Determination of Nitrofuran Metabolites in Aquatic Products

Government Laboratory

Introduction – Nitrofurans (硝基呋喃類)

- A **synthetic**, broad-spectrum **antimicrobial** agent.
- **Carcinogenic potential in animals**
- JECFA* considered that competent authorities should **prevent residues of certain kinds of nitrofurans** in food.
- **Prohibited** for use in food-producing animals in many countries.

Source: https://www.cfs.gov.hk/english/

* - Joint FAO/WHO Expert Committee on Food Additives

Introduction - CFS Press Release

Date	Matrix	Analyte	Concentration
2018-06-29	prepackaged frozen **tiger prawn**	AOZ	21 ng/g
2017-07-06	**Catfish**	SEM	NA
2017-06-06	fresh **shrimp**	AOZ	15 ng/g
2017-05-16	**Tiger Grouper**	AOZ	1.6 ng/g

Trace of nitrofuran metabolite found in prepackaged frozen tiger prawn sample June 29, 2018
https://www.cfs.gov.hk/english/press/20180629_6994.html

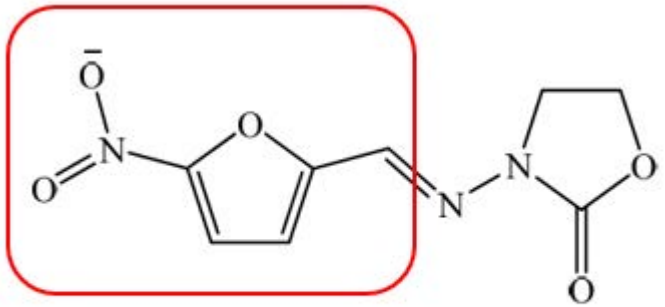
Nitrofurans Metabolites Residues in Catfish 6 Jul 2017
https://www.cfs.gov.hk/english/whatsnew/whatsnew_fst/whatsnew_fst_Nitrofuans_metabolites_residues_in_Catfish.html

CFS finds trace of nitrofuran metabolite in fresh shrimp sample June 6, 2017
https://www.cfs.gov.hk/english/press/20170606_0861.html

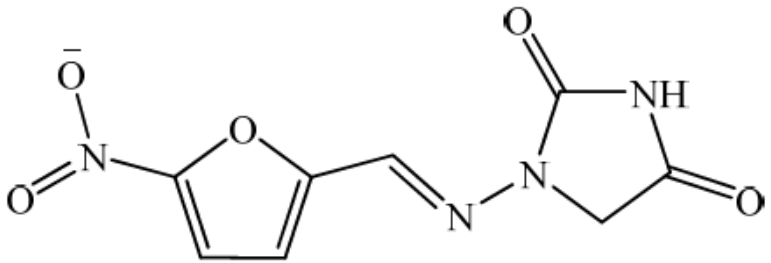
Nitrofurans in Tiger Grouper 16.5.2017
https://www.cfs.gov.hk/english/press/20170516_0846.html

Source: https://www.cfs.gov.hk/english/press/

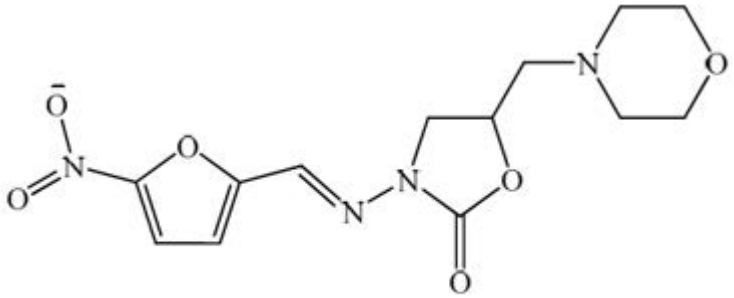
Introduction - Nitrofurans



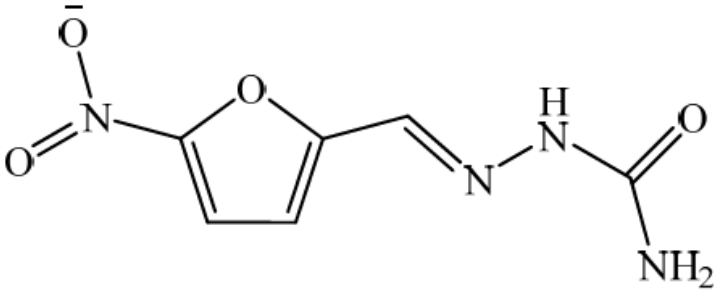
Furazolidone



Nitrofurantoin



Furaltadone

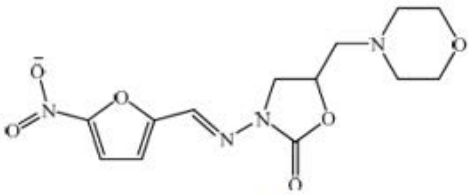


Nitrofurazone

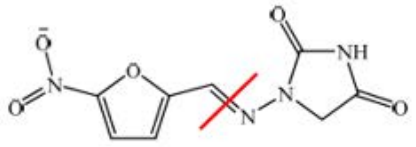
Common Nitrofurans and its metabolites

Parent Drug

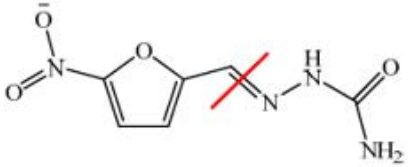
Furazolidone (呋喃唑酮)



Furaltadone (呋喃它酮)

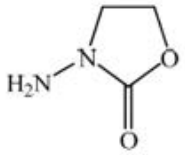


Nitrofurantoin (呋喃妥因)

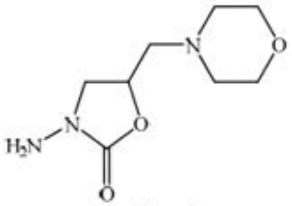


Nitrofurazone (呋喃西林)

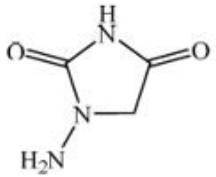
Monitored Marker Residue



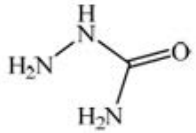
3-amino-2-oxazolidinone (AOZ)



3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ)



1-aminohydantoin (AHD)



Semicarbazide (SEM)

Regulatory Level of Nitrofurans - Hong Kong

CAP 132AF - Harmful Substances in Food Regulations

Press Release - CFS finds trace of nitrofuran metabolite in fresh shrimp sample

> "*Nitrofurans are a family of chemical compounds which have broad-spectrum antimicrobial activities. Although there is evidence suggesting that nitrofurans might cause cancer in animals, there is not yet sufficient data confirming that they can cause cancer in humans.* ***JECFA (Joint FAO/WHO Expert Committee on Food Additives) considered that competent authorities should prevent residues of certain kinds of nitrofurans in food.*** *Nitrofurans have been prohibited for use in food-producing animals in many countries*."

Source: https://www.cfs.gov.hk/english/press/20170606_0861.html

Regulatory Level of Nitrofurans - Codex

Risk Management Recommendations (RMR) for Residues of Veterinary Drugs

- Furazolidone (Metabolite: AOZ)
- Nitrofural (Metabolite: SEM)

"In view of the JECFA conclusions on the available scientific information, there is no safe level of residues of furazolidone or its metabolites in food that represents an acceptable risk to consumers. ***For this reason, competent authorities should prevent residues of furazolidone in food***. This can be accomplished by not using furazolidone in food producing animals."

Source: MRLs and RMRs for Residues of Veterinary Drugs in Foods, CX/MRL 2-2018

Regulatory Level of Nitrofurans - EU

Table 2	
Prohibited substances	
Pharmacologically active substance	MRL
Nitrofurans (including furazolidone)	MRL cannot be established

Source: COMMISSION REGULATION (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin

Standard / Published Method

- 中華人民共和國國家標準
  - GB/T 20752-2006　猪肉,牛肉,雞肉,猪肝和水產品中**硝基呋喃類**代謝物殘留量的測定
  - GB/T 21311-2007　動物源性食品中**硝基呋喃類**藥物代謝物殘留量檢測方法
- U. S. Food and Drug Administration
  - *Simultaneous Determination of **Nitrofuran Metabolites** and Chloramphenicol in Shrimp with a Single Extraction and LC-MS/MS Analysis*, Journal of AOAC International Vol. 98, No. 3, 2015
- National Measurement Institute Australia / National Institute of Metrology Thailand
  - Preparation and characterisation of certified reference materials for furazolidone and nitrofurazone metabolites in prawn, Accred Qual Assur (2015) 20:401–410

Guideline - CODEX

GUIDELINES FOR THE DESIGN AND IMPLEMENTATION OF NATIONAL REGULATORY FOOD SAFETY ASSURANCE PROGRAMME ASSOCIATED WITH THE USE OF VETERINARY DRUGS IN FOOD PRODUCING ANIMALS

CAC/GL 71-2009

Intended to provide the overarching principles and guidance for governments on the design and implementation of national and trade related food safety assurance programmes for residues of veterinary drugs.

Marker Residues - Target:

- *"Because the nitrofuran parent compounds can only be detected in animal tissues and products for a short period after treatment of the animals, monitoring of nitrofuran residues in livestock based on the identification of the parent compounds is not appropriate".*

Nitrofurans	Metabolites AMOZ, AHD, SEM, AOZ	Poultry Meat, Aquaculture products, Muscle/meat, Milk, Eggs	1 ppb MRPL for all

* - MRPL: Minimum Required Performance Limit

Source:

1. Community Reference Laboratories GUIDANCE PAPER (7 December 2007)
2. Scientific Opinion on nitrofurans and their metabolites in food, EFSA Journal 2015;13(6):4140

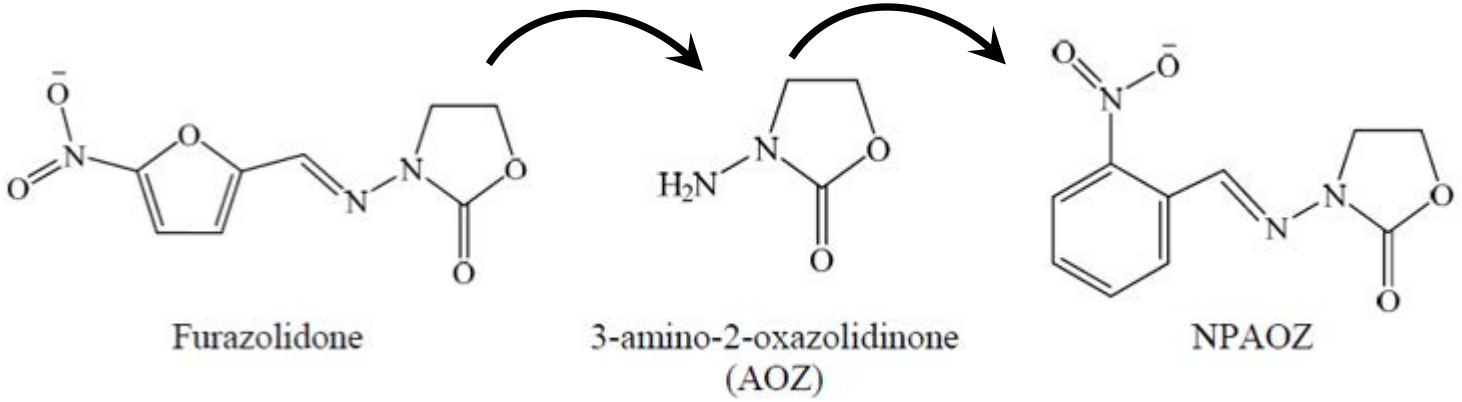
Methods - General Approach

1. Homogenization
2. Sample weighing
3. Hydrolysis & Derivatization
4. Extraction
5. Clean-up
6. LC-MS/MS

Hydrolysis & Derivatization:

1. Nitrofurans are rapidly metabolised, occurring in animal tissues as protein-bound metabolites
2. Acid hydrolysis and nitrobenzaldehyde derivatization of the released marker metabolites.

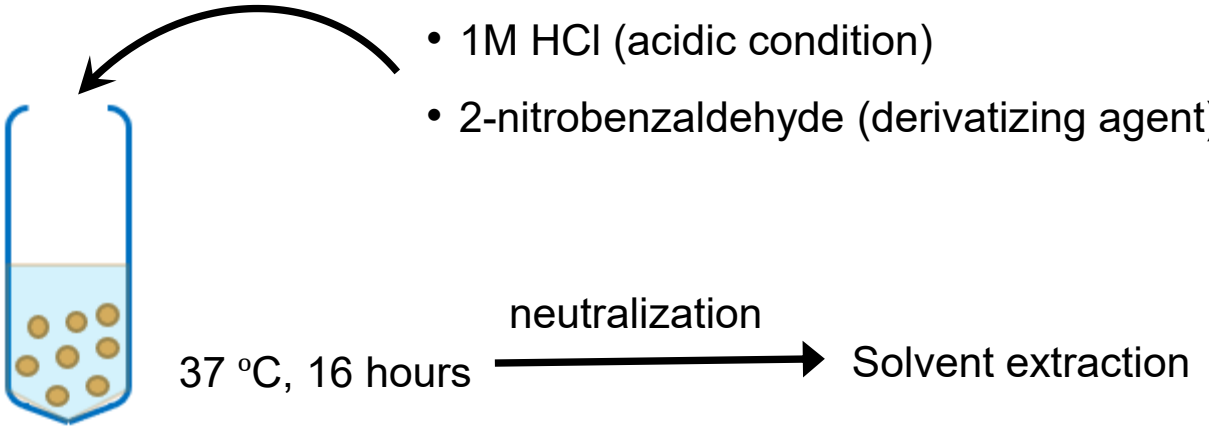
example:



Source: Scientific Opinion on nitrofurans and their metabolites in food, EFSA Panel on Contaminants in the Food Chain (CONTAM), EFSA Journal 2015;13(6):4140

Why derivatization?

- Condition: Acidic hydrolysis (1M HCl) and derivatization with 2-nitrobenzaldehyde
- Due to the relatively high noise in the low mass range and their nonspecific fragmentation behavior, the sensitivity of detection of the analytes by themselves is poor.
- After attaching the nitrophenyl moiety, their detection sensitivities by LC-MS/MS were improved.



Sample preparation for HPLC analysis

1. Homogenization
2. Sample weighing
3. Hydrolysis & Derivatization
4. Extraction
5. Clean-up : Liquid-Liquid Extraction / Solid Phase Extraction
6. LC-MS/MS

Experimental Condition of LC-MS/MS analysis - LC Condition

Injection volume	:	10 µL
Flow rate	:	0.3 mL/min
Column	:	Waters Acquity BEH C18 column (2.1 mm 9 100 mm, 1.7 µm)
Column temp.	:	50 °C
Mobile Phase / Solvent program	:	a gradient of acetonitrile (10 % to 29 % over 7 min) in aqueous 0.2 % formic acid

Source: *Preparation and characterisation of certified reference materials for furazolidone and nitrofurazone metabolites in prawn*, Accred Qual Assur (2015) 20:401–410

Experimental Condition of LC-MS/MS analysis - MS/MS condition

Nitrofurans*	MRM	
	Precursor ion (m/z)	Fragment ion (m/z)
AOZ	236	104
		134
AMOZ	335	262
		291
AHD	249	134
		178
SEM	209	166
		192

* - derivatized with nitrobenzaldehyde

Applications of the method - Aquatic Products

- Aquatic Animal Products
  - Fish
  - Shellfish
    - Molluscs
    - Crustaceans
- Derived Edible Products of Aquatic Animal Origin

Source: CAC/GL 71-2009

Internal Standard:

Isotope-labeled standard readily available:

- AOZ-d_4
- AMOZ-d_5
- AHD-$^{13}C_3$
- SEM-$^{15}N_2$, ^{13}C

Certified Reference Material:

Australia - NATA				
NMIA MX012B: Nitrofuran Marker Residues in Freeze-Dried Prawn				
Certified values				
Analyte	CAS No.	Mass fraction as supplied (ng/g)	Mass fraction reconstituted (ng/g)	Coverage Factor (k)
AOZ	80-65-9	30.2 ± 1.8	4.53 ± 0.27	2.09
SEM	563-41-7	70.3 ± 3.1	10.5 ± 0.5	2.23

Source: https://www.industry.gov.au/sites/default/files/nmi/chemical-reference/mx012b_2020_01.pdf

Proficiency Testing Program:

Proficiency test program is available from Fapas®.

Item Code	Matrix	Analytes	Product Code	Approx. Size
02438	Fish Muscle	nitrofuran metabolites	FCVD12-SEA13	20 g
02438b	Blank Fish Muscle	BLANK	BLVD12-SEA2	20 g

Analytes

AHD (bound), AHD (total), AMOZ (bound), AMOZ (total), AOZ (bound), AOZ (total), SEM (bound), SEM (total), Total Nitrofuran Metabolites

Source: www.fapas.com/

Sources of SEM other than nitrofurazone:

1. Natural occurrence of SEM has been reported in crustaceans (甲殼類): shrimps/prawns and crayfish

2. Azodicarbonamide
   - use as a blowing agent in foamed plastic gaskets
   - use as flour additive

3. Hypochlorite treatment - Carrageenan (卡拉膠, an extract from a red seaweed)

Source:

1. Scientific Opinion on nitrofurans and their metabolites in food, EFSA Journal 2015;13(6):4140

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Eliminating sources of SEM other than nitrofurazone:

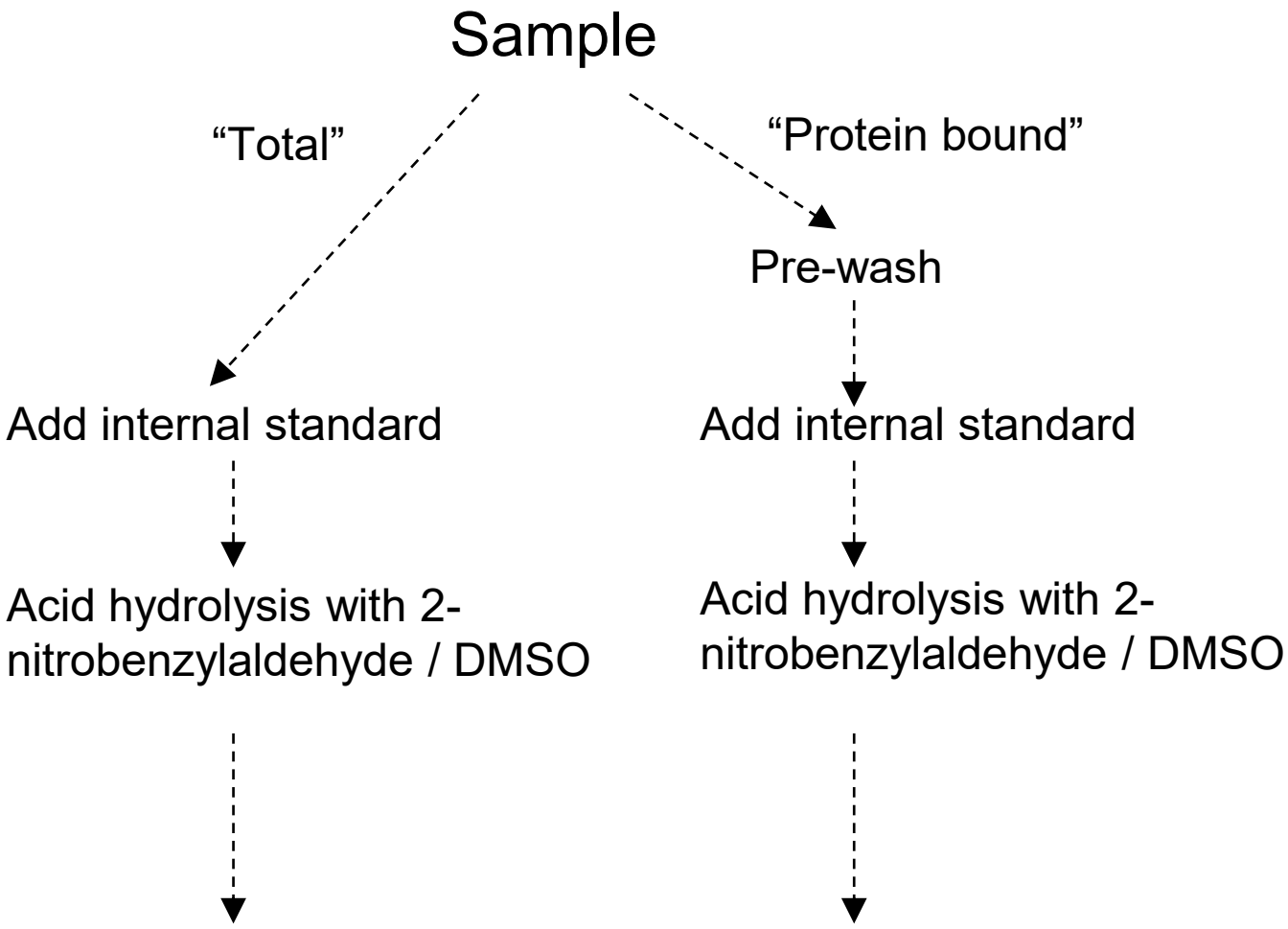
Purpose: Differentiate “protein-bound” SEM from “unbound” SEM

- Screening: “total” SEM (bound + unbound)

- Confirmation: Include a pre-washing step before analysis
  - Eg. Rinse with alcohol (GB/T 21311-2007)
    - 10 mL, 50% Methanol in water
    - Sonicate, Centrifuge, and Decant

Source:
1. GB/T 21311-2007 動物源性食品中硝基呋喃類藥物代謝物殘留量檢測方法

Eliminating sources of SEM other than nitrofurazone:



Nitrofuran Metabolites: Future

COMMISSION REGULATION (EU) 2019/1871

of 7 November 2019

on reference points for action for non-allowed pharmacologically active substances present in food of animal origin and repealing Decision 2005/34/EC

- Reference points for action: 1 ng/g ---> 0.5 ng/g
- Effective after 28 November 2022