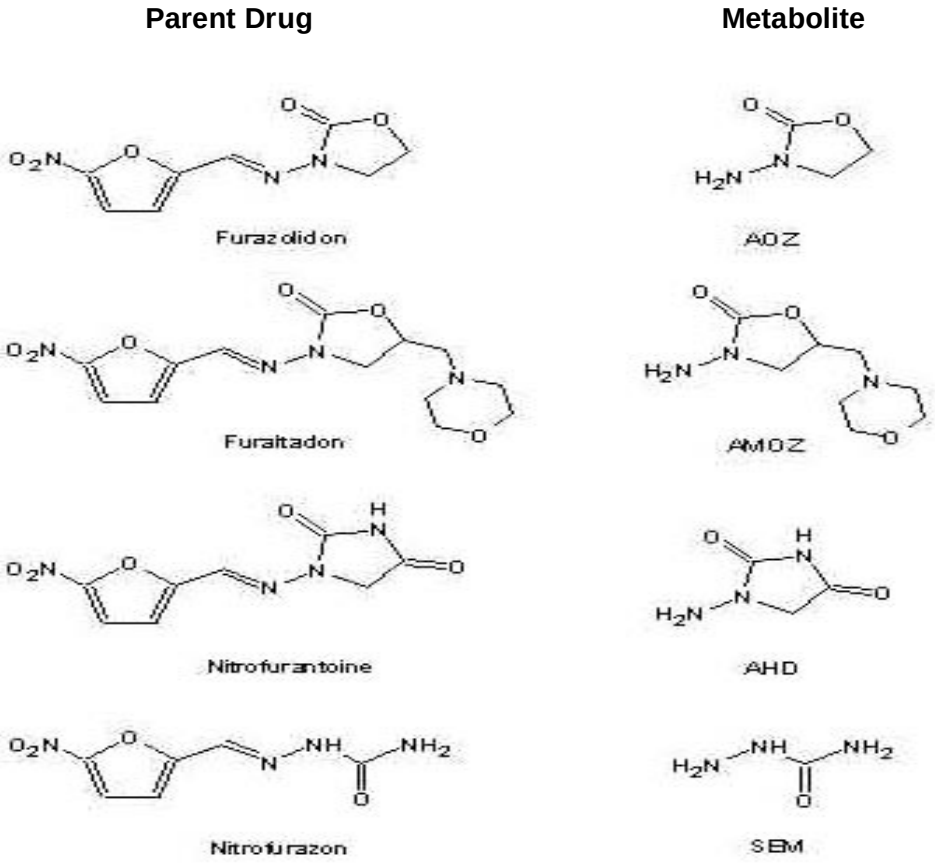


Figure 2.5: Structure of the nitrofuran antimicrobials and their free major metabolites.
*Source: Leitner et al. (2001)*

Draisci *et al*., (1997) and Di Corcia and Nazzari, (2002) reported that furazolidone metabolite, AOZ, forms a covalent binding to cellular protein. The covalent bonding between AOZ and the cellular protein is called azomethine bond. These bonds are hydrolysable by acid hydrolysis to release AOZ molecules (Hoogenboom, 1991; Horne *et al*., 1996), subsequently cleaved to ethylene (Hunder *et al*., 1987).

### 2.7.1 AOZ Residence Time in Animal Tissue

Tissue-bound metabolites are formed after treatment with the parent drug, furazolidone (Conneely *et al*., 2002). It is covalently bound to cellular protein in the tissues, such as liver, of the treated animals (Draisci *et al*., 1997; O'Keeffe *et al*., 1999). Due to the presence of these covalent bonding, the rate of elimination of furazolidone metabolites from the animal body is dependent on the rate of turnover of tissue protein,