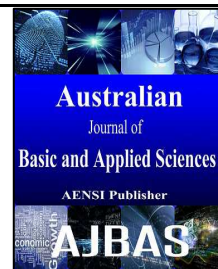




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### Detection of Furazolidone Metabolites in Cultured Shrimps in Penang Market

<sup>1,2</sup>Nurdiana Zainuddin, <sup>1</sup>Zakaria Ismail, <sup>1</sup>Wan Ismahanisa Ismail, <sup>1</sup>Muhammad Nabil Fikri Roslan, <sup>1</sup>Nor Hafeeda Rosdan, <sup>1</sup>Nor Raihan Mohammad Shabani, <sup>2</sup>Hasni Arshad, <sup>3</sup>Razak Lajis

<sup>1</sup>Universiti Teknologi Mara, Medical Laboratory Technology, Faculty of Health Sciences, 13200 Kepala Batas, Penang, Malaysia

<sup>2</sup>Universiti Sains Malaysia, Health Toxicology, Advance Medical and Dental Institute, 13200 Kepala Batas, Penang, Malaysia

<sup>3</sup>Universiti Sains Malaysia, National Poison Centre, 11800 Minden, Penang, Malaysia.

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#### ABSTRACT

**Background:** Furazolidone is a synthetic antibiotic in nitrofurans family which commonly used as a broad antibacterial and antiprotozoal agent for animal and human. However Malaysia prohibits the use of nitrofurans antibiotic (furazolidone, nitrofurazone, furaltadone, and nitrofurantoin) in the animal feed since 1996. But it is widely used in other countries as feed additives for poultry, cattle, farmed fish, turkey and aquaculture products. The metabolite *3-amino-2-oxazolidinone* (AOZ) is rapidly produce after ingestion of furazolidone and present for many week in body making them be a good indicator for illegal used of furazolidone antibiotic. **Objective:** To detect the presence of AOZ, determine its concentration and find the sources of cultured shrimp that contains AOZ in Penang markets. **Results:** twenty five cultured shrimp have been collected from Penang market and no AOZ have been detected in cultured shrimp in Penang markets. **Conclusion:** Cultured shrimp in Penang markets is safe from furazolidone and AOZ.

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#### INTRODUCTION

One of the emerging problems in human nutrition is the presence of antibiotics residue in foods of animal origin (Ahmed *et al.*, 2008). Antibiotics is used to prevent and treat diseases, promotes growth, vaccination and other management practice (YIBAR *et al.*, 2013). Nitrofurans, such nitrofurazone, Furazolidone and nifurpirinol (Furanace) were thought to hold promise for aquaculture at one time. Furanace has since been totally banned for its carcinogenic potential, while the use of the other two has been severely limited. In Malaysia, all three are still available for use by the aquaculture industry, although they are not extensively used. Administration is through the feed at a rate of 3-5 g/kg. The drug is sold at licensed shops at about \$36-40/kg (Fofonoff *et al.*, 2003).

Nitrofurans (furazolidone, furaltadone, nitrofurantoin and nitrofurazone) is rapidly metabolized resulting in very stable metabolites (Table 1) which have been linked with carcinogenic, mutagenic and teratogenic effects in humans for long exposure (Leston *et al.*, 2011). The half-life of furazolidone have been reported less than a few hours. The residue extensively formed in the liver,

kidney and muscle of the animal (YIBAR *et al.*, 2013). Improper use of nitrofurans and incorrect withdrawal time after treatment of shrimps could lead to the presence of antibiotic residues in shrimps and provoke allergic reaction in some hypersensitive individuals. Other effects of improper use of nitrofurans are drug-resistant pathogenic bacterial strains, bone marrow depression, aplastic anaemia, gastrointestinal and liver disturbances (Wang, 2010, Vass *et al.*, 2008b, Romich, 2005).

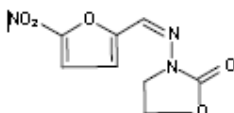
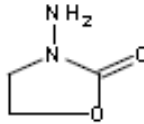
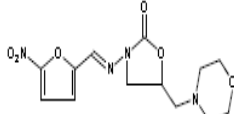
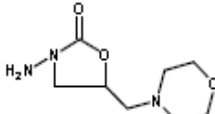
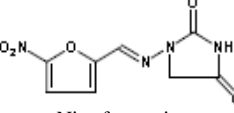
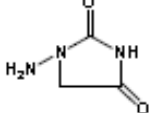
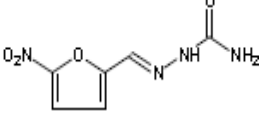
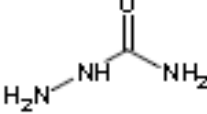
Foods including milk, meat and honey containing antibiotics residue is not allowed to import or export to Malaysia and Europe. Nitrofurans and nitrofurans metabolites has been banned in food and food stuff in many countries include Thailand, Philippines, Australia, Brazil and United States. This antibiotic and its metabolites is banned because of potential carcinogenic, mutagenic and teratogenic to animals and human (Ee, 2003, Chew, 2008, Vass *et al.*, 2008). The effects of parents nitrofurans seem related to the nitrofurans residue. The inhibition of carbohydrate metabolism in human nervous tissue may be the mechanism responsible for nitrofurans neurotoxicity. The use of nitrofurans in feed animal product result in drug residues in animal tissue and animals' product and may result in bioaccumulation

**Corresponding Author:** Nurdiana Zainuddin, Faculty of Health Sciences, Universiti Teknologi Mara, 13200 Kepala Batas, Penang, Malaysia.  
Tel: +601-24871320; E-mail: nurdiana\_zainuddin@yahoo.com

in feed animal tissue and human. Concerning the toxicological properties of this antibiotic, mutagenic activity has been observed in yeast, fungi, bacteria and sub-mammalian systems (Ee, 2003). In addition,

nitrofurans have been shown to cause tumorigenic and cytotoxic in rats and mice and mammalian cell culture (Ee, 2003, Vass *et al.*, 2008).

**Table 1:** Nitrofurans antibiotic and its metabolites.

Nitrofurans antibiotic	Nitrofuran metabolites
 <p>Furazolidone</p>	 <p>3-amino-2-oxazolidinone(AOZ)</p>
 <p>Furaltadone</p>	 <p>3-amino-5-morpholinomethyl-2 oxazolidinone (AMZO)</p>
 <p>Nitrofurantoin</p>	 <p>1-aminohydantoin (AHD)</p>
 <p>Nitrofurazone</p>	 <p>Semicarbazide (SEM)</p>

Therefore antibiotics used to prevent or treat animal feed are controlled through guidelines, with the marketed product regulated by the food Act 1983 and Food Regulation 1985. In Malaysia, the parents nitrofurans and nitrofurans metabolites in feed product such as chickens, eggs, and poultry product has been monitored by the Ministry of Health Malaysia. While aquaculture product such as fish, shrimp and prawn for exported was monitored by

Department of Fisheries Malaysia. However import alert from the United State Food and Drug Administration (FDA) in 2009 till 2015 shown that shrimp and prawn products exported have been detained without physical examination due nitrofurans and AOZ. Most of the detained products were imported from Perak, Penang and Selangor (Table 2).

**Table 2:** Import alert on nitrofuran contamination from 2009 to 2015 (FDA, 2015).

Year	Manufacturer state	Number of import alert
2009	Penang	1
	Perak	1
	Selangor	1
2010	Perak	1
2012	Penang	2
	Perak	1
2013	Penang	1
	Perak	2
2014	Penang	3
	Perak	1
2015	Penang	11
	Perak	1
	Kedah	1

The National Institute of Public Health and Environmental Protection (INCHEM) reported the administration of furazolidone has significant increase in incidence of mammary gland adenocarcinoma and benign and malignant combined mammary neoplasm in females' rats. In addition, high dose of furazolidone caused cell epithelium and carcinoma and neural astrocytoma in males' rats.

Furthermore, both sexes' rats shown increased incidence of sebaceous gland adenomas and thyroid adenomas for high dose (Ee, 2003).

The objective of this study is to provide the current abuse of furazolidone, find the sources and determine the level of AOZ in cultured shrimp sold in local market Penang.

**Methodology:****Sample collection and storage:**

All cultured shrimps sold in Penang market were collected. The source of the shrimp sold such as state and area was obtained from seller. Only cultured shrimps used in this study and wild species shrimp were used as control negatives and control positives. The samples then was place in ice in prior of transportation to the laboratory. Then the shells were peel and washed using distilled water. Samples then were keeping at  $-20^{\circ}\text{C}$  and covered with aluminium foil until homogenization. Then the freezed shrimps were homogenized until mince. The homogenized sample was transferred to 50 mL fulcon tube and stored at  $-20^{\circ}\text{C}$  until used for extraction.

**Control negative and control positive:**

For control negative, one gram of homogenized wild shrimp was continued with the extraction method. The control positive was prepared by spiked one gram of homogenized wild shrimp with  $25\mu\text{L}$  of 20ppb spiking standard and leaved to dry for 30 minutes before used for extraction.

**Extraction:**

Standard operating procedure from kit was followed. Homogenized samples were thaw and were weight. One gram of homogenized sample was added with 4 mL of distilled water, 0.5 mL of 1 M HCl and  $100\mu\text{L}$  of 10 mM 2-nitrobenzoic aldehyde (in DMSO). Then sample was mixed well by vortex for 2 minutes at 2500 rpm. The samples were incubated at  $55^{\circ}\text{C}$  for 2 hrs 15 min. Then the cooled mixture was added with 5 mL of 0.1 M  $\text{K}_2\text{HPO}_4$ , followed by 0.4 mL of 1M of NaOH and 5 mL ethyl acetate and vortex for 30 seconds. The process continues with centrifuge at 3,000 g for 10 minutes at room temperature and aliquot 2.5 mL of the ethyl acetate at the upper layer into a glass tube. Then the ethyl acetate was dried under a slow stream of nitrogen at  $60^{\circ}\text{C}$ . Dried samples then were added with 1 mL of n-hexane and 1 mL of dilution buffer. Mixtures were vortex for 1 minute and further with centrifugation at 3000 g for 10 minutes. The  $50\mu\text{L}$  lower aqueous layer was took and continued to the assay protocol (Romer Labs Singapore, 2012).

**Detection of AOZ:**

The Enzyme-Linked Immunosorbent Assay (ELISA) was used to determine the level of AOZ in shrimps muscle. The standard operating procedure from the kit was followed. Triplicate of  $50\mu\text{L}$  of AOZ standards (0 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.2 ng/mL, 0.50 ng/mL, and 1.50 ng/mL), control positive, control negative and shrimps sample was pipette into micro wells. Then  $25\mu\text{L}$  of the AOZ-HRP conjugate solution followed with  $25\mu\text{L}$  of the AOZ antibody solution was added into all the wells. The plate was seal and assay was continue by mix the contents thoroughly, by moving the plate for at least 1

minute. The plate was incubating at room temperature for 1hour. Incubation process was end by discard all solutions from the micro titre plate wells. Unbound reagent was removing in washing steps. Washing steps was performed by dispense  $300\mu\text{L}$  of the diluted wash buffer into each well, moved the plate in a circular motion and discarded the contents and plate was tap onto a clean, roll tissue paper to dry and remove as much of the remaining liquid in the wells as possible. Wash process was performed in triplicate. Assays were continued by adding  $150\mu\text{L}$  of TMB substrate solution into all wells and seal the plate. Then it was incubate for 30 minutes in the dark at room temperature. The assay protocol was end by adding  $50\mu\text{L}$  of stop solution to all wells and the optical density values were immediately read at 450 nm measuring wavelength and 630 nm reference wavelength using microplate reader. The reading then was transferred to the spreadsheet provided from the company to determine the concentration of the AOZ in the samples (Romer Labs Singapore, 2012).

**Result:****Distribution of cultured shrimp in Penang Market:**

Twenty five cultured shrimps sample was obtained from ten publics market and two FAMA market within April to May 2013. Figure 1 shows the market that sold the cultured shrimp in Penang. From total of 25 cultured shrimps sold in Penang Markets, 92 % of shrimps are obtained from public markets and only 8% of shrimps obtained from FAMA market in mainland Penang.

**Source of shrimp and AOZ status:**

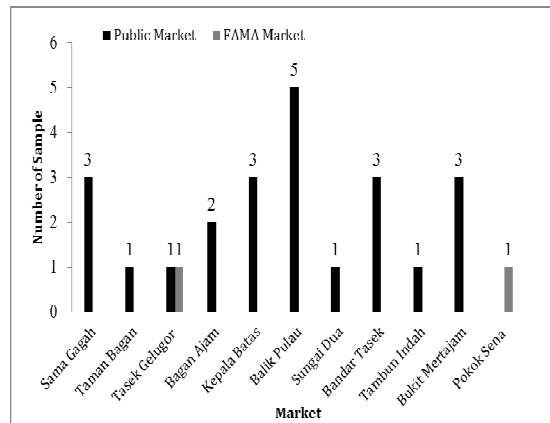
This study shows that, three states were supplied the cultured shrimp for Penang market are Penang (76%), Kedah (8%) and Perak (8%). In Penang, Forty percent of cultured shrimps in Penang are from Nibung Tebal followed by Bukit Mertajam (20%), Balik Pulau (16%), Tanjung Piandang (8%). Kedah have supplied cultured shrimps from Pantai Merdeka (4%) and Alor Setar (4%). However 8% is from unknown source and there is 4% of mixed sample from Sungai Udang and Tambun. All sample show negative for the presence of AOZ in the shrimps muscle. Figure 2 shows the sources area and detection of AOZ.

**Discussion:**

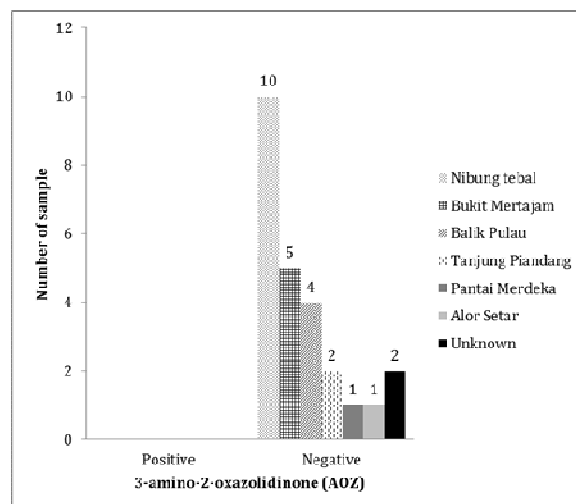
The nitrofurans are considered as drugs of Annex IV of EEC Reg.2377/90 and cannot be used in feed animal product (Ee, 2003). The illegal used of nitrofurans has measured by nitrofurans residue level in tissue. There is no maximum residue level (MRL) value set for nitrofurans metabolites presence in feed animal product (Ee, 2003, Vass *et al.*, 2008). Therefore feed animal products should not contain any nitrofurans metabolites in blood, tissue or products. However, shrimps from Asian countries have been reported to contain nitrofurans residues and

the AOZ were detected at 0.2 to 150 ng/g (Tittlemier *et al.*, 2007). United Kingdom and Italy have notified the presence of nitrofurans metabolites in prawn imported from Malaysia. Furazolidones metabolites (AOZ) have been reported presence in raw frozen farmed black tiger prawns (*Penaeus monodon*). In Penang, this study shows none of the cultured shrimps

from any sources have been detected for the presence of furazolidone antibiotic and AOZ. The limit of detection for ELISA assay used in this study is 0.1 ppb to 0.3 ppb. So presence of AOZ lower than 0.1 pb cannot be detected.



**Fig. 1:** Area and the number of cultured shrimp samples were collected.



**Fig. 2:** Detection of AOZ in shrimp supplied from different area.

The furazolidone antibiotic is rapidly metabolized in body and form nitrofurans metabolite called AOZ. The rapid metabolized of this antibiotic cause falsely negative for detection of furazolidone. However the metabolites present for many week in body making them be a good indicator for illegal used of furazolidone antibiotic (Cheng *et al.*, 2009, Vass *et al.*, 2008, Cooper and Kennedy, 2007).

Many research showed that AOZ is remained after cooking process such as grill and boiling. It is also increase with the temperature as proved by an experiment on the effect of cooking on AOZ. The result showed that the level of AOZ is higher in boiled eggs compared to uncooked eggs (YIBAR *et al.*, 2013). This showed that the AOZ is stable in high

temperature and continue to pose a health risk even after cooking process.

The Furazolidone have been reported to cause nausea and vomiting due to drug absorbed on central nervous system rapidly rather than from gastrointestinal. Other adverse effect related to human are skin rashes, eosinophilia and drug fever, renal failure due to peripheral neuritis, pneumonitis, hepatotoxicity, blood dyspraxia and teeth discoloration (Ee, 2003). Other Studies suggested that cell exposed to furazolidone caused irreversible damage to the DNA of human epithelial cells (HEp-2) and hormone disturbances due to reflecting endocrine dysfunction (Vass *et al.*, 2008).

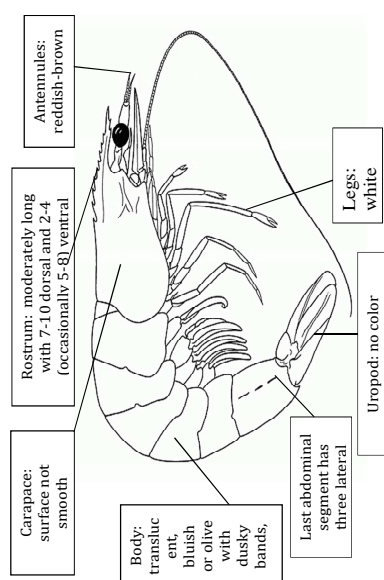
According to Artun *et al.*, boiling process may enhanced the efficiency of AOZ extraction and boiled

sample should be used for analysis of AOZ level in order to obtain more reliable and more predictive results (YIBAR *et al.*, 2013).

ELISA is suitable for the screening the presence of drugs include furazolidone and AOZ. ELISA is a highly sensitive and specific method. This method uses an antibody with broad specificity and enzymes that require rigorously controlled temperature, pH, humidity and non-toxic chemicals. Electrochemical sensor, microfluidic system with chemiluminescence, high performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC-MS/MS) are the successful method for detecting the veterinary drug such as chloramphenicol, ciprofloxacin, nitrofurans and nitrofurans metabolites (Zhai *et al.*, 2015.).

Twenty five cultured white shrimps have been collected for this study. There are two types of cultured white shrimps in Malaysia which is *P.merguensis* and *P.vannamei* (Jabatan Perikanan

Malaysia, 2011). Identification of species for this study was failed. Fresh shrimps must be used for species identification. However cultured shrimps collected for this study have been confirmed as white shrimps and most likely *P.vannamei*. *P.vannamei* has special characteristics as shown in figure 3. This is supported by the report from the Jabatan Perikanan Malaysia that *P.merguensis* was not cultured in Malaysia in 2011 (Fisheries and Aquaculture Department. 2013). In addition, white shrimp is more preferred in local market due to the lower price than the tiger prawn. According to Malaysia Fisheries Department 2011, Penang is the second highest state that produces *P.vannamei* with production 10,975.76 tonnes followed by Perak with 10,038.04 tonnes. This factor contributed in the distribution of sample collection. Results shown that seventy six per cent of cultured shrimps collected were supplied from Penang area followed by Perak and Kedah 8% respectively.



**Fig. 3:** Characteristic of *P.vannamei* (Food and Agriculture Organization of United Nations).

### Conclusion:

ELISA shows none detected of furazolidone and AOZ in cultured shrimp sold in Penang. This study shows cultured shrimp in Penang market is safe from the use of furazolidone and AOZ and safe to consume. Improvement can be made for this research by using boiled shrimp. Boiled sample is proved to obtain more reliable and more predictive results. However more sensitive method such as Liquid Chromatography can be used to confirm the presence and concentration of the furazolidone and AOZ in the sample. Further research on the presence nitrofurans and its metabolite in the cultured and farmed animal should be done to ensure the safety of the food consume in Malaysia. This is due to the report shows nitrofurans and other antibiotic have been added in

the cultured or farmed animals such as catfish, chicken and tilapia in other country.

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