Non-Targeted Approach towards Determination of Novel Markers for Nitrofurazone Abuse in Shrimp Farming

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Abstract:

Antibiotic use in farming operations can have repercussions when it comes to antibiotic resistant bacteria and to antibiotic residues consumed by the general population. Nitrofurazone is a synthetic broad-spectrum antibiotic of the nitrofuran family that has been banned since the early 1990's by most regulatory agencies due to increasing evidence of carcinogenicity. Due to the quick metabolism of nitrofurans by host bodies, their detection relies on the analysis of their common metabolites. In the case of nitrofurazone, semicarbazide is used to monitor its use in farm animals. However, it has been shown that semicarbazide can occur due to sources other than nitrofurazone abuse, and of particular importance, it can occur naturally in shrimp and other crustaceans. This comes to question the validity of semicarbazide analysis for nitrofurazone monitoring.

No studies had discussed the possibility of thermal degradation of nitrofurazone up to date. This study monitored the degradation of nitrofurazone at 100°C at two pH levels (7 and 1.3) with a "dilute and shoot" non-targeted methodology in an HPLC-QTOF system in an attempt to better understand the stability of the different bonds within the molecule. This allowed not only to verify that nitrofurazone does degrade significantly at 100°C, but also to monitor the appearance of different entities that could be correlated with thermal treatment. Ten compounds were found to increase with cooking time and their fragmentation patterns were studied to attempt to determine their structures. The Agilent Molecular Structure Correlator software provided matches for six of these ten compounds. For two of these there was structural similarity with the parent compound, and for one a theoretical degradation mechanism was developed to discuss the degradation of nitrofurazone into [(Furan-2-yl)methylidene]hydrazine. However, once a standard for this compound was obtained, its retention time and fragmentation pattern did not match the entity observed in the degradation experiment. Despite not confirming any entity with a standard, this experiment still discovered several masses that increase as thermal treatment of nitrofurazone increases.

A non-targeted metabolomic experiment was designed to compare the observed metabolites between shrimp treated with nitrofurazone and control shrimp using an HPLC-QTOF system. Four extractions were tested with the shrimp matrix and ultimately an acetonitrile/water

(80:20, v/v) extraction was selected for all sample analysis. Data treatment revealed multiple entities that increased with nitrofurazone treatment but ultimately a single molecule that was present in all treated samples and absent in all control samples was not found. This meant that a replacement for semicarbazide was not found using this extraction and data treatment but does not necessarily signify that it does not exist. More non-targeted extractions should be tested in the search of a replacement for semicarbazide. Despite this, the differences between control and treated shrimp were exploited to build a Partial Least Squares statistical model that could potentially differentiate between treated and control shrimp. Initial cross-validation of the model showed that it had up to 98% accuracy in differentiating between the sample groups, but when an external validation sample set was used to test the model, it was unable to differentiate between sample groups. This result is likely due to extraction and analysis variability and a larger sample set would be necessary to minimize this in order to build a more robust model. Overall, this work has tested the application of a non-targeted methodology for the discovery of a new metabolite to monitor NFZ abuse. Although this metabolite was not identified, it still allowed to rule out a solvent mix for this purpose and allowed to better understand the conditions necessary for the development of a robust model capable of differentiating between treated and control samples.

Résumé:

L'utilisation d'antibiotiques dans l'élevage animal peut avoir des répercussions quant au développement de bactéries résistantes aux antibiotiques et aux résidus d'antibiotiques qui seraient consommés par la population générale. Le Nitrofurazone est un antibiotique synthétique à large spectre de la famille des nitrofuranes qui a été interdit par la plupart des autorités sanitaires depuis le début des années 1990 à cause de preuves croissantes de leur cancérogénicité. À cause du métabolisme rapide des nitrofuranes par les organismes traités, leur détection repose dans l'analyse de leurs métabolites les plus communs. Dans le cas du nitrofurazone, le métabolite est le semicarbazide. Néanmoins, il a été prouvé que le semicarbazide peut être formé depuis sources autres que le traitement avec le nitrofurazone, et en particulier, il peut apparaitre de façon naturelle dans les crevettes et autres crustacés. Ces aspects remettent en question la validité de l'analyse du semicarbazide pour la surveillance du nitrofurazone.

À date, aucune étude n'a été publiée dans la littérature à propos de la dégradation thermique du nitrofurazone. Dans cette étude, la dégradation du nitrofurazone a été évaluée dans l'eau à 100°C a deux pH (7 et 1.3) par une méthodologie non-ciblée reposant sur l'injection directe dans un système HPLC-QTOF pour comprendre la stabilité des différentes liaisons de la molécule en question. Ceci a permis non seulement de vérifier qu'en effet le nitrofurazone se dégrade à 100°C, mais que plusieurs entités chimiques apparaissent en corrélation avec le traitement thermique. Dix composés dont l'intensité augmente avec le temps de cuisson ont été trouvés, et leur modèle de fragmentation a été étudié pour essayer de déterminer leurs structures. Le logiciel Molecular Structure Correlator d'Agilent a permis d'attribuer une structure pour six des dix composés. Pour deux d'entre eux, une relation avec la structure du nitrofurazone a pu être suggérée, incluant un mécanisme théorique qui aurait expliquer la dégradation du nitrofurazone en [(Furan-2-yl)methylidene]hydrazine. Néanmoins, une fois que le standard de cette molécule a été obtenu, le temps de rétention et le modèle de fragmentation ne correspondaient pas à ceux de la molécule trouvée dans la dégradation thermique. Bien que les

structures exactes ne puissent être présentement confirmées, cette expérience a mis en évidence plusieurs composés qui augmentent avec le traitement thermique de nitrofurazone.

Une étude non-ciblée a été conçue pour comparer les métabolites observés entre des crevettes traitées avec nitrofurazone et des crevettes du traitement contrôle, en utilisant un système HPLC-QTOF. Quatre extractions ont été testées dans la matrice des crevettes et ultimement une extraction avec acétonitrile/eau (80:20, v/v) a été choisie pour analyser les échantillons. L'analyse de données a révélé plusieurs entités qui augmentaient avec le traitement de nitrofurazone, mais ultimement aucune des molécules présente dans tous les échantillons traités n'est complétement absente dans tous les échantillons contrôles. Ceci veut dire qu'une alternative au semicarbazide n'a pas été trouvée utilisant la présente extraction et le traitement de données, même si cela ne veut pas dire qu'elle n'existe pas. D'autres extractions non-ciblées devraient être testées dans le futur. Par ailleurs, les différences dans les métabolomes extraits pour les crevettes traitées ont été exploitées pour développer un modèle à base de Partial Least Squares qui pourrait potentiellement différentier entre des crevettes traitées et des crevettes contrôles. Une validation croisée initiale a vérifié que le modèle avait une exactitude de jusqu'à 98% pour différentier les types d'échantillons, mais quand une validation avec un groupe externe fut testée, il ne pouvait plus différentier les groupes d'échantillons. Ceci est probablement à cause de la variabilité dans l'extraction et dans l'analyse de données et un groupe d'échantillonnage plus grand pourrait minimiser cette erreur et permettrait de construire un modèle plus robuste. Globalement, ce travail a testé l'application d'une analyse non-ciblée pour la découverte d'un nouveau métabolite pour la surveillance du nitrofurazone. Même si ce métabolite ne fut pas identifié, cela a permis d'exclure ce mélange de solvants pour ce propos et de mieux comprendre les conditions nécessaires pour le développement d'un modèle robuste capable de différencier entre des échantillons traités et contrôle.

Contribution of Authors:

The contribution of authors and their contribution to the different articles is as follows:

Pablo Elizondo is the MSc candidate who designed and carried out all experiments besides verification of SEM levels in shrimp shells for Chapter 3 in consultation with the supervisor. He performed all data analysis and prepared all the manuscripts for revisions by the supervisor. He performed the literature review in Chapter 1.

Dr. Stéphane Bayen is the thesis supervisor under whose guidance all the research was conducted. He received and revised drafts of all the manuscripts for publication.

Richard Zhu was the summer undergraduate student who carried out the experiments for measuring semicarbazide levels in shrimp shells for validation in Chapter 3 under the supervision of Pablo Elizondo.

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List of Abbreviations:

ACN: acetonitrile
ADC: azodicarbonamide
AH: 1-aminohydantoin
AM: D3-acetylmorphine
AMOZ: 3-amino-5-morpholinomethyl-1,3-oxazolidone
AOZ: 3-amino-2-oxazolidone
APCI: atmospheric pressure chemical ionization
CPD: compound
DMF: dimethyl formamide
DMSO: dimethyl sulfoxide
DNPH: 2,4-dinitrophenylhydrazine
DPH: D3-diphenylhydramine
EC: European Commission
EIC: extracted ion chromatogram
ESI: electrospray ionization
EtOAc: ethyl acetate
EU: European Union
F2MH: [(Furan-2-yl)methylidene]hydrazine
FTD: furaltadone
FZD: furazolidone
GC-MS: gas chromatography – mass spectrometry
HCl: hydrochloric acid
HPLC: high performance liquid chromatography
HRMS: high resolution mass spectrometry
IARC: International Agency for Research on Cancer
LC-MS: liquid chromatography – mass spectrometry
LDA: linear discriminant analysis
MC: micronucleus
MPP: mass profiler professional software
MRL: maximum residue levels
MRPL: minimum required performance limit
MSC: molecular structure correlator software
NaCl: sodium chloride
NaOH: sodium hydroxide
NBA: 2-nitrobenzaldehyde
NF: 5-nitro-2-furaldehyde
NFT: nitrofurantoin
NFZ: nitrofurazone
OPLS-DA: orthogonal partial least squares – discriminant analysis
PCA: principal component analysis
PEG: polyethylene glycol
PLS-DA: partial least squares – discriminant analysis

QTOF: quadrupole-time-of-flight
QuEChERS: quick easy cheap effective rugged and safe
RASFF: Rapid Alert System for Food and Feed
RRF: relative response factor
RT: retention time
SEM: semicarbazide
SPE: solid phase extraction
SRM: single reaction monitoring
TBE: targeted batch extraction
TIC: total ion chromatogram

Introduction:

With the growing world population, a higher demand for food products has led to an upscaling of farm operations. This has been observed in all areas of agriculture, including the switch from fishing to aquaculture. However, the increased density of production has in turn led to new problems, in particular the need to control disease and infections in farmed animals and crops. The use of veterinary drugs and pesticides in food production has been a way to improve outputs and reduce disease in large scale crop and animal production. In some cases, these drugs are even used for their "growth-promoting" or "prophylactic" properties (Hoenicke et al. 2004). This means that they can be pre-emptively used to promote healthy animal growth without observed infection or symptoms. This has begun to worry some in the scientific and public health communities as to the possibility of generating antibiotic resistant microorganisms, and the implications that this may have in human health (Samanidou and Evaggelopoulou 2007).

The increase in veterinary drugs use has also peaked curiosity in these sectors since the turn of the 20th century as to what traces or metabolites of the used drugs might still be present in the product that is being eaten by consumers. Tests were then usually run to assess the toxicity of the different parent drugs and their metabolites *in vitro* and *in vivo*. The results of such occurrence and toxicity studies are then often used by governing and importing bodies to establish maximum residue levels (MRL) for different compounds. When a drug is deemed as not safe for humans, there can also exist a "zero-tolerance" for given metabolites (Love et al. 2011). In the late 2000's there was an increase in non-compliant imported seafood samples worldwide, but since total analysis numbers were not always available, it is possible that the increase in veterinary drug violations was merely a result of increased frequency of testing, as well as more sensitive laboratory analysis techniques (Love et al. 2011).

Nitrofurans are antimicrobial agents that have been banned from use since the early 90's in most Western countries. However, their effectiveness and availability result in many farming and aquaculture operations continuing to use them. The consequence of this is that regulatory agencies must continue to monitor the abuse of these "zero-tolerance" antibiotics in local and imported products. Since nitrofuran drugs metabolize rapidly in animals (Vass, Hruska, and Franek 2008; Chu et al. 2008), efforts have been made to develop analytical methods to monitor

their main metabolites. Semicarbazide (SEM) is currently the metabolite used by regulatory agencies to monitor the abuse of nitrofurazone (NFZ), but in recent years it has been shown to occur naturally in shrimp and other crustaceans (Saari and Peltonen 2004; Van Poucke et al. 2011). This has led to a concern in the occurrence of false positives during analysis. The purpose of the present study was to use a non-targeted approach to detect a new metabolite capable of replacing SEM, or to develop a new methodology that would be capable of differentiating between shrimps treated with NFZ and untreated shrimps.

Chapter 1: Literature Review of Nitrofurazone and Semicarbazide

1.1 Nitrofuran Drugs:

Nitrofurans are a group of synthetic broad-spectrum anti-microbial agents that have 5-nitro-2-furaldehyde as a common moiety. The family includes the drugs nitrofurazone, furaltadone, furazolidone, and nitrofurantoin amongst others. These drugs are effective anti-microbial agents and were routinely used in farming and aquaculture during the second half of the 20th century (Stadler et al. 2015). Given their instability, importance has been given to the breakdown products of the parent compounds, in particular to the side chains of the 5-nitro-2-furaldehyde ring which differentiate the different antibiotic agents of this class (Vass, Hruska, and Franek 2008). The structures of the four most common nitrofurans and their main metabolites are shown in Figure 1.1.

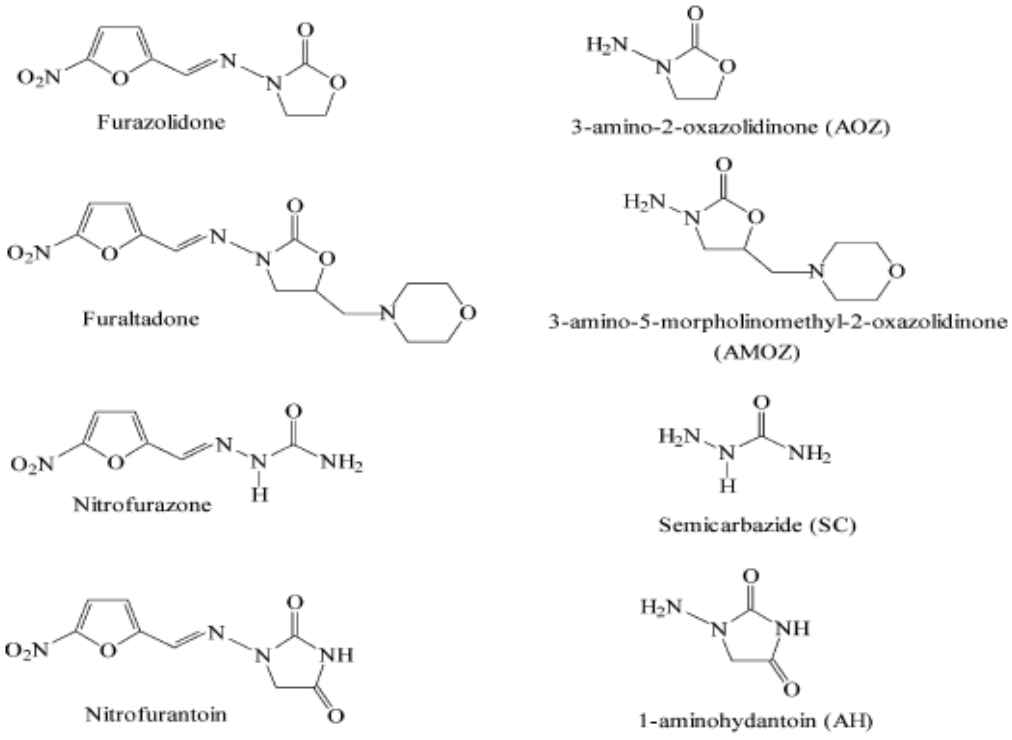


Figure 1.1: Structure of the four main Nitrofuran antibiotics Furazolidone (FZD), Furaltadone (FTD), Nitrofurazone (NFZ), and Nitrofurantoin (NFT) and their main metabolites 3-amino-2-oxazolidone (AOZ), 3-amino-5-morpholinomethyl-1,3-oxazolidone (AMOZ), 1-aminohydantoin (AH), and semicarbazide (SEM) Reproduced from (Chu and Lopez 2005)

1.1.1 Occurrence:

In their review of occurrence of veterinary drug residues in different seafood products by four inspecting bodies throughout the early 21st century, Love et al. (2011) determined a high percentage of non-conforming samples being due to nitrofuran drugs. Their results are summarized in Figure 1.2 below. In their review of nitrofurans, Vass et al. (2008) summarize the data from the Rapid Alert Systems for Food and Feed (RASFF) of the EU between 2007 and 2008 with regards to non-compliance due to nitrofuran abuse in different products. The most common infractions are due to AOZ or SEM detection in shrimps or prawns (41.5% and 36.4% respectively). Their results are summarized in Figure 1.3 below. More recent reports on nitrofuran contamination in seafood are not available. As it can be seen from both these figures, the cases of nitrofuran contamination are prevalent in the context of shrimp, and high frequency of SEM and AOZ suggest an abuse of nitrofurazone and furazolidone respectively.

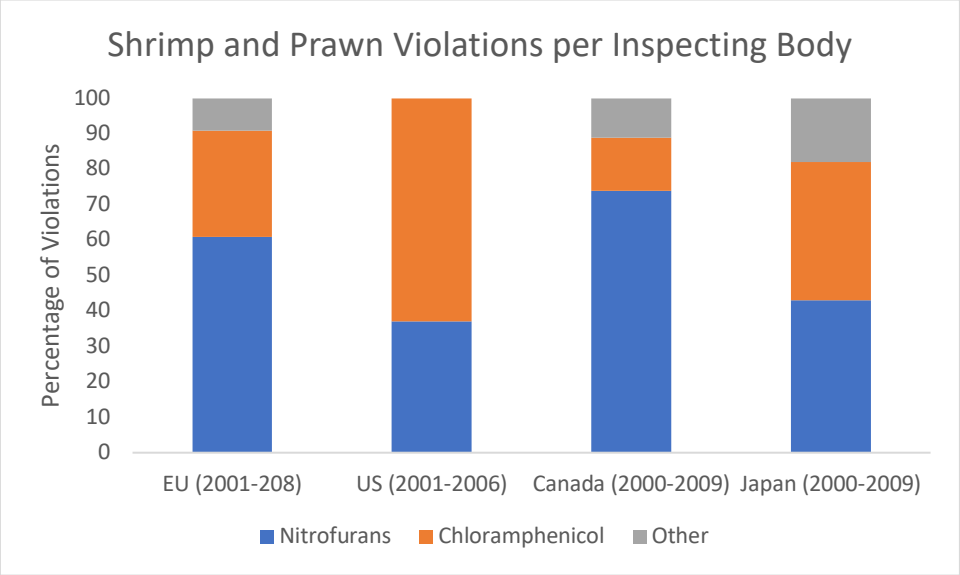


Figure 1.2: Percentage of nitrofuran and chloramphenicol violations in shrimps and prawns from four different inspecting bodies: EU (n=545), US (n=27), Canada (n=239), Japan (n=205) (Love et al. 2011)

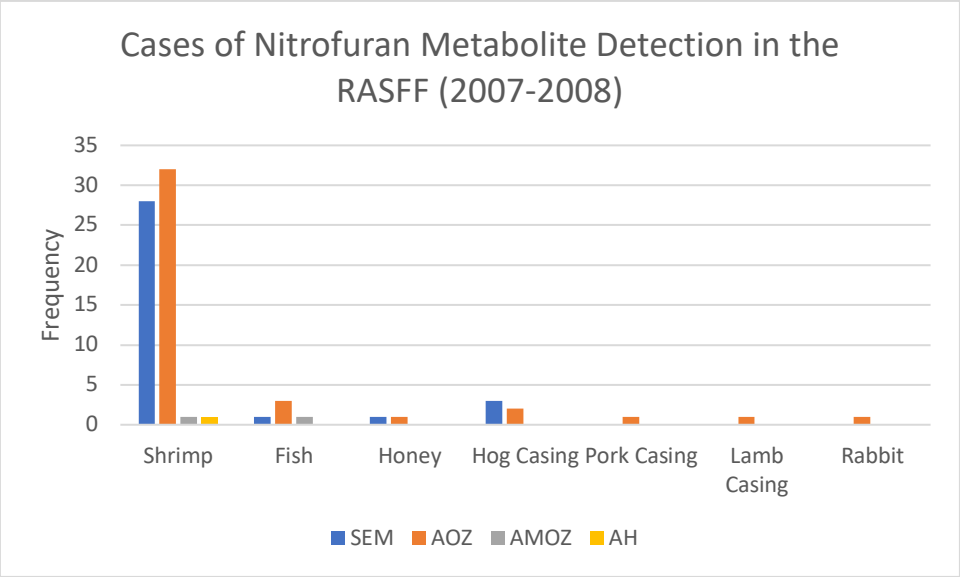


Figure 1.3: Summary of nitrofuran non-compliant samples by food product from RASFF between 2007 and 2008 (Vass, Hruska, and Franek 2008)

Nitrofurazone (NFZ) will be the focus of this literature review in light of the current issues associated with its detection in food products, discussed in Section 1.4 of this review. It is a pale-yellow crystalline powder with melting point/degradation at 240°C, limited solubility in water, and molecular mass of 198.14 g/mol (HSDB 2003). Its most known metabolite, semicarbazide (SEM), is a white crystalline powder with melting point/degradation at 175-185°C, very soluble in water, and a molecular mass of 111.54 g/mol (HSDB 2005). NFZ is administered to treat gastrointestinal and dermatological infections including salmonellosis in chicken farming (Stadler et al. 2015). It has been particularly used in shrimp and poultry farming for its growth-promoting properties and to prevent bacterial infections (Van Poucke et al. 2011).

1.1.1 Toxicity:

In the late 1970's and early 1980's several studies began to show the possible carcinogenic side effects of some of these drugs and their metabolites. It appears to be the nitro group that is responsible for their mutagenic and carcinogenic properties, and it has been demonstrated that enzymatic reduction of its structure is necessary before mutations are observed (Olive and McCalla 1977). These researchers suggested that tissues with higher

concentration of nitrofuran-reducing enzymes such as the liver and kidney would be more susceptible to nitrofuran toxicity. They also noted that decreasing oxygen concentrations in the cell suspensions led to significantly higher degrees of toxicity, with highest toxicity was observed in anaerobic conditions under nitrogen (Olive and McCalla 1977).

In his review, McCalla (1983) identified four main pathways by which the nitro group can become genotoxic in mammals and cultured mammalian cells. Principally, reduction by type I and II nitroreductase, formation of superoxide, and co-oxidation by prostaglandin synthetase. High doses of nitrofurazone (750ppm in feed over 15 weeks) have also been associated with a 98% decrease in sperm counts in Swiss CD1 mice (Vass, Hruska, and Franek 2008). However, the limited amount of carcinogenic data on animals and inadequate evidence in humans made NFZ be classified as a group 3 carcinogen by the IARC: “not classifiable as to its carcinogenicity to humans” (IARC 1997).

Given the quick degradation of the parent compound, it is important to consider the available data on toxicity of its most analyzed metabolite: SEM. In their review on SEM, Tian et al. (2014) discuss the toxicological data with respect to cumulative toxicity, chromosome aberration toxicity, endocrine toxicity, and genotoxicity. They conclude that SEM provides “moderate accumulation of toxicity” (Tian, Sang, and Wang 2014) and gave positive results in the chromosome aberration test, revealing some mutagenic activity. However, SEM resulted in no statistically significant differences in micronucleus (MC) formation and is not considered genotoxic. Hirakawa et al. (2003) also looked at DNA damage caused by SEM in the presence of Cu(II). They mention SEM showed no or little mutagenicity in the *Salmonella*-microsome test, but that oxidative DNA damage was observed in the presence of Cu(II). They attribute this to the formation of nitrogen radicals derived from the SEM but had no conclusive evidence to show it. Similarly to NFZ, the IARC classified SEM as a group 3 carcinogen due to lack of evidence, but this has not been reviewed since the mentioned studies were published (IARC 1998). Despite this, there seems to be a general consensus that the estimated exposure levels of SEM are well below concentrations discussed in carcinogenic effect studies and it does not pose a major risk for humans (Radovnikovic et al. 2013).

1.1.2 Regulations:

The fear of the carcinogenic evidence led the European Commission (EC) to classify nitrofurans in their list of pharmacologically active compounds for which maximum residue levels (MRLs) cannot be set in 1990, and finally to ban them entirely in 1993 for use in livestock animals and aquaculture products (EC 1990, 1993). A similar ban was observed in Canada in 1994 where 5-nitrofuran compounds were banned for use in any food-producing animal (Canada 2017). Once the regulations were passed it became necessary to develop standardized analytical methods for the screening of these drugs in food products.

Many authors identified a problem with the analysis of the parent compounds in food samples given their instability. Zhang et al. (2017) associated the quick depletion to low stability and photosensitivity of the parent compounds. In their review, Vass et al. (2008) stated that analysis of parent nitrofuran structures in food products was not adequate for evaluation of contamination and risk. Other studies have confirmed that despite the parent drugs depleting in less than 24 hours from the test animal's system, the metabolites remained in the system and were detectable up to 56 days after a single dose (Chu et al. 2008). For this reason, most analytical methods were developed to analyze for the metabolites of the parent drugs using the side chains that differentiate each drug as the analyte. For the four major nitrofurans screened: FZD, FTD, NFT, and NFZ; the four analyzed metabolites are AOZ, AMOZ, AH, and SEM respectively (Vass, Hruska, and Franek 2008). In 2003, the EC set a minimum required performance limit (MRPL) for the analysis of nitrofuran metabolites of 1 μg/kg (EC 2003), and anything that is non-compliant according to these specifications is discarded.

1.1.3 Controlled Exposure Studies and Stability:

Several tests have been run on tracking nitrofuran administration and metabolite formation in different matrices, but special attention has been given to administration to aquatic animals in this review. In cases where the drug was administered via water bath, the concentrations used were between 10 mg/L for 8 hours (Chu et al. 2008) and 50 mg/L changing water daily for one week (Van Poucke et al. 2011). Only two studies using oral administration were found. In the first, the four main nitrofuran drugs were dissolved in DMF and diluted with

polyethylene glycol (PEG) to 2 mg/mL and were given to catfish in the form of a gel capsule at the dose of 1 mg/kg body weight (Chu et al. 2008). The second case was for furazolidone, but proposed an interesting oral dosing method, in which 4 g of furazolidone powder were mixed with 1 kg of shrimp feed, and the medicated feed was then coated with 30g of water, and then with 30 g of cod liver oil before being frozen at -20°C until being used (Douny et al. 2013). The shrimp were then fed medicated feed four times a day for 7 days, followed by commercial feed for another 28 days prior to sampling (Douny et al. 2013). The corresponding measured metabolite concentrations with these doses are summarized in Table 1.1 below. The study by Douny et al. (2013) was not included in this table due to lack of a calculation determining dose concentration (lack of control of how much feed was given to each shrimp).

Reference	Nitrofuran Drug	Mode of Administration	Administered Dose	Time After Measurement	Metabolite Concentration	Units
Chu et al. 2008	FZD	Water	10mg/L 8hrs	NA	203	ng/g
Chu et al. 2008	NFZ	Water	10mg/L 8hrs	NA	18	ng/g
Chu et al. 2008	FTD	Water	10mg/L 8hrs	NA	36	ng/g
Chu et al. 2008	NFT	Water	10mg/L 8hrs	NA	2	ng/g
Van Poucke et al. 2011	NFZ	Water	50mg/L 1 week	NA	>50	µg/kg
Chu et al. 2008	NFZ	Oral	1mg/kg bw	1 day	31.1	ng/g
Chu et al. 2008	NFZ	Oral	1mg/kg bw	7 days	11	ng/g
Chu et al. 2008	NFZ	Oral	1mg/kg bw	10 days	15	ng/g
Chu et al. 2008	NFZ	Oral	1mg/kg bw	14 days	12.5	ng/g
Chu et al. 2009	NFZ	Oral	1mg/kg bw	28 days	11.5	ng/g
Chu et al. 2010	NFZ	Oral	1mg/kg bw	56 days	8	ng/g

Table 1.1: Summary of Correlation between dose and measured metabolite concentration

One study by Cooper et al. (2007) measured the stability of the different nitrofuran drugs in tissues and in standard solutions over 10 months. For the purpose of this review, only the data on SEM will be reported. They stored both pig liver and muscle tissues with known metabolite concentrations at -20°C for 8 months and measured metabolites at 2, 4 and 8 months. There was no significant decrease in concentration over the 8 months (remained within 95% of the original value). The standard solutions were prepared in two different concentrations (1.0 mg/mL and 10.0 ng/mL) in methanol and were stored at 4°C for 10 months. Measurements were done at 2, 6, 8 and 10 months. There was no significant decrease of the stock solution at 1.0mg/ml over the 10 months, but the working solution at 10.0 ng/mL showed a drop in concentration after storage

at this temperature, where the line of regression predicts a 5% drop in concentration after 4 months (Cooper and Kennedy 2007). For this reason, the authors suggest that stock solutions be kept for no longer than 10 months at 4°C, and that working solutions be kept for no longer than 4 months (Cooper and Kennedy 2007). Stability studies at lower temperatures or during longer storage times were not found.

Studies have been run by several scientists in an attempt to discover the photodegradation products nitrofurazone. The first approach was done by Quilliam et al. (1987) by treating NFZ solutions in water with fluorescent and tungsten lamps for different times and analyzing the results with HPLC-UV. They found that there were two main products observed, the first being the cis isomer of NFZ, and the second was bis(5-nytrofurylidene) azine. De Luca et al. (2010) further explored the kinetics of photodegradation. They concluded that NFZ quickly isomerizes to its syn counterpart, and that that in turn undergoes degradation to a series of colored compounds which were difficult to characterize. They also note that an increase in the rate constant of degradation is observed with increasing light power and with decreasing NFZ concentration. Thermal degradation studies were not found for this compound and will be the first step in the development of the proposed project. Tian and Bayen (2018) have discussed this type of methodology in their analysis of thermal degradation products of chloramphenicol.

1.1.4 Current Issues:

Despite being banned in most countries, nitrofurans are still used by farmers in some places due to their efficacy and affordability. For this reason, many border control and regulatory agencies monitor imported products for metabolite residues. Several articles discuss that the number of non-conforming samples with respect to most nitrofurans have decreased since the legislations were put into place in the mid 1990's, but this does not hold true for nitrofurazone and its metabolite semicarbazide (Radovnikovic et al. 2013; Stadler et al. 2015). In their review about nitrofurans, Vass et al. (2008) show tables of the notifications of the Rapid Alert Systems for Food and Feed (RASFF) for nitrofurans in Europe between 2007 and 2008, and 36.4% of the cases were due to SEM detection in shrimp.

Natural Occurrence of SEM in Aquaculture Products:

NFZ and its metabolite SEM have recently become a cause for concern within the scientific community due to reports showing the occurrence of SEM in products where there is no evidence nitrofurazone abuse. One of the first of such studies was the discovery of naturally occurring SEM in samples of crayfish in Finland (Saari and Peltonen 2004). In this study, the authors first observed SEM in cooked store-bought crayfish samples and decided to obtain samples from non-professional fishermen who caught crayfish from rivers not near aquaculture farms. They were surprised to find that even in these samples, where nitrofurazone abuse could be discounted, they were still finding levels of SEM above the MRPL of 1 μg/kg set by the European Commission. It is confusing that these authors did not test for the occurrence of SEM in raw samples, particularly since in their conclusions they state that the question of whether SEM is formed during the cooking process "remains unanswered" (Saari and Peltonen 2004). A simple run of their procedure without the cooking step could have answered their lingering question, and reviewers should have pointed that out.

Throughout the beginning of the 21st century, more reports of the natural occurrence of SEM in aquaculture products came to light. In particular, there has been a rise of shrimp and crustacean products where SEM has been detected. Van Poucke et al. (2011) looked into the possibility of the SEM detected originating from the shell of *Macrobrachium Rosenbergii* prawns. They observed that Belgium was the country with highest number of SEM detections within the EU, and that they were also the only member state where the whole shrimp, rather than just the muscle, was being analyzed. After analyzing whole and peeled shrimp for SEM, they concluded that, although the mechanism was unknown, the shell did contain a higher concentration of SEM than the muscle tissue. McCracken et al. (2013) further explored this research topic by measuring the distribution of SEM in the muscle of shrimp. They also explored the effect of location of shrimp fishing with respect to aquaculture farms in the river to determine if the occurrence of SEM could be due to residual nitrofurazone from aquaculture farms upstream from the fishing sites. They concluded that location showed no statistically significant difference in SEM concentrations, but that there was a significant difference in the concentration of SEM between the inner and outer muscle meat. They suggest that there could exist migration from the

epidermis to the meat even when the exoskeleton is removed prior to analysis. These authors hypothesize that shrimp may ingest high amounts of "unusual amino acids from algae" (McCracken et al. 2013) like gigartinine which they have shown can yield small amounts of SEM when reacted under conditions used to analyze for SEM.

Prompted by the two previous studies and a growing concern of the non-specificity of SEM as a marker for nitrofurazone, Hui et al. (2015) studied the concentrations of SEM in *Macrobrachium Nipponese* prawns throughout their growth stages. For this experiment they bought egg-carrying prawns from a professional aquafarm where culturing was carried out under standard conditions. The larvae hatched and were held in three separate tanks to have three sample replicates. Larvae samples were collected at 1, 4, 6, 9, 12, 15 days and analyzed for SEM content separately in head, muscle, and shell at each stage. Their results showed two trends: the concentrations of SEM are highest in the shell, then the head, and lowest in the muscle; and the total concentration of SEM increased significantly between the 12^{th} and 15^{th} days and is associated with the metamorphosis of larvae into post-larvae and the appearance of the shell (Hui, Zhang, and Yu 2015). This conclusion is in accordance with the results observed by the two previous studies mentioned, and further suggests that SEM is not the most appropriate metabolite for monitoring NFZ use in shrimp products.

Other Sources of SEM in Food Products:

On top of all the natural occurrence studies, several studies began to show that SEM presence in food samples could be due to contamination from other compounds. Hoenicke et al. (2004) studied the effect of hypochlorite treatment on formation of SEM. After finding that the concentration of SEM in carrageenan increased significantly during the bleaching step, they decided to test the effect of hypochlorite on other samples. They tested the formation of SEM in various food products (eg. shrimp, chicken, milk, gelatin, carrageenan, etc.) at 4 hypochlorite concentrations (0.015%, 0.05%, 1%, and 12% active chlorine) as well as a non-treated sample. Their results show that there is little to no difference in SEM content up until the 1% active chlorine samples, and a significant increase in the 12% active chlorine treated samples for most of the food products. Following these results, they decided to treat solutions of pure nitrogen-

containing substances with hypochlorite at 0.015% and 12% active chlorine to attempt to determine which of them could result in SEM formation. Figure 1.4 summarizes the results from this section of their experiment. Interestingly, they showed that solutions of arginine and creatine could degrade and form SEM with no hypochlorite treatment (Hoenicke et al. 2004). This research is of particular importance with respect to shrimp since guidelines on shrimp farming suggest the use of hypochlorite as a sanitizing agent during tank cleaning between harvests (Joshua et al. 2002).

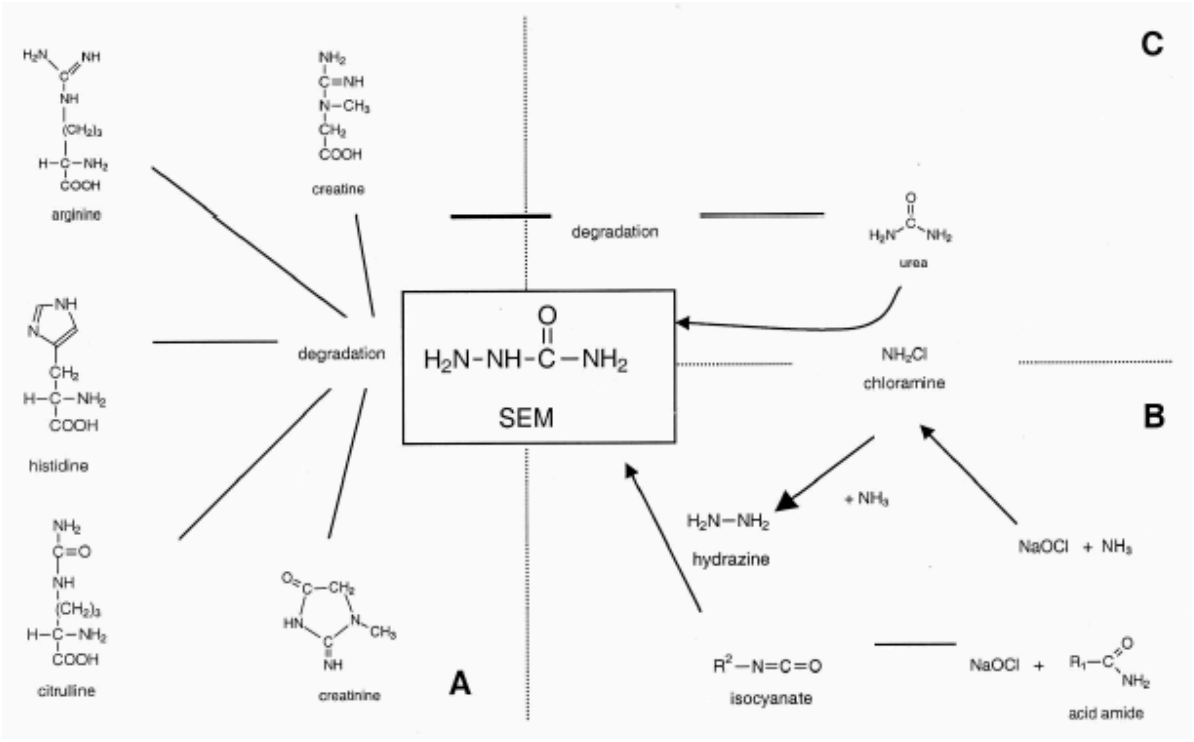
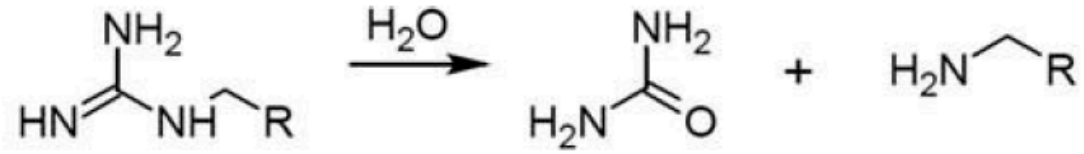
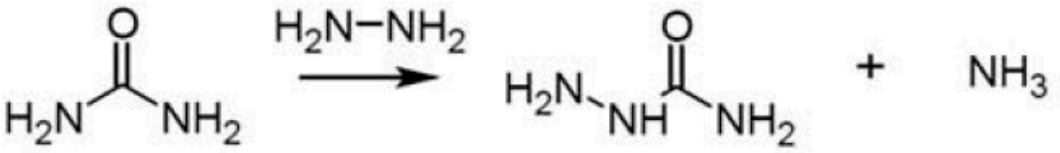


Figure 1.4: Reactions with hypochlorite leading to SEM formation from several nitrogen-containing species. (A) shows SEM formation from degradation of nitrogen-containing species with amidino or ureido residues, (B) shows reaction of hydrazine with isocyanate, and (C) shows reaction of urea with chloramine. Reproduced from (Hoenicke et al. 2004)

Azodicarbonamide (ADC) has also been identified as a source of SEM in food products. It is commonly used as a foaming agent for sealants and as an additive for flour bleaching and maturing (Pereira, Donato, and De Nucci 2004). It has been shown that under heat treatment, ADC can decompose into gases, primarily nitrogen and carbon dioxide, and leave behind residues

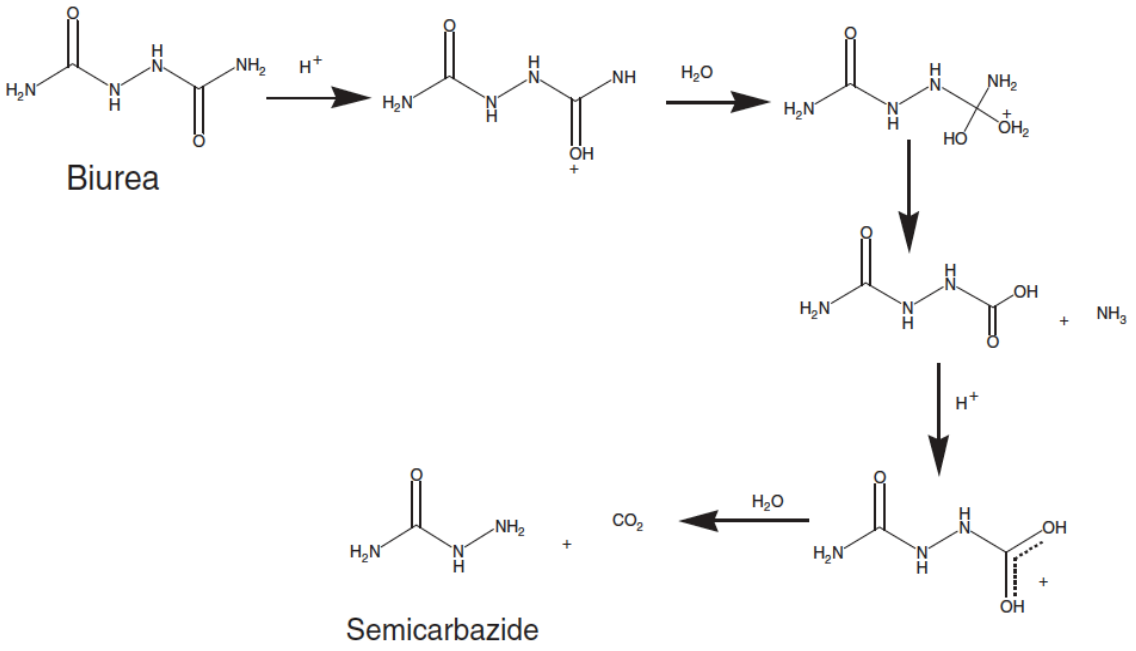
of biurea, urazole, and cyanuric acid (Vass, Hruska, and Franek 2008). Several researchers have identified biurea as a precursor for SEM and have proposed mechanisms for the reaction. Stadler et al. (2015) have proposed a Hofmann-like rearrangement in dairy products where a guanidinum group undergoes hydrolysis/oxidation to afford urea. Urea can then react with hydrazine to afford SEM and ammonia. This mechanism can be applied in part to biurea from ADC assuming cleavage of nitrogen-nitrogen bond in acidic conditions and is illustrated in Figure 1.5a. Pereira et al. (2004) proposed a different mechanism for the formation SEM in ADC treated flour via two hydrolysis reactions of biurea, releasing ammonia, and finally carbon dioxide to afford SEM. This mechanism is illustrated in figure 1.5b. Chu et al. (2005) found that after spiking shrimp samples with SEM, and adding a pre-washing step to SEM analysis, about 95% of non-tissue-bound SEM was removed. This still means that traces of SEM from non-bound sources can be observed despite a pre-washing step. However, Hoenicke et al. (2004) also mention the fact that SEM is known to "react rapidly with substances harbouring a carbonyl moiety" (Hoenicke et al. 2004) and this suggests that some fraction of SEM from other sources such as hypochlorite treatment or ADC treatment might end up bound to the shrimp tissue and not be affected by a pre-washing step.





urea SEM

a)



b)

Figure 1.5: Mechanisms of SEM formation from urea (a) Reproduced from (Stadler et al. 2015) and from biurea (b) Reproduced from (Pereira, Donato, and De Nucci 2004)

A new study run by Tian et al. (2016) has tried to map the SEM concentrations in different parts of Laizhou bay in western China. This is a large aquaculture zone and is the "chief-shellfish-producing region in China" (Tian et al. 2016). To do this they established 15 locations within the bay and took water and sediment samples at each of them. They took shellfish samples at most but due to uneven fishing conditions they were unable to get shellfish samples for all 15 locations. To date, this is the only paper that has analyzed the dispersion of SEM in the environment and the direct effect on the bioaccumulation of it in animals of the area. Their results showed that there are higher concentrations of SEM near estuaries and they attributed this to human activities upstream in the rivers, including agricultural, industrial, and domestic settlements. They also observed that SEM concentrations in shellfish were both generally higher than water concentrations, and higher in locations where the water SEM concentration was higher. These results suggested that there is a correlation between water SEM content and degree of bioaccumulation in shellfish and is the first attempt to show this in literature (Tian et al. 2016). Further tests should be run in regions where natural occurrence of SEM in shrimp and other

animals has been observed in an attempt to rule out the effect of water contamination from the analysis.

What all the examples in this section have in common is that SEM has been detected and quantified in samples where the illegal use of NFZ could be discounted. All this information is briefly summarized in Figure 1.6 below. The piling evidence brings us, as well as many of the previously cited authors, to the conclusion that SEM should no longer be considered an appropriate marker for NFZ abuse. The advances in analytical methods and data analysis software, as well as a better understanding of the metabolomics approach to food safety (coined "foodomics" by Alejandro Cifuentes in 2009) have allowed for a non-targeted analytical approach to determining new metabolites and simultaneous multi-residue analyses. The purpose of this project is to apply the non-targeted metabolic fingerprinting analytical approach to NFZ treated *Penaeus Vannamei* shrimp and compare the results to a control group in an attempt to discover a new metabolite to use as a marker for NFZ abuse in aquaculture.

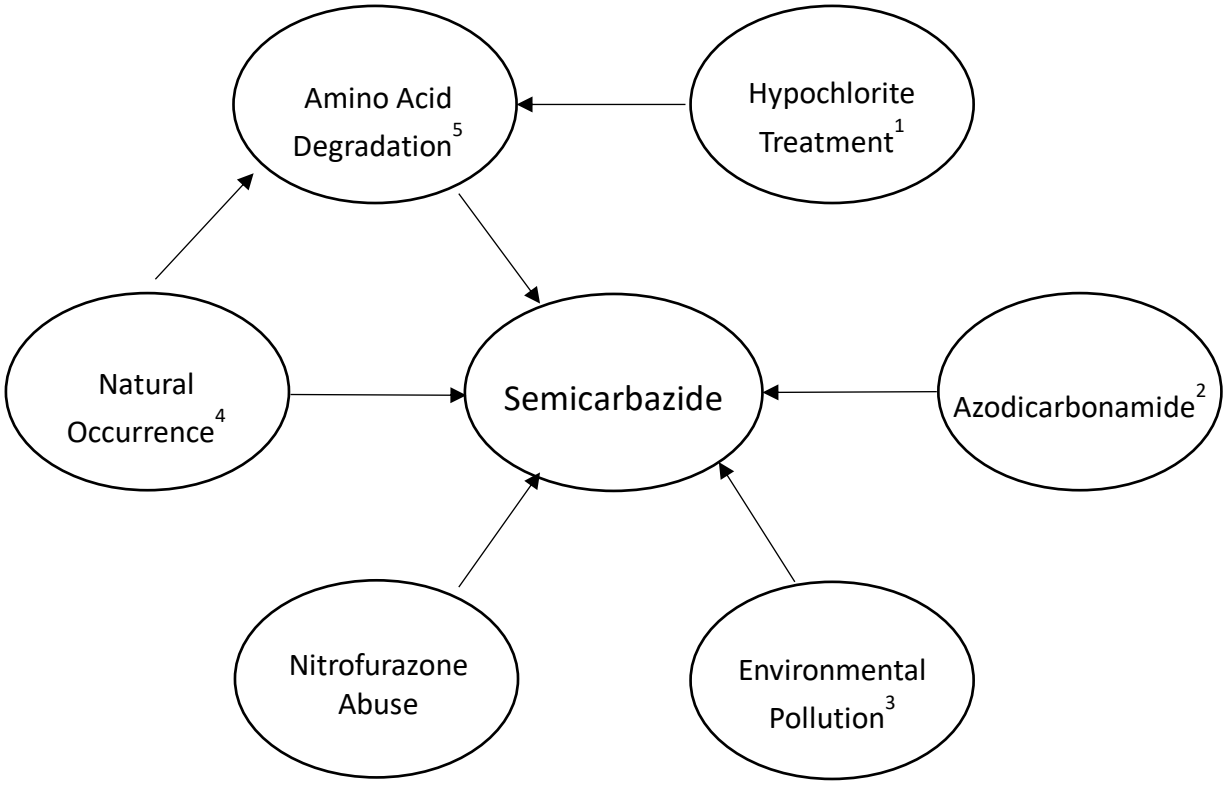


Figure 1.6: Sources of SEM in food products. 1 and 5 are conclusions from (Hoenicke et al. 2004), 2 is from the conclusions of (Pereira, Donato, and De Nucci 2004; Stadler et al. 2015; Vass, Hruska, and Franek 2008), 3 is from the conclusions of (Tian et al. 2016), and 4 is from the conclusions of (Saari and Peltonen 2004; McCracken et al. 2013; Van Poucke et al. 2011; Hui, Zhang, and Yu 2015)

1.2 Methodology:

Having determined that discovering a new metabolite of NFZ has become imperative in the monitoring of abuse of this antibiotic, this chapter will focus on the existing analytical methods for metabolite analysis. First will be a discussion of the current approaches to new metabolite discovery, in particular the non-targeted metabolic fingerprinting approach. Then there will be a discussion on the current methodology for detecting SEM in food products, followed by a discussion on the current approaches to using other metabolites for NFZ abuse monitoring, none of which have used a non-targeted approach.

1.2.1 Introduction to "Omics" Technologies:

Since the human genome project was completed in the beginning of the 21st century, there has been an explosion of technologies with relation to characterizing changes in different levels of cells' and organisms' biological functions. Antignac et al. (2011) mention that the appeal of these technologies is to explore the complexity of life with unrestrictive analytical methods. In his review, Cifuentes (2012) discusses the three so called "omics" technologies: genomics, proteomics, and metabolomics; and the potential of their application for food analysis into what he calls "foodomics". He defined genomics as the study of the mechanisms of gene expression and the search for characterization and quantitation of genes in a given organism. Similarly, proteomics is the search for characterization and quantitation of proteins, and metabolomics is that for metabolites (Cifuentes 2012). A visual representation of the different levels of biological functions and their relation to omics technologies is given below in Figure 1.7. Patti et al. (2012) discussed that metabolites make for the easiest connection to phenotype given that they are "downstream biochemical end products" (Patti, Yanes, and Siuzdak 2012). They argued that a single genetic mutation could be related to multiple metabolic pathways and that protein structure can be insufficient to infer its function in the biological system as a whole. Most applications of omics technologies have been in the health sciences sector, but as Cifuentes (2012) mentioned in his review, there is more and more pressure for nutritional sciences and food science to introduce these techniques into their workflow. Evidence of this is the increasing number of publications since the term foodomics was introduced in 2009, shown in Figure 1.8a which was generated by performing a quick search in Web of Science and EbscoHost with the topic "foodomics". A similar search using the terms "metabolomics" and "food" was done to account for those papers not necessarily using the term "foodomics" and is results are shown in Figure 1.8b.

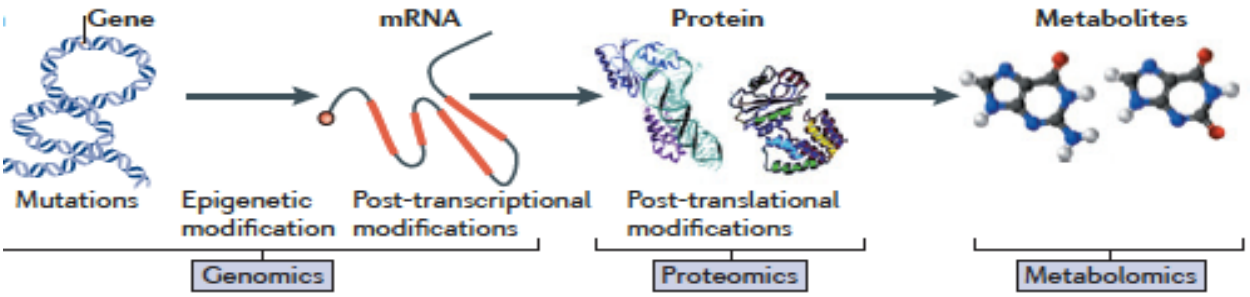
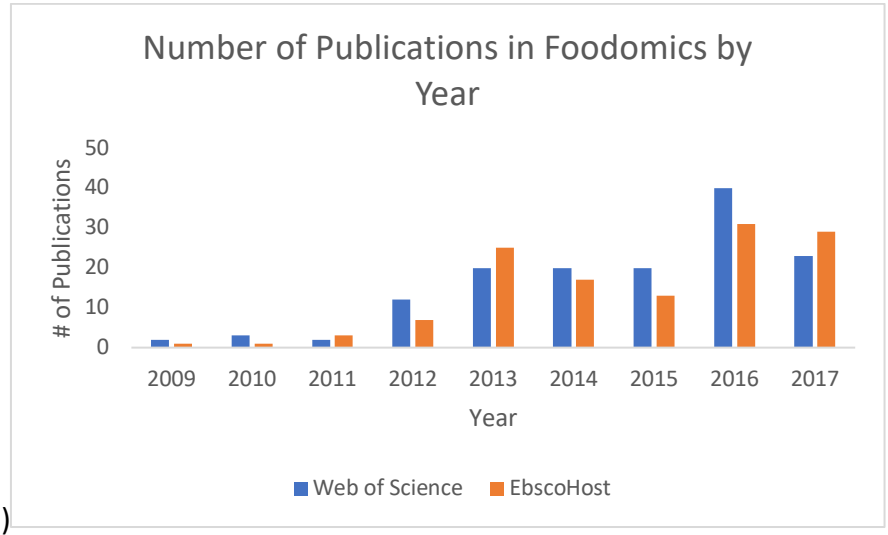


Figure 1.7: Relation between different omics technologies and monitored substances Reproduced from (Patti, Yanes, and Siuzdak 2012)

a)



b)

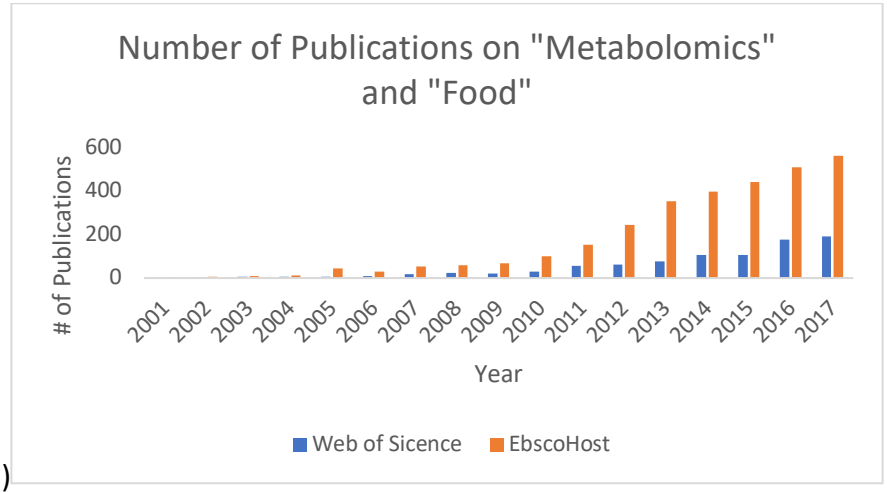


Figure 1.8: Graphical representation of increasing number of publications when doing a search in Web of Science and EbscoHost with the term “foodomics” (a) and with the terms “metabolomics” and “food” (b)

Of particular interest for this review is the approach in metabolomics known as metabolic fingerprinting, which is the topic of the review by Antignac et al. (2011) but is also discussed in detail in the review by Cifuentes (2012). This application of metabolomics is great tool for analyzing changes in metabolic patterns in response to drug exposure or disease (Antignac et al. 2011). It does not attempt to identify all the metabolites at first but is a good first step in a non-targeted analytical approach for identifying differences in the metabolic spectrum between treated and non-treated samples (Antignac et al. 2011; Cifuentes 2012; Hu and Xu 2013). It is often described as a hypothesis-generating approach rather than a hypothesis-driven approach, as targeted or even metabolic profiling are considered (Patti, Yanes, and Siuzdak 2012).

1.2.2 Overview of Targeted vs. Non-Targeted Analysis:

Within the different omics technologies there are two main approaches for sample and data treatment: targeted and non-targeted analysis. These two differ greatly in the approach to sample extraction and clean-up, and in the amount of generated data and the ways to treat this data. Recent advances in High-Resolution Mass Spectrometry (HRMS) and bioinformatics have made the non-targeted approach more feasible and has allowed for higher throughput of samples and data analysis (Cifuentes 2012; Patti, Yanes, and Siuzdak 2012; Hu and Xu 2013; Turnipseed, Lohne, and Boison 2015). The two approaches are discussed in more detail below, but a quick comparison of the workflow between targeted and non-targeted metabolomics is shown below in Figure 1.9.

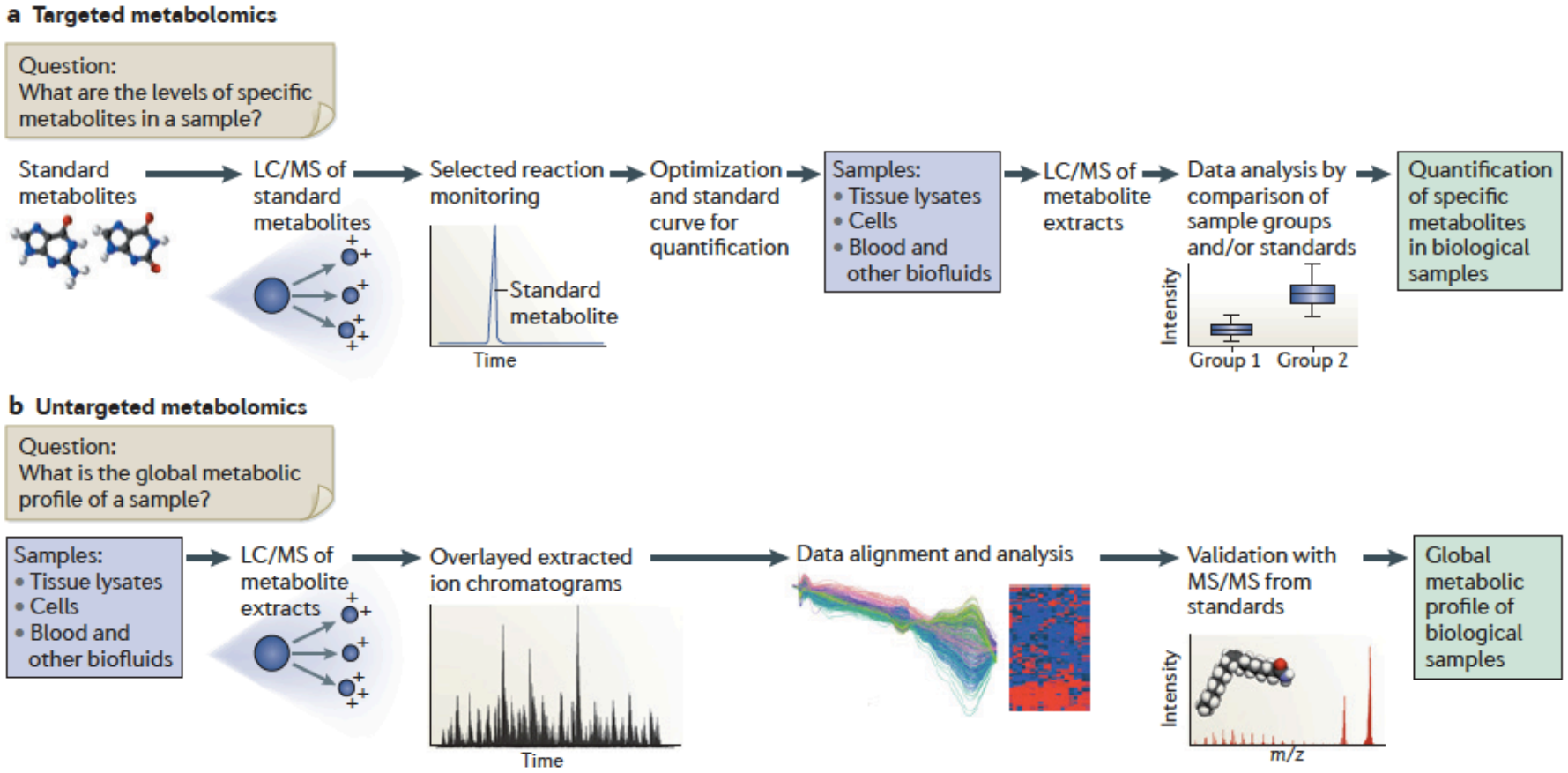


Figure 1.9: Comparison of the workflow in targeted (a) and non-targeted (b) metabolomics studies Reproduced from (Patti, Yanes, and Siuzdak 2012)

Despite the term *metabolomics* being relatively new, the use of targeted analysis of metabolites has been well documented over the last century and can be a useful way to monitor disease or abuse of veterinary drugs in food production (Patti, Yanes, and Siuzdak 2012). Targeted analysis refers to analysis where a specific list of metabolites, or analytes of interest, are selected prior to method development, and the method is built around measuring levels of the specific compounds in samples (Tengstrand et al. 2013; Patti, Yanes, and Siuzdak 2012). This is very useful when the metabolite associated to a specific disease or drug treatment is known, verified, and unique to that process. Extraction procedures are therefore designed specifically for the analyte of interest (eg. polar solvent for polar analyte) and the samples are cleaned-up using various purification techniques (liquid/liquid extractions and SPE). The main advantage of this approach is that the specific extractions and sample clean-up result in fewer interfering species and high sensitivity of analysis, in particular when mass spectrometry (MS) is used as a detection method (Hu and Xu 2013). This is the approach used for the analysis of side-chain metabolites for most of the nitrofuran drugs (AOZ, AMOZ, AH), but as discussed in the previous chapter, this is no longer appropriate for SEM as a way for monitoring NFZ. However, to this day, the targeted SEM analytical method is still the standard method for NFZ monitoring in food products (main methods for such analysis are discussed in the following section). The main disadvantage of targeted methods is that by only measuring what is expected to be there, we remained closed to the possibility that an unknown adulterant or contaminant may be present in the sample (Knolhoff and Croley 2016), as was the example of melamine adulteration of milk samples from China, which was not expected and was not tested for until symptoms were apparent (Tengstrand et al. 2013).

With the advances in HRMS technology and bioinformatics, and more cases where a non-biased approach to data analysis could reveal new compounds of interest both for mechanistic studies and monitoring purposes, the field of non-targeted analysis has been growing steadily in the last decades (Patti, Yanes, and Siuzdak 2012; Cifuentes 2012). In essence, a non-targeted approach refers to developing a sample preparation method as broad as possible in hopes to determine the whole metabolome of a particular sample. There are however problems associated with this approach, in particular due to the lack of standardized methods for sample

preparation and data treatment (Antignac et al. 2011). Minimal sample preparation is often preferred in order to avoid loss of any metabolites of interest, and for this reason a "dilute and shoot" approach can be optimal if the matrix is not too complex (Antignac et al. 2011). It is generally recognized that no single analytical method can detect all metabolites in a sample due to the high complexity of metabolites mixtures in most matrices (Cifuentes 2012; Knolhoff and Croley 2016). For this reason, sample extractions should be done with solvents of varying polarities in an attempt to get a better representation of the metabolites in the sample. Often, doing both polar and non-polar extraction to observe both families of metabolites is proposed (Patti, Yanes, and Siuzdak 2012; Hu and Xu 2013), but this should be optimized for each experiment and matrix studied. This can result in a very convoluted sample, and although sample clean-up with techniques such as solid phase extraction (SPE) or liquid/liquid extraction can be done, the extensive clean-up can potentially remove compounds of interest and should be used sparingly (Knolhoff and Croley 2016).

The extracts are most often analyzed by mass spectrometric methods such as quadrupole-time-of-flight (QTOF) or Orbitrap detectors given their high sensitivity and possible determination of highly accurate masses which can help in elucidation of metabolite structures (Patti, Yanes, and Siuzdak 2012; Antignac et al. 2011). However, nuclear magnetic resonance (NMR) is sometimes used in these experiments as well despite showing lower sensitivity (Antignac et al. 2011). If electrospray ionization (ESI) is used as the ion source, special attention must be given to ion suppression, and choice of an adequate internal standard can be imperative for repeatable results (Hu and Xu 2013). The use of tandem LC-MS and GC-MS systems has greatly increased the capability of the non-targeted approach since it adds another separation step as well as a further verification of metabolite identity by retention time comparison with standards (Hu and Xu 2013).

Data analysis and treatment are often considered the rate limiting step of non-targeted analysis given the generation of large datasets per sample (Hu and Xu 2013). Here is where the advances of bioinformatics become very useful in the automation of parts of this process (Patti, Yanes, and Siuzdak 2012). The use of dedicated software programs (XCMS, MassProfiler, Markerlynx, Markerview…) for the sorting of data (filtering, non-linear retention time alignment)

and of robust metabolite libraries (METLIN, Human Metabolome Database, SciFinder, PubChem, ChemSpider…) for metabolite identification from high accuracy masses become key in the data treatment of a non-targeted experiment (Hu and Xu 2013; Knolhoff and Croley 2016). Following this, statistical analysis tests are performed to determine which peaks could be of interest. Different tests are used for particular purposes. Univariate methods like Student's t-test can be used as a first screening step to determine significant differences between data sets (Antignac et al. 2011). Most commonly, unsupervised principle component analysis (PCA) and supervised partial least squares-discriminant analysis (PLS-DA) are used for data sorting in metabolomics studies (Hu and Xu 2013). Finally, verification of the structures of potential candidates by comparison with MS/MS data and retention times of standards is required to have an unambiguous determination of structure (Hu and Xu 2013). Figure 1.10 below summarizes the steps taken in the metabolite-identification workflow of a non-targeted study. Table 1.2 below summarizes the effects that different parameters that could affect the results of the non-targeted approach.

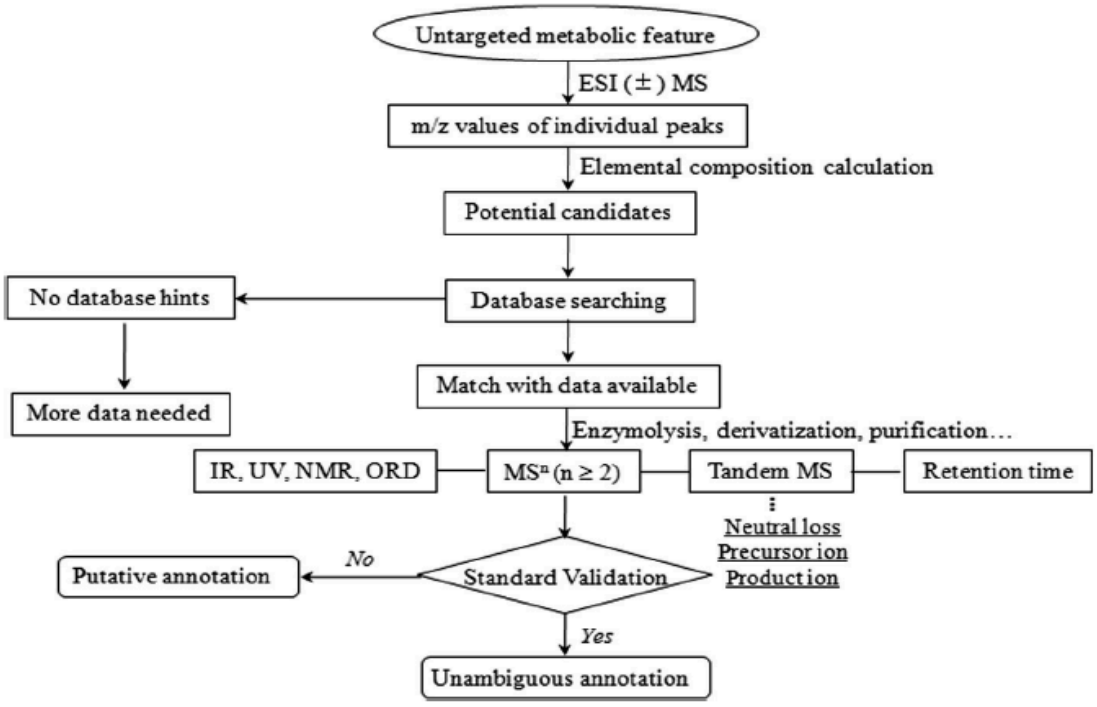


Figure 1.10: Workflow for identification of metabolites in non-targeted analysis Reproduced from (Hu and Xu 2013)

Step	Effect
Treatment Methods	Results from water treatment versus oral dosing could be very different in terms of levels of measured metabolites in tissue
Storage of Sample	The wrong storage conditions might cause degradation of metabolites prior to analysis
Extraction Solvent	Choice of extraction solvents determine which types of metabolites will be observed. Crucial to attempt as broad an extraction as possible in order to see the largest amount of metabolites possible
Purification Steps	Purification steps might be necessary, but must consider what might be lost during the process of purification, maybe some metabolites as well.
Ionization Method	Ionization methods can affect the observed metabolites, in particular with ESI where matrix effect can be significant, and must be monitored using standard addition. Some metabolites might not be readily ionized by some ionization methods
Detector	Type of detector influences what is observed and with what resolution.
Mode of Detection	Running the analysis in positive or negative mode will yield different results, and experiment should be run in both modes in order for a more full metabolome to be observed
Data Analysis	This is the most crucial step in this type of experiments. If the data is treated without care, misrepresentation of peaks can lead to unnecessary purchasing of standards and erroneous attribution of peaks

Table 1.2: Parameters that could influence results in non-targeted approach

1.2.3 Current Approaches to Non-Targeted Extractions:

As discussed in the previous section, most non-targeted approaches aim to minimize sample treatment in order to maximize the number of features that can be observed after HPLC-MS analysis. Several efforts have been done to standardize extraction methods, but no consensus has been reached. Some authors use the QuEChERS (Quick Easy Cheap Effective Rugged and Safe) approach to sample treatment which is generally as follows: extraction of representative sample with organic solvent, followed by anhydrous salt and NaCl for removing water and help in phase separation. The sample is then centrifuged, and the supernatant is transferred to a centrifuge tube with reactant that can bind to fatty acids (eg. Z-Sep or Primary Secondary Amine (PSA)),

vortexed and centrifuged again. The supernatant is then evaporated to dryness and reconstituted in mobile phase (Villar-Pulido et al. 2011). Slight variations of this procedure are shown in another group in which they use DPX tips for automation of the process (Jia et al. 2017). Table 1.3 below summarizes these procedures as well as a few other examples of even simpler non-targeted extractions using acetonitrile, methanol, and water as extraction solvents (Baduel et al. 2015; Dasenaki and Thomaidis 2015; Nacher-Mestre et al. 2013). These other methods usually do not include addition of a compound for binding of fatty acids but include a freezing step in which fatty acids and some proteins are precipitated.

Reference	Amount of Sample (g)	Extraction Solvent	Volume (ml)	Centrifuge	Freezing Protein Precipitation	Compounds of Interest	De-fatting or extra notes
Nácher-Mestre et al. 2013	5	Acetonitrile/Water (80:20) 0.1% HCOOH	10	4500rpm 10min	2hr min	Furaltadone Furazolidone	
Dasenaki et al. 2015	1	2ml 0.1% EDTA 0.1% formic 2ml ACN 2ml MeOH	6	4000rpm 10min	12hr	N/A	5ml Hexane
Villar-Pulido et al. 2011	10	Acetonitrile 0.1% HCOOH 4g anhydrous MgSO4 1.75g NaCl	10	3700rpm 3min	none	N/A	250mg PSA (primary secondary amine) after first centrifugation, with 750mg of nahydrous MgSO4 again, then centrifuged again at 3700rpm for 3 mins. Finally sample is filtered through 0.45um PTFE filters
Jia et al. 2017	1	Acetonitrile/Water (84:16) 100ul of 0.1M EDTA, 1% HCOOH 1.0g anhydrous MgSO4 and 0.3g Sodium Acetate	10	2264g 5min		Nitrofurazone	Uses DPX tips tro transfer extract to filter salts prior to centrifugation. Addition of Z-Sep+ at this step.
Baduel et al. 2015	10	Acetonitrile 4g anhydrous MgSO4 1g NaCl	10	3500rpm 10min	4hr min at -20	N/A	Transfers 500ul after freezing and adds 1ml ACN (0.1% Formic acid) and filters through Captiva ND lipid cartridge

Table 1.3: Summary of non-targeted extraction methods

1.2.4 Model Building Approaches to Sample Classification:

Some authors discuss the importance of statistical models in the application of non-targeted analysis as a way to discriminate between different groups (Antignac et al. 2011). This could prove useful in discriminating between treated and control shrimp in the present study. The biggest risk in using predictive models is that they are often prone to chance-correlations and overfitting (Rubingh et al. 2006). This risk becomes even greater as multivariate data

becomes megavariate. The authors define a set having 10 times more variables than samples as megavariate. Cross-validation is a way to reduce the risk of overfitting, as it utilises a part of the dataset to build a model and then tests the model on the remaining fraction of samples. A separate test using a new data set, or double cross-validation, is recommended to determine the predictability for future data (Rubingh et al. 2006) but has not been observed in all publications discussing specific applications of models.

Righetti et al. (2016) discussed the application of Principal Component Analysis (PCA) models, Partial Least Squares Discriminant Analysis (PLS-DA) models, and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) models in the differentiation between different types of ancient grains. They cross validated their model using the "leave one third out" method as described by Rubingh et al. (2006), however they did not run a double cross-validation, nor a test sample set outside the original sample set for testing the predictability of the model. Martinez Bueno et al. (2018) discussed the application of Linear Discriminant Analysis (LDA) models in the differentiation between organic and non-organic tomatoes. In this study, they did run the prediction model on an external data set of store-bought tomatoes but did not discuss the original training of the model with cross-validation. On the other hand, Novotna et al. (2012) discussed a "leave one out" methodology for cross validation of their model to identify between organic and conventional tomatoes and peppers, but did not discuss an external sample set for double cross-validation.

1.2.5 Current SEM Targeted Methods:

Since before the legislative decision to ban nitrofuran drugs for use in food producing animals in the 1990's, many efforts for standardization of analytical methods for their metabolites had been done. After evidence of the quick degradation of the parent compounds *in vivo,* the focus shifted to determining an efficient way to measure long lasting metabolites in animal tissue.

Original UV Detection Method:

The first study to mention analysis of the intact side chain metabolites was done slightly before the prohibition and was looking at the depletion time of furazolidone in pig tissues. In it, Hoogenboom et al. (1991) tested various conditions for the hydrolysis of tissue bound AOZ and subsequent derivatization with 2-nitrobenzaldehyde (NBA). Although they did not discuss the choice of derivatizing agent, they tested different HCl concentrations and hydrolysis/derivatization times and temperatures. The derivatization reaction is described in Figure 1.11 and the whole analytical procedure was as follows: 1 g of tissue was frozen in liquid nitrogen and pulverized. It was then washed with 6 mL fractions of methanol (3X), ethanol (2X), and diethyl ether. Pellets were then re-suspended in water and treated with NBA (50mM in dimethyl sulfoxide (DMSO)) and HCl. The solution was left in a water bath to incubate and was then extracted twice with 2 mL of ethyl acetate. The solvent was evaporated under nitrogen gas at 40°C and re-dissolved in 0.25 mL of acetonitrile-water (1:1 v/v) prior to HPLC analysis. They used a C18 column and acetonitrile-water (3:7, v/v) as their mobile phase. Detection of compounds was done at 275 nm. After testing various conditions, they concluded that the general procedure should be "2mL of incubation mixture containing 5-10 mg of protein in 0.1 N HCl and 0.5 mM NBA, incubated over 20hr at 37°C" (Hoogenboom et al. 1991). A big advantage of this study is that they also treated the pigs with ^{14}C-furazolidone, some in the furan ring, and some in the AOZ side chain. They used this to confirm the origin of the tissue-bound metabolite AOZ and be able to undoubtedly attribute it to FZD use.

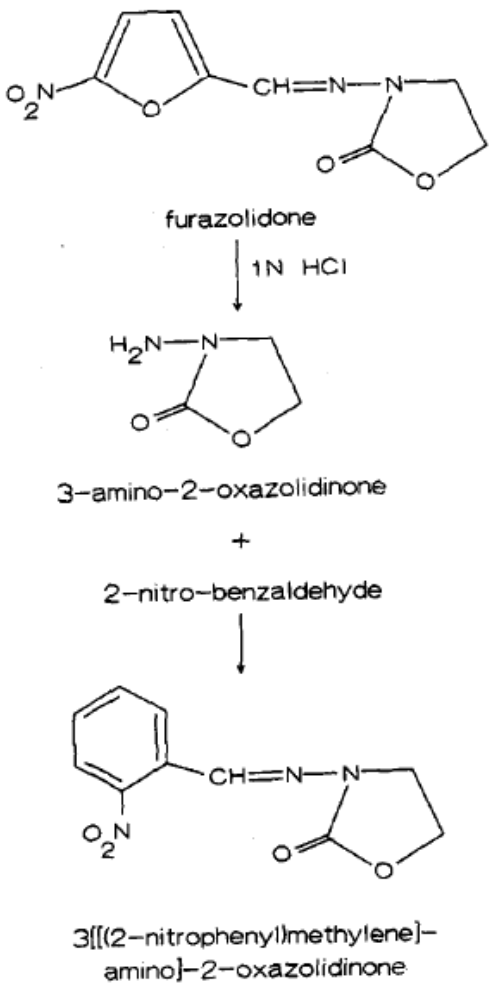


Figure 1.11: Proposed mechanism for the derivatization of furazolidone side chain AOZ by 2-nitrobenzaldehyde (NBA) resulting in 3-(2-nitrobenzylidenamino)-2-oxazolidinone (NPAOZ) (Hoogenboom et al. 1991)

The aforementioned method was later confirmed by the same research group to function for the determination of tissue bound metabolites of the other main nitrofuran drugs: FTD, NFZ, and NFT (Hoogenboom and Polman 1993). Figure 1.12 shows the four main nitrofuran drugs, their metabolites, and the structure of the nitrophenyl derivatives used during analysis. It is a variation of this method that is still commonly seen in most studies and by most regulatory laboratories analyzing for nitrofuran residues. The studies mentioned in the "current issues" section of this review all use a modification of this extraction procedure, except they use different MS detection systems rather than UV-detection. This is done due to greater accessibility of MS

technology in the present as well as lower detection limits available making it easier to abide by the European Commission MRPL of 1 µg/kg (EC 2003). The main changes from the original extraction procedure have been the addition of an SPE step for sample purification (Valera-Tarifa et al. 2013), and the removal of the extensive initial washing step for total residue analysis. A washing step with methanol is introduced when only tissue-bound metabolites are being measured. Given the other sources of SEM in shrimp products, McCracken et al. (2013) suggest that only tissue-bound SEM should be used for the determination of NFZ abuse, since total metabolite analyses could produce false-positive results. They also suggest that the shell should be removed before grinding of sample in order to minimize SEM residues occurring naturally in the samples.

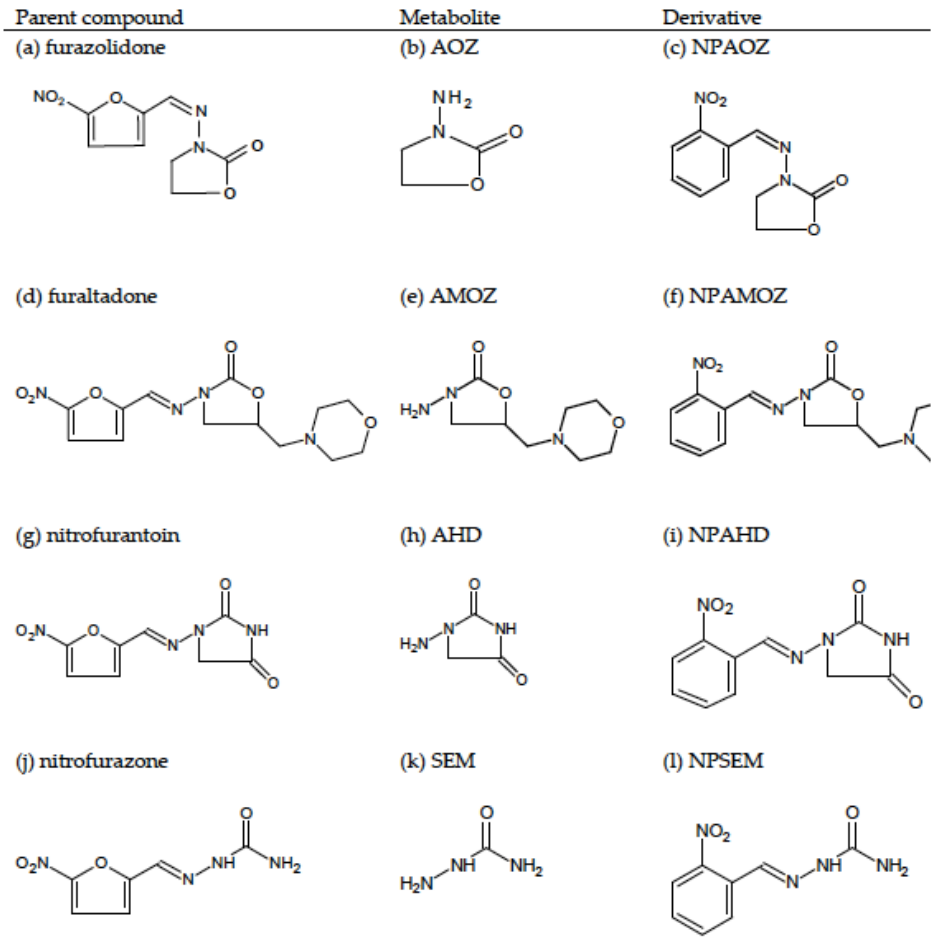


Figure 1.12: Structure of the main nitrofuran drugs, their main metabolites, and their derivatives after treatment with NBA Reproduced from (Vass, Hruska, and Franek 2008)

Modified MS Detection:

A method variation of the original Hoogenboom et al. (1991) extraction by Chu et al. (2005) is the most often cited extraction procedure when analysis of nitrofuran drugs in shrimp is performed. The authors found that using this method SEM could be detected in samples up to 56 days after NFZ treatment. Their detailed extraction procedure accounts for more standardized volumes as well as addition of a filtration step, and liquid-liquid extraction purification steps and is as follows (Chu and Lopez 2005): 2 g of shrimp tissue were mixed with 5 mL of aqueous 50% methanol and homogenized. They were then centrifuged at 3000 rpm for 5 minutes, and the supernatant was decanted and discarded. The pellets were then sequentially washed with 5 mL of ethyl acetate (EtOAc) and then ethanol by vortex and then centrifuge. The supernatants were once again decanted and discarded (these steps ensure that only tissue bound metabolites are measured). At this point, the stock nitrofuran standard solution was used to generate a four-point calibration standard curve in blank tubes. 10 mL of 0.125 M HCl and 400 µL of freshly prepared 50 mM NBA solution in DMSO were added and the samples were vortex mixed and was incubated in a 37°C water bath for 14-16 hours. Afterwards, the solution pH was adjusted to 7.1-7.5 with 1 mL of 0.1 M K_2HPO_4 and then addition of 0.8 M aqueous NaOH dropwise. Tissue samples were centrifuged at 3000 rpm for 5 minutes at 4°C and the supernatants were decanted into polypropylene tubes equipped with a filtering frit in an SPE manifold. Pellets were washed twice with 3 ml of water, vortexed, centrifuged, and washes were added to their respective tubes in order to avoid analyte loss. All samples were adjusted to 20-22 mL with Milli-Q water. One quarter of a teaspoon of NaCl (to avoid foaming) and 10 mL hexane (to remove lipid material and excess NBA) were added at this point and gently hand-mixed. The solutions were centrifuged for 10 minutes at 3000 rpm and the top hexane layer was discarded. The aqueous layer was then extracted 3 times with 4ml of ethyl acetate (EtOAc) and centrifuged at 3000rpm for 5 minutes. The EtOAc fractions were combined in polypropylene tubes and washed with 1 mL of Milli-Q water, which was then pipetted out. The solvent was evaporated at 40°C and the residues were dissolved in 200 µL of HPLC mobile phase (55% Methanol- 45% 20 mM Ammonium Acetate). Final extracts were filtered through 0.2 µm filters into auto-sampler vials (Chu and Lopez 2005). It is

interesting however that unlike most other literature found, these authors decided to use atmospheric pressure chemical ionization (APCI) rather than electrospray ionization (ESI) prior to mass-spectrometric (MS) analysis. They argue that “in [their] laboratory, [they] found the APCI interphase more sensitive and more rugged toward matrix effects than ESI” (Chu and Lopez 2005). Most other articles found use ESI in positive mode for analysis of SEM or other nitrofuran metabolites and some use SPE as a purification step in the extraction. This is summarized in Table 1.4 below for most of the experiments done on shrimp found and in some other food matrices.

Article	Matrix	Detected Substance	Detection Method	Pre-wash Solvent	Extration Solvent	HPLC Mobile Phases	Column Used	Ionization Method	SPE Cartridge or Filter Used (if applicable)	% Recovery (if available)
Hoogenboom et al. 1991	Pig Liver	AOZ	UV	Methanol, Ethanol, Diethyl Ether	Ethyl Acetate	Acetronitrile-Water (3:7 v/v)	C18-spher	N/A	N/A	N/A
Hoogenboom et al. 1993	Pig Hepatocytes	AOZ, AMOZ, SEM, AH	UV	Methanol, Ethanol	Ethyl Acetate	0.01M Potassium Phosphate Buffer-Acetonitrile (75:25 v/v)	C18 Hypersil 5 ODS	N/A	N/A	N/A
Saari et al. 2004	Crayfish Muscle	SEM	Ion Trap MS	Some with Methanol	Ethyl Acetate	0.1% Acetic Acid in Water (A), 0.1% Acetic Acid in Acetonitril-Water (90:10 v/v)	C18	(+) ESI	N/A	65-120
Chu et al. 2005	Shrimp Muscle	AOZ, AMOZ, SEM, AH	Triple-Quad MS	50% Aqueous Methanol, Ethyl Acetate, Ethanol	Ethyl Acetate	Methanol (A) 20mM Ammonium Acetate (B)	Inertsil ODS-3	(+) APCI	Filtering Frits	N/A
Chu et al. 2008	Channel Catfish Muscle	AOZ, AMOZ, SEM, AH	Triple-Quad MS	70% Aqueous Methanol, Ethyl Acetate, Ethanol	Ethyl Acetate	Methanol (A) -20mM Ammonium Acetate (B)	Inertsil ODS-3	(+) APCI	Filtering Frits	N/A
Van Poucke et al. 2011	Prawn Muscle and Shell	SEM	Triple-Quad MS	50% Aqueous Methanol, 75% Aqueous Methanol, Methanol, Water	Ethyl Acetate	0.1% Acetic Acid Water (A) 0.1% Acetic Acid Acetonitrile-Water (90:10 v/v)	C18	(+) ESI	N/A	N/A
McCracken et al. 2011	Shrimp Muscle and Shell	SEM	Triple-Quad MS	50% Aqueous Methanol	N/A	N/A	C18	(+) ESI	N/A	N/A
Valera-Tarifa et al. 2013	Shrimp Muscle	AOZ, AMOZ, SEM, AH	Triple-Quad MS	None	Ethyl Acetate	Methanol (A) 10mM Ammonium Formate (B)	C18	(+) ESI	Some Oasis HLB, Some C18	73-103
Hui et al. 2015	Shrimp Muscle	SEM	Orbitrap MS	Some 50% Aqueous Methanol, 75% Aqueous Methanol	Ethyl Acetate	Methanol (A) 2mM Ammonium Acetate with 0.1% Formic Acid	C18	(+) ESI	N/A	N/A

Table 1.4: Summary of different analytical techniques for nitrofuran metabolites detection

1.2.6 Current Targeted Methods on Other Metabolites:

In light of the current issues with respect to occurrence of SEM in shrimps and other products with no evidence of NFZ use, some research groups have begun to look at alternative ways to confirm the origin of SEM or other metabolites to determine the use of NFZ. Although there have been some advances, none of the developed methods are stand-alone and usually require SEM analysis in tandem as verification. This results in increased costs and times of analysis given that two experiments must be run rather than a single one. The three alternative methods are discussed below. Although they all propose interesting solutions to this problem, none has attempted a non-targeted approach which could help in the discovery of novel metabolites associated to NFZ abuse in shrimp and will be the purpose of this study. However, the results of these experiments as well as their extraction techniques will be considered when analyzing the data of the non-targeted experiment by looking out for the masses of the different metabolites that they have studied.

Detection of Biurea:

The first method can be used to determine if the SEM detected by the traditional SEM targeted method mentioned in the previous section is due to the use of azodicarbonamide (ADC) as a flour additive or a sealing agent (Mulder, Beumer, and Van Rhijn 2007). The authors discuss that pre-derivatization wash with solvent might not be enough to differentiate between SEM originating from NFZ or ADC because SEM from ADC can covalently bind to proteins during the baking process of breaded products (Mulder, Beumer, and Van Rhijn 2007). They evaluated the literature on the breakdown mechanism of ADC and established that biurea could be a good compound to analyze for its monitoring in flour coated products given that ADC is almost quantitatively converted to biurea during dough preparation (Pereira, Donato, and De Nucci 2004; Mulder, Beumer, and Van Rhijn 2007). Figure 1.13 below shows the simplified mechanism for formation of SEM from ADC. With this in mind, they developed a method to measure the amount of biurea in food samples and correlate this to the presence of SEM from ADC. The extraction procedure performed was as follows: fresh, coated, and processed samples were

extracted with a dimethylformamide (DMF)-water mixture. Since DMF does not readily evaporate, an aliquot of the extract was diluted in ACN and injected into the LC-MS/MS system (Mulder, Beumer, and Van Rhijn 2007). The mass transition measured during single reaction monitoring (SRM) was from *m/z* 119>76. DMF extracted biurea with high efficiency (>80%) when compared to other solvents tested and the method was able to detect biurea concentrations as low as 10 µg/kg. They do mention encountering problems with ion suppression, and that sample filtration with 30 kD membranes was unsuccessful in lowering suppression observed. Further tests showed that a dilution factor of 200 minimized the effects of ion suppression in their matrix. Correlating their results with measured concentrations of SEM, they found that if SEM in a product is from ADC origin, the minimum measurable concentration of biurea would be 20 µg/kg for every 1 µg/kg of SEM (Mulder, Beumer, and Van Rhijn 2007). Although this can be a useful method, it still requires analysis with the SEM targeted method, and does not differentiate between the other 4 possible sources of SEM in shrimp samples shown in Figure 1.6.

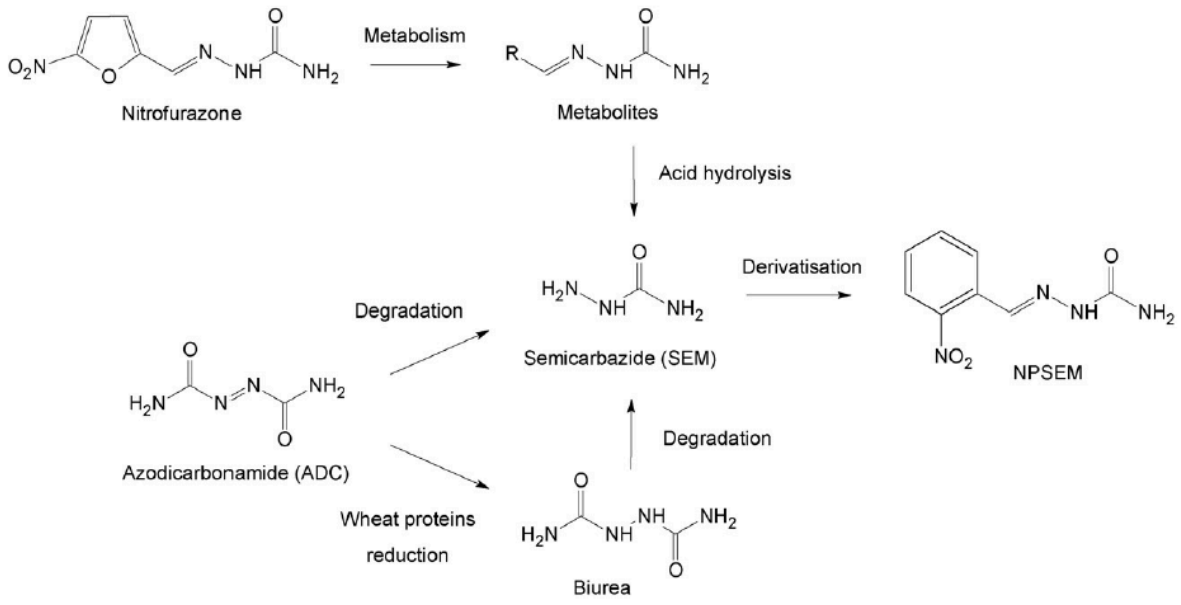


Figure 1.13: Formation of SEM from nitrofurazone and from azodicarbonamide Reproduced from (Mulder, Beumer, and Van Rhijn 2007)

Detection of Cyano-Metabolite:

The second method is a derivation from the earlier work done by Vroomen et al. (1987) before they determined that the side-chain tissue bound metabolites could be the most efficient way to monitor nitrofuran use in farm and aquaculture products. They discovered that an open-chain cyano-derivative of the parent drug furazolidone could be measured in the muscle of pigs 2 hours after the last dosing of furazolidone. The structure of this metabolite is shown in figure 1.14 below. Their original procedure consisted of an aqueous extraction of pulverized muscle tissue followed by purification using a Merck Extrelut® 1 column and elution with EtOAc. Following this, the extract was filtered and run through an HPLC system with detection by absorption at 254 nm (Vroomen et al. 1987). After the discovery of the longer lasting side-chain metabolites by the same research group in 1991, this cyano-derivative was slightly put aside. However, in the recent search for alternative markers for nitrofurazone monitoring, Wang et al. (2010) found that the equivalent cyano-derivative of nitrofurazone could be detected in the muscle of channel catfish up to 2 weeks after the final dosing had taken place. The pathway for the metabolic reduction into the cyano-metabolite of NFZ is shown in Figure 1.15 below. Their extraction procedure involves shaking the homogenized muscle tissue with a 50 mM phosphate buffer (pH 7.4) for 2 hours and then centrifuging to separate solids. The supernatant was then washed with hexane and centrifuged again. After discarding the hexane layer, the aqueous layer was loaded onto a Waters Oasis HLB SPE cartridge and washed with water. The cyano-metabolite was finally eluted with methanol and dried under nitrogen. Finally, the sample was dissolved in 0.1% acetic acid water and analyzed via LC-MS/MS in both positive and negative ESI modes in a triple quadrupole/linear ion trap (QTRAP) hybrid mass spectrometer (Wang et al. 2010). The metabolite was monitored by selected reaction monitoring (SRM) with the transition *m/z* 167>124, and the procedure was estimated to have a recovery of 92%. This is a promising alternative biomarker for confirming NFZ use in channel catfish but has a relatively low half-life (approximately 81 hours) (Wang et al. 2010) when compared to the 56 days of detection of SEM with the method by Chu et al. (2008). Moreover, it has only been confirmed in channel catfish and never in shrimp muscle. The authors also comment that accurate quantitation could not be performed due to a lack of a commercial standard for generation of a calibration curve.

Monitoring for the mass of the open chain metabolite in shrimp tissue for confirmation will be performed in the current study.

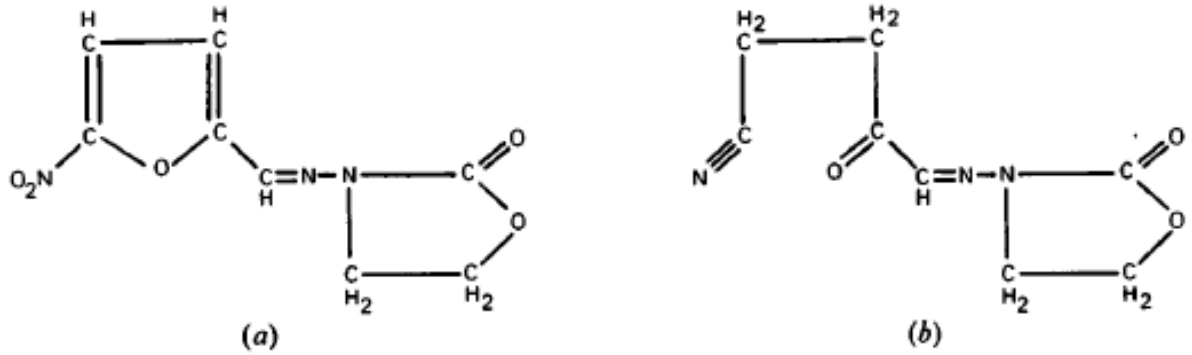


Figure 1.14: (a) Furazolidone and (b) its open-chain cyano-derivative Reproduced from (Vroomen et al. 1987)

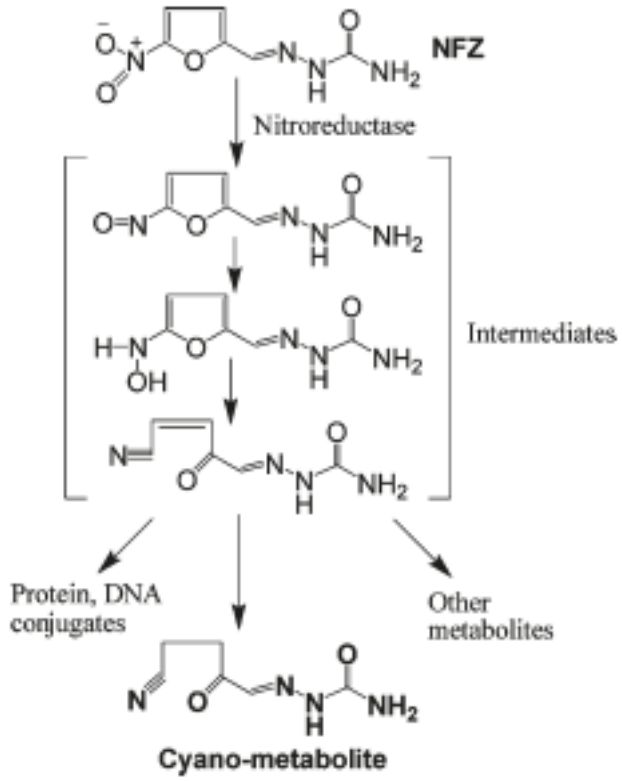


Figure 1.15: Metabolic pathway for reduction of NFZ into its cyano-metabolite Reproduced from (Wang et al. 2010)

Detection of 5-nitro-2-furaldehyde (NF):

The third and final alternative method to be discussed is the most recent and probably the most promising. Zhang et al. (2015) stipulate that the hydrolysis of the known SEM side chain should result in the release of 5-nitro-2-furaldehyde (NF) from the parent compound. This is shown in Figure 1.16 below. Although this does not correspond to the proposed mechanism with regards to the formation of the open-chain cyano-derivative mentioned before, they managed to detect NF in aquaculture samples treated with NFZ in concentrations around one order of magnitude smaller than measured SEM concentrations. Moreover, they confirmed that there was no detection of NF in shell of crab caught from the sea where SEM concentrations were measured and associated to natural occurrence, but small traces of NF were still measured in shell of shrimp bought from a market (Zhang et al. 2015). Their extraction procedure was as follows: 1.0 g of sample was mixed with 0.2 M HCl and 1.0 mg/L solution of 2,4-dinitrophenylhydrazine (DNPH, derivatizing agent) by vortex mixing and ultrasonification for 30 minutes. The pH of the mixture was then adjusted to 7.5 with K_2HPO_4 and extracted with 2X4ml portions of ethyl acetate. After centrifugation, the supernatant was decanted and evaporated prior to reconstitution in ACN/water. The solution was finally filtered through 0.2 µm PTFE filters prior to HPLC injection. MS/MS detection was carried out in negative ESI mode by monitoring the transition 320 → 273m/z (Zhang et al. 2015). In a later report, the same research group introduced an SPE step with Waters® Oasis PRiME HLB cartridges after derivatization and used QTOF-MS instead of triple quadrupole MS (Zhang et al. 2017). Despite their positive results, it is important to mention that the NF moiety is common to all nitrofuran drugs, and therefore this analysis must be run in tandem with the traditional side-chain metabolite analysis to confirm that the NF observed is in fact from NFZ and not any other nitrofuran. In case other nitrofuran side-chain metabolites are present, this method would be insufficient to determine if the SEM present was due to NFZ abuse or because of any of the possible sources of SEM previously discussed. Table 1.5 below briefly summarizes the metabolites discussed in this section.

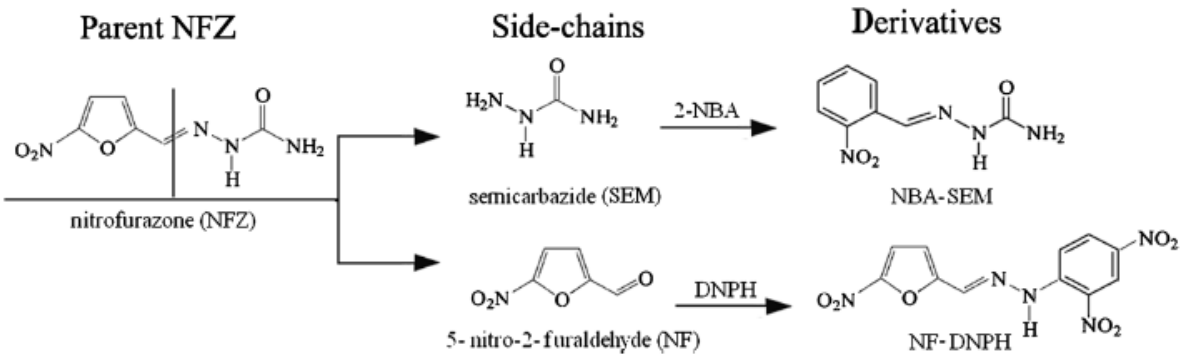


Figure 1.16: Breakdown of NFZ resulting in SEM and NF and their derivatization for analysis Reproduced from (Zhang et al. 2015)

Article	Metabolite	Extraction Solvent	Mass Transition Observed
Mulder et al. 2007	Biurea	Dimethylformamide/ Water	119 → 76m/z
Wang et al. 2010	Cyano-derivative	Phosphate buffer	167 → 124m/z
Zhang et al. 2015	NF-DNHP	Ethyl Acetate	320 → 273m/z

Table 1.5: Brief summary of other metabolite approaches to NFZ monitoring

1.3 Conclusion:

Large amounts of shrimp and other aquaculture products are often discarded due to contamination by SEM and the assumption that this originates from NFZ abuse. In light of the overwhelming evidence of SEM occurrence from sources other than NFZ abuse, it becomes imperative to develop novel analytical methods for monitoring the use of this banned veterinary drug. Previous attempts for the discovery of a new metabolite for these purposes have been made, but so far, they have all resulted in possible confirmatory methods. Moreover, none have utilized the non-targeted approach to sample preparation and data analysis. However, the

resulting metabolites of some of these experiments are promising and will be kept in mind and monitored during the data analysis of the current research project.

The proposed research will focus on the development of a non-targeted analytical method for the discovery of a new metabolite for monitoring nitrofurazone abuse in aquaculture, particularly in shrimp farming. The metabolic fingerprinting approach will be the first step in determining metabolome differences between samples of nitrofurazone treated *Penaeus Vannamei* shrimp and non-treated samples grown under identical conditions. The data treatment approaches discussed in this review will be utilized to identify potential candidates for differentiation between treated and non-treated samples and confirmed by the use of standards whenever available. The purpose of this project is to identify a novel metabolite specific to nitrofurazone abuse or a repeatable way to differentiate between treated and control shrimps in order to prevent the unnecessary disposal of product which causes both economic and ecological problems for all those involved.

1.4 References:

Antignac, J. P., F. Courant, G. Pinel, E. Bichon, F. Monteau, C. Elliott, and B. Le Bizec. 2011. 'Mass spectrometry-based metabolomics applied to the chemical safety of food', TrAC Trends in Analytical Chemistry, 30: 292-301.

Baduel, C., J. F. Mueller, H. Tsai, and M. J. Gomez Ramos. 2015. 'Development of sample extraction and clean-up strategies for target and non-target analysis of environmental contaminants in biological matrices', J Chromatogr A, 1426: 33-47.

Chu, P.S., and M.I. Lopez. 2005. 'Liquid Chromatography-Tandem Mass Spectrometry for the Determination of Protein-Bound Residues in Shrimp Dosed with Nitrofurans', Journal of Agricultural and Food Chemistry, 53: 6.

Chu, P.S., M.I. Lopez, A. Ann, K.R. El Said, and S.M. Plakas. 2008. 'Residue Depletion of Nitrofuran Drugs and Their Tissue-Bound Metabolites in Channel Catfish (Ictalurus punctatus) after Oral Dosing', Journal of Agricultural and Food Chemistry, 56: 5.

Cifuentes, A. 2012. 'Food Analysis: Present, Future, and Foodomics', ISRN Analytical Chemistry, 2012: 1-16.

Cooper, K. M., and D. G. Kennedy. 2007. 'Stability studies of the metabolites of nitrofuran antibiotics during storage and cooking', Food Addit Contam, 24: 935-42.

Dasenaki, M. E., and N. S. Thomaidis. 2015. 'Multi-residue determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs using liquid chromatography-tandem mass spectrometry', Anal Chim Acta, 880: 103-21.

De Luca, M., S. Mas, G. Ioele, F. Oliverio, G. Ragno, and R. Tauler. 2010. 'Kinetic studies of nitrofurazone photodegradation by multivariate curve resolution applied to UV-spectral data', Int J Pharm, 386: 99-107.

Douny, C., Widart, J., De Pauw, E., Silvestre, F., Kestemont, P., Thi Tu, H., Thanh Phuong, N., Maghuin-Rogister, G., and Scippo, M.-L. 2013. 'Development of an analytical method to detect metabolites of nitrofurans', Aquaculture, 376-379: 54-58.

European Commission. 1990. "Council Regulation (EEC) No 2377:90 Laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin." In, edited by European Commission.

European Commission. 1993. "Council Regulation (EEC) No 2901/93 amending Annexes I, II, III and IV to Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin." In, edited by European Commission.

European Commission. 2003. "Commission Decision amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin." In, edited by European Commission.

Government of Canada. 2017. 'List of Banned Drugs'. https://www.canada.ca/en/health-canada/services/drugs-health-products/veterinary-drugs/list-banned-drugs.html.

Hirakawa, K., Midorikawa, K., Oikawa, S., and Kawanishi, S.. 2003. 'Carcinogenic semicarbazide induces sequence-specific DNA damage through the generation of reactive oxygen species and the derived organic radicals', Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 536: 91-101.

Hoenicke, K., R. Gatermann, L. Hartig, M. Mandix, and S. Otte. 2004. 'Formation of semicarbazide (SEM) in food by hypochlorite treatment: is SEM a specific marker for nitrofurazone abuse?', Food Addit Contam, 21: 526-37.

Hoogenboom, L. A., and T. H. Polman. 1993. "Simultaneous detection of protein bound residues of nitrofuran drugs furazolidone, furaltadone, nitrofurazone and nitrofurantoin." In EuroResidue II, 6. Veldhoven, Netherlands.

Hoogenboom, L. A., M. Van Kammen, M. C. Berghmans, J. H. Koeman, and H. A. Kuiper. 1991. 'The Use of Pig Hepatocytes to Study the Nature of Protein-Bound Metabolites of Furazolidone: A New Analytical Method for Their Detection', Food and Chemical Toxicology, 29: 8.

Hu, Chunxiu, and Guowang Xu. 2013. 'Mass-spectrometry-based metabolomics analysis for foodomics', TrAC Trends in Analytical Chemistry, 52: 36-46.

Hui, Y., Zhang, X, and Yu, H-J. 2015. "Investigation on the Natural Occurrence of Semicarbazide in Macrobrachium Nipponese prawns." In International Symposium on Material, Energy and Environment Engineering. Changsha, China.

IARC. 1997. 'Nitrofural (Nitrofurazone)'. http://www.inchem.org/documents/iarc/vol50/09-nitrofural.html.

IARC. 1998. 'Semicarbazide Hydrochloride'. http://www.inchem.org/documents/iarc/vol12/semicarbazidehydrochloride.html.

Jia, W., X. Chu, J. Chang, P. G. Wang, Y. Chen, and F. Zhang. 2017. 'High-throughput untargeted screening of veterinary drug residues and metabolites in tilapia using high resolution orbitrap mass spectrometry', Anal Chim Acta, 957: 29-39.

Joshua, K., A. Sujatha, L.V. Ramana, E. Carolin, V. Supraba, and H. Dinesh Kumar. 2002. "Use of various chemicals in shrimp hatcheries and its sustainability in hatchery Technology." In National Workshop on Aquaculture Drugs. Cochin University of Science and Technology.

Knolhoff, A. M., and T. R. Croley. 2016. 'Non-targeted screening approaches for contaminants and adulterants in food using liquid chromatography hyphenated to high resolution mass spectrometry', J Chromatogr A, 1428: 86-96.

Love, D. C., S. Rodman, R. A. Neff, and K. E. Nachman. 2011. 'Veterinary drug residues in seafood inspected by the European Union, United States, Canada, and Japan from 2000 to 2009', Environ Sci Technol, 45: 7232-40.

Martinez Bueno, M. J., F. J. Diaz-Galiano, L. Rajski, V. Cutillas, and A. R. Fernandez-Alba. 2018. 'A non-targeted metabolomic approach to identify food markers to support discrimination between organic and conventional tomato crops', J Chromatogr A, 1546: 66-76.

McCalla, D.R. 1983. 'Mutagenicity of Nitorfuran Derivatives: Review', Env. Mutagenesis, 5: 21.

McCracken, R., B. Hanna, D. Ennis, L. Cantley, D. Faulkner, and D. G. Kennedy. 2013. 'The occurrence of semicarbazide in the meat and shell of Bangladeshi fresh-water shrimp', Food Chem, 136: 1562-7.

Mulder, P. P., B. Beumer, and J. A. Van Rhijn. 2007. 'The determination of biurea: a novel method to discriminate between nitrofurazone and azodicarbonamide use in food products', Anal Chim Acta, 586: 366-73.

Nacher-Mestre, J., M. Ibanez, R. Serrano, J. Perez-Sanchez, and F. Hernandez. 2013. 'Qualitative screening of undesirable compounds from feeds to fish by liquid chromatography coupled to mass spectrometry', J Agric Food Chem, 61: 2077-87.

Novotna, H., O. Kmiecik, M. Galazka, V. Krtkova, A. Hurajova, V. Schulzova, E. Hallmann, E. Rembialkowska, and J. Hajslova. 2012. 'Metabolomic fingerprinting employing DART-TOFMS for authentication of tomatoes and peppers from organic and conventional farming', Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 29: 1335-46.

Olive, P.L., and D.R. McCalla. 1977. 'Cytotoxicity and DNA Damage to Mammalian Cells by Nitrofurans', Chemical-Biological Interactions, 16: 11.

Patti, G. J., O. Yanes, and G. Siuzdak. 2012. 'Innovation: Metabolomics: the apogee of the omics trilogy', Nat Rev Mol Cell Biol, 13: 263-9.

Pereira, A. S., J. L. Donato, and G. De Nucci. 2004. 'Implications of the use of semicarbazide as a metabolic target of nitrofurazone contamination in coated products', Food Addit Contam, 21: 63-9.

Quilliam, M. A., McCarry, B.E., Hoo, K.H., McCalla, D.R., and Vaitekunas, S. 1987. 'Identification of the photolysis products of nitrofurazone irradiated with laboratory illumination', Canadian J. of Chem, 65: 1128-32.

Radovnikovic, A., E. R. Conroy, M. Gibney, J. O'Mahony, and M. Danaher. 2013. 'Residue analyses and exposure assessment of the Irish population to nitrofuran metabolites from different food commodities in 2009-2010', Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 30: 1858-69.

Righetti, L., J. Rubert, G. Galaverna, S. Folloni, R. Ranieri, M. Stranska-Zachariasova, J. Hajslova, and C. Dall'Asta. 2016. 'Characterization and Discrimination of Ancient Grains: A Metabolomics Approach', Int J Mol Sci, 17.

Rubingh, C. M., S. Bijlsma, E. P. Derks, I. Bobeldijk, E. R. Verheij, S. Kochhar, and A. K. Smilde. 2006. 'Assessing the performance of statistical validation tools for megavariate metabolomics data', Metabolomics, 2: 53-61.

Saari, L., and K. Peltonen. 2004. 'Novel source of semicarbazide: levels of semicarbazide in cooked crayfish samples determined by LC/MS/MS', Food Addit Contam, 21: 825-32.

Stadler, R. H., L. Verzegnassi, W. Seefelder, and L. Racault. 2015. 'Why semicarbazide (SEM) is not an appropriate marker for the usage of nitrofurazone on agricultural animals', Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 32: 1842-50.

Tengstrand, E., J. Rosen, K. E. Hellenas, and K. M. Aberg. 2013. 'A concept study on non-targeted screening for chemical contaminants in food using liquid chromatography-mass spectrometry in combination with a metabolomics approach', Anal Bioanal Chem, 405: 1237-43.

Tian, L., and S. Bayen. 2018. 'Thermal degradation of chloramphenicol in model solutions, spiked tissues and incurred samples', Food Chem, 248: 230-37.

Tian, W. R., Y. X. Sang, and X. H. Wang. 2014. 'Semicarbazide - from state-of-the-art analytical methods and exposure to toxicity: a review', Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 31: 1850-60.

Tian, X., Y. Xu, X. Song, X. Gong, Y. Liu, Q. Zhou, Z. Wang, and C. Xia. 2016. 'Temporal and spatial distribution of semicarbazide in western Laizhou Bay', Mar Pollut Bull, 112: 393-98.

Turnipseed, S. B., J. J. Lohne, and J. O. Boison. 2015. 'Review: Application of High Resolution Mass Spectrometry to Monitor Veterinary Drug Residues in Aquacultured Products', J AOAC Int, 98: 550-8.

Valera-Tarifa, N. M., P. Plaza-Bolaños, R. Romero-González, J. L. Martínez-Vidal, and A. Garrido-Frenich. 2013. 'Determination of nitrofuran metabolites in seafood by ultra high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry', J. of Food Comp. and Anal., 30: 8.

Van Poucke, C., C. Detavernier, M. Wille, J. Kwakman, P. Sorgeloos, and C. Van Peteghem. 2011. 'Investigation into the possible natural occurence of semicarbazide in Macrobrachium rosenbergii prawns', J Agric Food Chem, 59: 2107-12.

Vass, M., K. Hruska, and M. Franek. 2008. 'Nitrofuran antibiotics: a review on the application, prohibition and residual analysis', Veterinarni Medicina, 53: 32.

Villar-Pulido, M., B. Gilbert-Lopez, J. F. Garcia-Reyes, N. R. Martos, and A. Molina-Diaz. 2011. 'Multiclass detection and quantitation of antibiotics and veterinary drugs in shrimps by fast liquid chromatography time-of-flight mass spectrometry', Talanta, 85: 1419-27.

Vroomen, L. H., M. C. Berghmans, P. Hekman, L. A. Hoogenboom, and H. A. Kuiper. 1987. 'The elimination of furazolidone and its open-chain cyano-derivative from adult swine', Xenobiotica, 17: 1427-35.

Wang, Y., E. L. Jester, K. R. El Said, A. Abraham, J. Hooe-Rollman, and S. M. Plakas. 2010. 'Cyano metabolite as a biomarker of nitrofurazone in channel catfish', J Agric Food Chem, 58: 313-6.

Zhang, S., Y. Guo, Z. Yan, X. Sun, and X. Zhang. 2015. 'A selective biomarker for confirming nitrofurazone residues in crab and shrimp using ultra-performance liquid chromatography-tandem mass spectrometry', Anal Bioanal Chem, 407: 8971-7.

Zhang, S., P. Li, Z. Yan, J. Long, and X. Zhang. 2017. 'Identification and quantification of nitrofurazone metabolites by ultraperformance liquid chromatography-quadrupole time-of-flight high-resolution mass spectrometry with precolumn derivatization', Anal Bioanal Chem, 409: 2255-60.

Connecting Statement I

In **Chapter 1** a literature review established that the use of SEM as a way to monitor the abuse of NFZ in shrimp farming is not appropriate and can lead to product loss. Non-targeted analysis and its application to food monitoring was reviewed, as well as the recent advances in the search for a new metabolite which could be used to replace SEM in monitoring NFZ abuse. It was also established that there is a knowledge gap when it comes to the thermal stability of NFZ.

Chapter 2 will introduce an application of non-targeted analysis aiming at determining possible thermal degradation products of NFZ at pH 1.3 and 7. The purpose of this is to better understand the stability of NFZ and provide possible candidate molecular features to investigate in an actual food matrix in the following chapter.

Chapter 2: Non-Targeted Mass Spectrometric Analysis for the Determination of Possible Thermal Degradation Products of Nitrofurazone

Abstract: Thermal degradation products of nitrofurazone have not been comprehensively studied in the literature. In this study, a non-targeted approach based on High Performance Liquid Chromatography High Resolution Mass Spectrometry (HPLC-HRMS) was developed to investigate the thermal degradation of nitrofurazone. Aqueous solutions of nitrofurazone (50 mg/L in ultrapure water) were prepared at two pH levels (7 and 1.3) and then heated to 100°C for up to 240 mins. Samples were then diluted in HPLC water and analyzed with HPLC coupled to a Quadrupole Time of Flight (HPLC-QTOF) mass spectrometer. Significant degradation of nitrofurazone occurred after 240 minutes in at least one condition. After peak alignment, data processing resulted in the identification of 8 and 2 new molecular features in the thermally treated solutions at pH 7 and 1.3, respectively. Further MS/MS analysis was applied to attempt to identify the structure of these features. One of the molecular features in treated solutions at pH 7 was tentatively identified as (1E)-(2-furylmethylene)hydrazine and was selected for further confirmation using an analytical standard of the pure compound. However, the comparison with the purchased standard proved that the feature was not (1E)-(2-furylmethylene)hydrazine.

2.1 Introduction:

Nitrofurazone (NFZ) belongs to the family of nitrofurans, a group of synthetic broad-spectrum anti-microbial agents that have 5-nitro-2-furaldehyde as a common moiety. These drugs were routinely used in farming operations throughout the second half of the 20th century (Stadler et al. 2015), however, due to increasing evidence of their carcinogenicity (Olive and McCalla 1977; McCalla 1983) they were banned for use in food producing animals by most regulatory agencies in the early 90's (European Commission 1993; Canada 2017). Despite this, many farmers continue to use them for their availability and efficacy. Love et al. (2011) show that shrimps and prawns

represent the largest percentage of non-conforming food products in Japan, EU, and Canada, mostly as a result of nitrofuran detection.

Nitrofurans metabolize rapidly in shrimp and other farm animals and are non-detectable within 12 hours of last treatment (Chu et al. 2008). Therefore, our ability to monitor them relies on the detection of their main metabolites in animal tissue. Semicarbazide (SEM) is currently used to monitor nitrofurazone abuse in animal farming (Hoogenboom and Polman 1993), but its suitability as a marker for nitrofurazone has recently been brought to question as it has been shown to occur due to the degradation of azodicarbonamide (Pereira, Donato, and De Nucci 2004), and through hypochlorite treatment (Hoenicke et al. 2004). Most importantly, there are several reports showing the natural occurrence of SEM in shrimps and other crustaceans (Saari and Peltonen 2004; McCracken et al. 2013; Van Poucke et al. 2011), demonstrating the need for a new metabolite to monitor the abuse of nitrofurazone, particularly in shrimp farming.

Few studies describe the photodegradation of NFZ, mostly into its syn-isomer and into bis(5-nytrofurylidene) azine (Quilliam et al. 1987; De Luca et al. 2010). However, there are no published studies which discuss the possibility of NFZ degrading thermally. Due to the quick metabolism of the parent drug in animal treatment, a thermal degradation product might not serve as a replacement for SEM as a marker of NFZ. Despite this, it could still provide important insight into the stability of the bonds in the antibiotic structure, which may prove useful in future studies looking for new metabolites of NFZ.

Non-targeted analysis has proven to be an extremely useful tool in the age of high-resolution analytical instruments (Patti, Yanes, and Siuzdak 2012). A broad extraction procedure with minimal clean-up can result in large data-sets capable of housing the answers to complicated questions (Cifuentes 2012). For this reason, a “dilute and shoot” methodology is often preferred as it can generate the most complete image of a sample (Antignac et al. 2011), and hence the largest data set. This has been used in the determination of thermal degradation products of other antibiotics in both aqueous solutions and incurred samples (Tian and Bayen 2018) but has also been used in more complex matrices such as honey (Von Eyken et al. 2019).

Data analysis and treatment are often considered the rate limiting step of non-targeted analysis given the generation of these large datasets (Hu and Xu 2013). The use of dedicated

software programs (XCMS, MassProfiler®, Markerlynx®, Markerview®…) for the sorting of data, and of robust metabolite libraries (METLIN, Human Metabolome Database, SciFinder, PubChem, ChemSpider…) for metabolite identification from high accuracy masses become key in the data treatment of a non-targeted experiment. (Knolhoff and Croley 2016)

The purpose of this study was to design a non-targeted thermal degradation study to determine novel degradation products of NFZ in water and mild acid and evaluate the power of the data bank generated in providing some possible mechanistic information.

2.2 Materials and Methods:

2.2.1 Chemicals:

HPLC grade Water, HPLC grade Acetonitrile, HPLC grade Methanol, 1N Hydrochloric Acid, and Optima LC/MS Formic Acid were all purchased from Fisher Scientific (Hampton, NH, USA). 5-Nitro-2-furaldehyde-semicarbazone (Nitrofurazone ≥97.0%), D3-Diphenylhydramine, D3-Acetylmorphine, and ^{13}C-^{15}N Labelled Nitrofurazone were purchased from Sigma Aldrich (St. Louis, MO, USA). An analytical standard of [(furan-2-yl)methylidene]hydrazine was purchased from ChemSpace. (Princeton, NJ, USA).

D3-Diphenylhydramine and D3-Acetylmorphine were used as injection internal standards (IS) and were added for a final concentration of 0.02 mg/L for all samples. A stock solution of 603.5 mg/L of nitrofurazone was prepared in methanol and was used for calibration curve. All stock solutions were stored in amber vials in the dark at -20°C until use.

2.2.2 Sample Preparation:

Based on Tian and Bayen's (2018) thermal degradation study of the antibiotic chloramphenicol, a similar study was designed for detecting thermal degradation products of Nitrofurazone (NFZ). All samples were prepared in triplicate for statistical relevance. Solutions of 5 mL of NFZ at 50 mg/L were prepared in HPLC grade water and in 0.1M HCl in 20 mL amber vials (measured pH of 7.5±0.2, and 1.2±0.1 respectively). The acidified samples were tested to account

for possible acidification during cooking. The samples were then heated to 100°C in a boiling water bath for 30 min (t1), 60 min (t2), 120 min (t3), and 240 min (t4) (in triplicates) to simulate normal cooking conditions. Samples of both pH values were kept as controls with 0 min of heating (t0). Blank HPLC grade water and 0.1 M HCl samples were also studied at t0 and t4 in triplicates. After heating, the samples were filtered through 0.22 µm filters and diluted ten times in HPLC grade water for injection (100 µL of sample, 850 µL HPLC Water, 50 µL Injection Positive Internal Standard (IS) stock solution to have final IS concentration of 0.02 mg/L).

Calibration samples of NFZ were prepared using a stock solution of 603.5 mg/L NFZ to final concentrations 0.02412 mg/L, 0.04824 mg/L, 0.4824 mg/L, 0.9648 mg/L, and 2.412 mg/L. They all contained a final IS concentration of 0.02 mg/L.

2.2.3 Instrumental Analysis:

Samples were analyzed using a 1290 Series LC System from Agilent Technologies (Santa Clara, CA, USA) equipped with an InfinityLab Poroshell 120 EC-C18 column (3.0 X 150 mm, 2.7 µm) fitted with an Ecplise Plus C18 guard column (2.1 × 5 mm, 1.8 µm) both from Agilent Technologies. The mobile phases were a mixture of HPLC grade water with 0.1% formic acid (Mobile Phase A) and HPLC grade acetonitrile with 0.1% formic acid (Mobile Phase B) with a flow rate of 0.3 mL/min. The solvent gradient in the 25-minute run was as follows: 0 min (95%A, 5%B), 1.00 min (95%A, 5%B), 15.00min (0%A, 100%B), 20.00 min (0%A, 100%B), and 20.10 min (95%A, 5%B). The LC system was coupled to a 6545 series Quadrupole Time of Flight Mass Spectrometer (Q-TOF-MS) from Agilent Technologies, equipped with a Dual AJS ESI ion source operating in positive ionization mode. The ESI was operating with a gas temperature of 325°C, and a drying gas flow of 5 L/min, a pressure of 20psig at the nebulizer and a sheath gas temperature of 275°C and flow rate of 12 L/min. The capillary voltage was set to 4000 V and the nozzle voltage to 2000 V. Finally, the MS TOF fragmentor was set to 175 V, and the skimmer at 65 V. For the tandem MS experiments, all parameters were set as in the original analysis, and selected peaks were set to fragment at 30 eV.

2.2.4 Data Treatment:

Using Agilent Masshunter Workstation software Quantitative Analysis B.07.01, the peaks for NFZ and for both injection internal standards were monitored (NFZ $[M+H]^+$: 199.0467 *m/z* at 7. ±0.05 min, D3-Diphenylhydramine (DPH) $[M+H]^+$: 259.1889 *m/z* at 9.3 min ±0.05 min, D3-6-Acetylmorphine (AM) $[M+H]^+$: 331.1734 *m/z* at 6.5 min ±0.05 min). With the calibration curve data, a Relative Response Factor (RRF) was calculated with each Internal Standard using the following equation (Equation 1):

$$RRF = \frac{\frac{Area\ NFZ}{Area\ IS}}{\frac{[NFZ]}{[IS]}} \qquad \text{Equation 1}$$

The average RRF can be used to calculate concentration of NFZ in the thermal degradation samples given that the areas for both peaks and the IS concentration are known. A summary of all the concentrations and areas can be found in Supplementary Table 2.1. The NFZ concentration was then subjected to a one tailed t-test using Microsoft Excel (Version 16.11.1) to determine if there was significant degradation between t0 and the different time points of the experiment. A p-value of 0.05 was set as the limit for acceptability. These results can be found in Supplementary Table 2.2 for both pH values.

Having observed statistically significant degradation in at least one time-point, the Q-TOF data was aligned with Agilent Masshunter Profinder software (version B.08.00) using guidelines for data treatment outlined by Von Eyken and Bayen (2019). Briefly, the peaks with counts above 200 were selected for extraction, setting gain or loss of protons as the only source of ions, and looking for any common organic molecules in the isotopic patterns. The retention time error was set to 0.00% + 0.3 minutes, and the mass error to 20.0 ppm +2 mDa. The aligned peak data was then analyzed using Agilent Masshunter Mass Profiler Professional (MPP) software (version B.14.8). Volcano plots were performed for all conditions comparing t0 vs t4 with a fold change cut-off of 1.0 and a p-value cut-off of 0.05.

2.2.5 Quality Assurance/Quality Control (QC/QC):

Quality Control (QC) samples generated by aliquoting 10 µL of each sample and followed the same data treatment as experimental samples with Profinder and MPP. Principal component analysis (PCA) was run during the set-up of the MPP experiment to ensure grouping of QC samples.

Mass accuracy of the NFZ standard and the injection IS was measured throughout to ensure accuracy of the data. Recoveries of calibration standards injected throughout the HPLC-QTOF run were calculated using the RRF of DPH to ensure intensities were consistent throughout the run.

2.3 Results and Discussion:

2.3.1 Method Validation and Quality Control:

The calibration curve corrected for the D3-diphenylhydramine area resulted in acceptable linearity (R^2=0.9785) considering the residuals graph (Supplementary Figure 2.1). Following quality control guidelines from Cajka and Fiehn (2016), the samples were randomized during the injection. QC samples were injected throughout the run. To ensure stability of the mass value the mass error was calculated in samples for the NFZ, DPH and AM, and the average error was -8.4 ppm, -6.4 ppm, and -0.6ppm respectively. Despite being a little over the expected error, it still falls within the mass extraction parameters of Profinder and Masshunter Qualitative and Quantitative analysis software. Due to the higher signal response and recovery of subsequent calibration standard injections, the RRF with D3-diphenylhydramine was chosen for the quantification in samples. The average RRF was 0.01173, with a relative standard deviation of 16.1%. The third calibration standard was reinjected every 10 samples during the HPLC-QTOF run to ensure stability of the intensity data. The recovery for these calibration samples using the RRF of DPH was 88%. Injection blanks and procedural blanks were analyzed and confirmed to be NFZ-free.

2.3.2 Potential Thermal Degradation Products:

Degradation kinetics were studied using the theoretical equation for first order kinetics below (Equation 2). A plot of ln[C] vs time (minutes) showed that the thermal degradation of NFZ seemed to obey first order kinetics, with a rate constant of $1.5X10^{-3}$ (Supplementary Figure 2.2) using Equation 2 below. [C] corresponds to the concentration of NFZ at any given time, $[C_0]$ to the concentration of NFZ at time 0, k is the rate constant, and t is the time in minutes. This rate constant is smaller than that reported by De Luca et al. (2010) of $8.6X10^{-2}$ for the photodegradation of NFZ. However, there have been no reports of the thermal degradation of NFZ to compare rate constants with.

$$\ln[C] = \ln[C_0] + k \times t \qquad \text{Equation 2}$$

Having determined that the peak area of NFZ decreased significantly with cooking (shown in Figure 2.1), the next step was to use MPP to determine what entities were increasing with cooking time. Through the use of the volcano plot tool of this software, entity lists were generated with entities that increased significantly from unheated samples to samples heated for 4 hours. The lists for the water and acidified experiments were then analyzed manually with the entity inspector tool of MPP to verify increasing peak size throughout heating experiment. A finalized entity list of interest was then re-extracted from the raw data with the Agilent Masshunter Workstation Qualitative Analysis (version B07.01) to confirm proposed entities from processed data. This finalized list was selected to run tandem MS analysis to attempt to elucidate their structures.

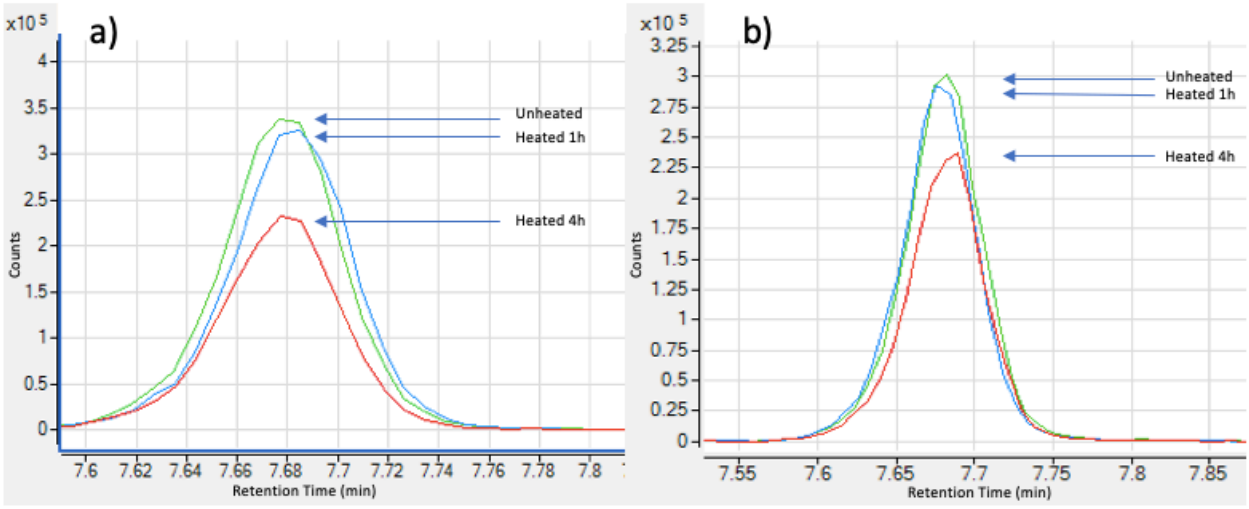


Figure 2.1: Extracted Ion Chromatograms (EIC) for Nitrofurazone peak ($[M+H]^+$ of 199.0467 m/z) in water (a) and acid (b) experiments

The targeted MS/MS data was used to generate formulas using Agilent Masshunter Workstation Qualitative Analysis software. The summary of this list of compounds with their proposed formulas and scores, as well as presence or absence in different sample groups can be found in Table 2.1. Despite generating a formula, the Agilent MassHunter Molecular Structure Correlator B.07.00 (MSC) software was not always capable of finding a structure that could match with the fragmentation pattern obtained using Chemspider as a reference library. However, compounds 1, 2, 5, 6, 8 and 10 obtained several matches which had to be narrowed down by similarity in structure to the parent compound and likeliness to occur given experimental conditions. The highest scored match for each of these compounds as well as the structure of the parent compound are shown in Figure 2.2. As can be noted in Table 2.1, only CPD10 was observed in both the acidified and the neutral pH experiments. It is expected that there would be different degradation products depending on the pH as many degradation mechanisms are affected by the presence or absence of free protons. However, it would have been expected that more degradation would occur at an acidic pH and this was not the case.

M+H	Name	RT	Suggested Formula	Mass Error (ppm)	Water (Unheated)	Water (Heated 4h at 100ºC)	Acid (Unheated)	Acid (Heated 4h at 100ºC)	Number of Matches in MSC
111.0916	CPD1	2.3	C6H10N2	6.2	ND	ND	ND	Yes (99.16)	292
111.0555	CPD2	2.9	C5H6N2O	3.4	ND	Yes (90.33)	ND	ND	192
206.0537	CPD3	4.8	C6H9N2O6	1.9	ND	Yes (79.19)	ND	ND	0
228.0335	CPD4	4.8	C6H5N5O5	33.9	ND	Yes (86.68)	ND	ND	0
389.1181	CPD5	4.8	C16H16N6O6	28.6	ND	Yes (88.1)	ND	ND	9
96.0443	CPD6	5.8	C5H5NO	6.4	ND	Yes (91.7)	ND	ND	50
127.0505	CPD7	5.8	C3H4N5O	-10.9	ND	Yes (78.97)	ND	ND	0
167.0449	CPD8	6.0	C7H6N2O3	7.7	ND	Yes (92.12)	ND	ND	300
189.0272	CPD9	6.0	C7H8O4S	-50.4	ND	Yes (80.76)	ND	ND	0
214.0465	CPD10	9.0	C7H7N3O5	-1.0	ND	Yes (86.68)	ND	Yes (86.68)	13

Table 2.1: Summary of the generated formulas for the chromatographic peaks of interest in both the water and acid degradation studies. If present in a sample group, the number in parenthesis represents the formula match score provided by the software. ND stands for not detected, and mean's peak height had a signal to noise ratio below 3.

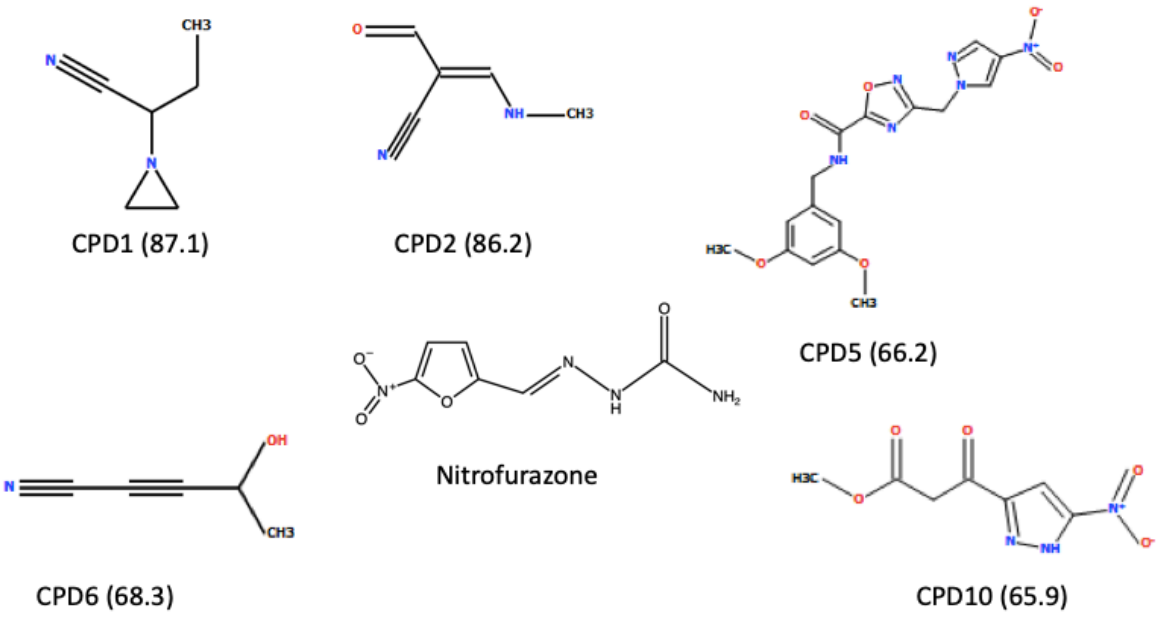


Figure 2.2: Top match for each of the compounds with matches in the Molecular Structure Correlator software as well as the parent structure of Nitrofurazone. In parenthesis is the structural match calculated by the software by comparing actual and theoretical spectral features in MS/MS spectra.

Given that no thermal degradation studies for nitrofurazone have been published in the literature, there was limited structural information to base the classification of proposed structures. Quilliam et al. (1987) and De Luca et al. (2010) proved that nitrofurazone is photolabile, and therefore experiments were carried out in amber vials with minimal exposure to light. Given that cooking happened inside the covered water bath, cooking time should not correlate to a higher exposure to light, and the structures proposed by these previous studies should not have been present in the list of entities having increased with cooking time. In fact, the mass of 279.0366 m/z, which would be equivalent to the $[M+H]^+$ ion of bis(5-nytrofurylidene)-azine, the main photo-degradation product discussed by these authors, is not observed in any sample in either the entity lists generated by MPP or by manual extraction of the raw data.

Of the structures proposed by the MSC, only compounds 2 and 10 provided a structure that seemed likely considering the structure of the parent nitrofurazone. The extracted ion chromatograms (EIC) of both these compounds contain an increasing signal with cooking time

can be found in Supplementary Figure 2.3. EIC's for all entities reported in Table 2.1 showed an increase in intensity with cooking time when extracted manually from the raw data, which confirms the data treatment with Profinder and MPP was successful in separating entities of interest. However, some entities in the list generated by MPP did not prove to be of interest once manual extraction of the raw data was performed, which comes to show that results from treated data should always be confirmed with the raw data.

[(Furan-2-yl)methylidene]hydrazine (F2MH) (Figure 2.3a), a proposed structure for CPD 2, could be thought of as nitrofurazone having lost the nitro and amide moieties. Its structural similarities made it stand out from the list of proposed structures, particularly since the reaction conditions could be enough to lead to this product. The degradation could be thought to occur in two distinct steps: the loss of the nitro group, and the hydrolysis of the amide releasing the hydrazine. Laali and Gettwert (2001) and Marziano, Traverso, and Cimino (1980) discussed the nitration of aromatic compounds. Theoretically, these reactions could occur in reverse and result in the loss of a nitro group from an aromatic ring. Their proposed reactions occur in slightly different conditions, mainly in the presence of a stronger acid, but could theoretically be applicable to aqueous systems. On the other hand, the hydrolysis of amides as described by Cox (2012) is a well understood process that occurs in the current experimental conditions. A diagram showing a proposed mechanism for this degradation can be seen in Supplementary Figure 2.4. Using the data generated by the non-targeted acquisition, extracted ion chromatograms for the masses of theoretical intermediate products could be observed as increasing with cooking time and are shown in Supplementary Figure 2.5. It is interesting to note, that although the intensity of these entities increases with cooking time they were not detected by the data treatment and therefore are not a part of Table 2.1. It is likely that this is due to the peak height filters of the initial Profinder treatment.

As for CPD 10, there are strong structural similarities between nitrofurazone and N'-acetyl-5-nitro-2-furohydrazide (Figure 2.3b), but the replacement of an α-amino group by a methyl group seems unlikely given the reaction conditions. For this reason, a straightforward mechanism cannot be proposed for this compound. The intensity of CPD10 was statistically higher in the acid treatment experiment ($8X10^3$ counts versus $3.5X10^3$ counts in water) which

suggests that the degradation mechanism leading to this product is catalyzed by the presence of free protons.

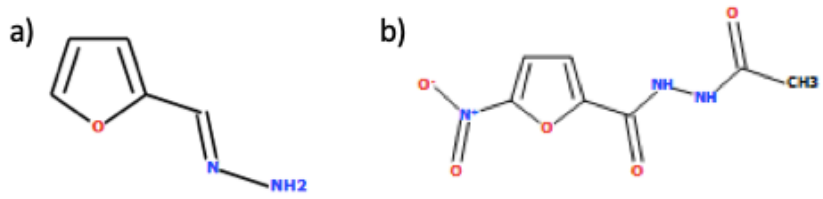


Figure 2.3: Structure of [(Furan-2-yl)methylidene]hydrazine (a) and N'-Acetyl-5-nitro-2-furohydrazide (b)

2.3.3 Confirmation with Labelled NFZ and Standard Comparison:

The thermal degradation methodology described was repeated for t0, t3 and t4 using ^{13}C-^{15}N-labelled nitrofurazone (See Figure 2.4 for structure) to have more insight into the degradation mechanism and possible degradation products. A peak for the $[M+H]^+$ of the labelled equivalent of CPD2 (113.0499 *m/z*) was observed at a similar retention time in this degradation study (Figure 4). More importantly, the original CPD2 peak of 111.0555 *m/z* at 2.9 minutes is no longer observed. The MS/MS spectra showed same main fragments with a change in mass of the molecular ion peak, and of the peak at 96.0302 *m/z* which shifted to 98.0254 *m/z*.

The absence of all the masses in Table 2.1 was confirmed in the thermal degradation of the labelled NFZ which suggests that they are indeed coming from NFZ. Moreover, an equivalent of CPD10 was observed in the degradation of the labelled NFZ with an $[M+H]^+$ mass of 217.0427 *m/z* coming out at exactly the same retention time of 9.05 minutes. The EIC chromatogram comparison of the labelled and the native standards for this compound can be found in Supplementary Figure 2.6. This further suggests that CPD10 is a degradation product of NFZ and further experiments should be done to attempt to identify its structure. Similarly, a peak which could correspond to CPD5 was observed with a +6 *m/z* shift ($[M+H]^+$ 395.1158 *m/z*) which could signal that CPD5 is due to the dimerization of NFZ, although the peak is 1000 times smaller in the

labelled standard than in the native standard degradation. Equivalent peaks for the other compounds in Table 2.1 were not observed.

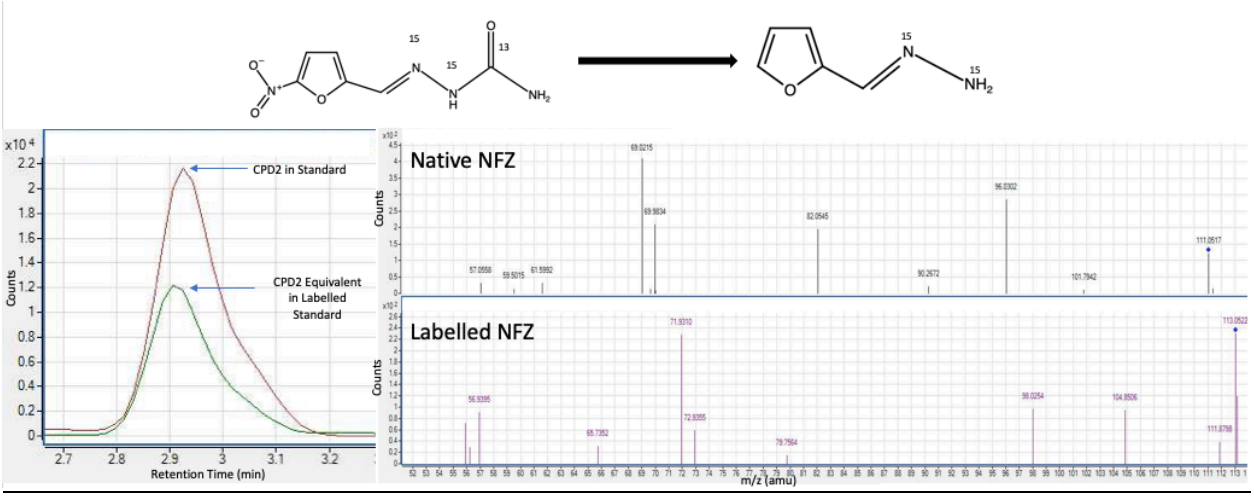


Figure 2.4: CPD2 chromatogram and MS/MS spectrum comparison between t4 for standard and labelled standard thermal degradation studies

This provided enough proof to justify purchasing a standard of F2MH. This standard was then analyzed with the same method to compare MS/MS spectra and retention times. However, the purchased F2MH standard did not match the RT or the fragmentation pattern when compared to the degradation product. The comparison of the RT and MS/MS fragmentation pattern can be observed in Figure 2.5 below.

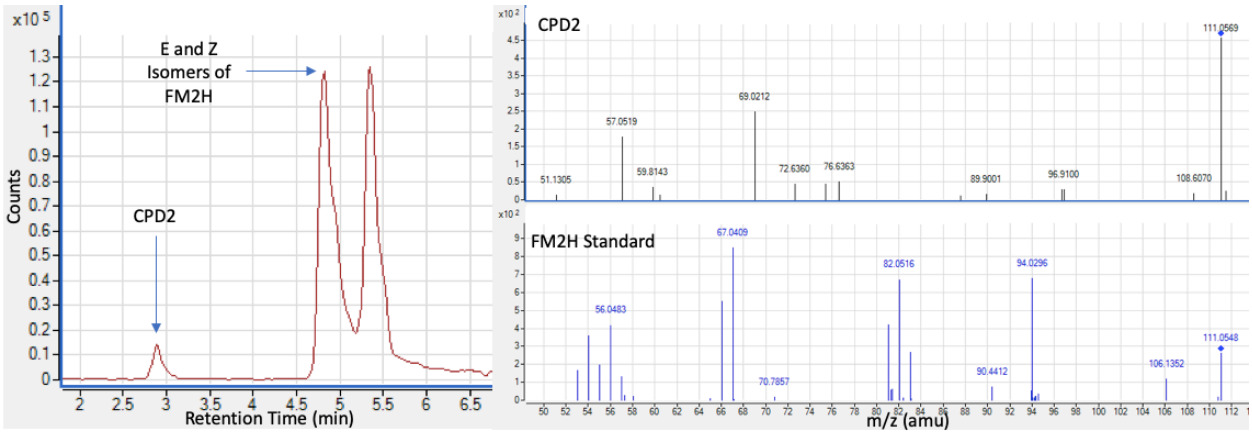


Figure 2.5: EIC and MS/MS spectra of thermal degradation sample spiked with pure FM2H

2.4 Conclusions:

Degradation of nitrofurazone occurred at 100°C in aqueous solutions cooked for different time periods. The use of a non-targeted workflow allowed for the detection and formula generation of 10 possible degradation products. Of these 10 formulas, the Molecular Structure Correlator managed to propose structures for 6. Given the high number of matches and the lack of other thermal degradation studies of nitrofurazone to guide in the classification of proposed structures, only entity was ultimately chosen to compare to a pure standard. CPD2 with monoisotopic mass of 110.0480 amu and retention time (RT) of 2.95 minutes was tentatively identified as [(furan-2-yl)methylidene]hydrazine, but comparison with a pure standard showed this to be false. If a proper model for RT prediction was commercially available and accurate, it could act as a secondary confirmation prior to purchasing standards and would save time and money in the type of workflow proposed. Despite not elucidating their structure, this study still found 10 entities which increased significantly with cooking time and shows that there is more that we could learn about the structural stability of nitrofurazone.

Nitrofurazone is metabolized quickly by animals and thermal degradation products are unlikely to be the answer in the search for a new metabolite for monitoring purposes. However, a better understanding of the stability of different covalent bonds within the molecule can give a hint in the search for a new metabolite capable of replacing semicarbazide. Overall, this study shows that a non-targeted workflow can be useful in the determination of novel degradation products. Moreover, it suggests that the large data set generated by a non-targeted approach can be useful in looking for possible intermediates of the degradation in hope of understanding mechanistic pathways.

2.5 References:

Antignac, J. P., F. Courant, G. Pinel, E. Bichon, F. Monteau, C. Elliott, and B. Le Bizec. 2011. 'Mass spectrometry-based metabolomics applied to the chemical safety of food', TrAC Trends in Analytical Chemistry, 30: 292-301.

Cajka, T., and O. Fiehn. 2016. 'Toward Merging Untargeted and Targeted Methods in Mass Spectrometry-Based Metabolomics and Lipidomics', Anal Chem, 88: 524-45.

Chu, P.S., M.I. Lopez, A. Ann, K.R. El Said, and S.M. Plakas. 2008. 'Residue Depletion of Nitrofuran Drugs and Their Tissue-Bound Metabolites in Channel Catfish (Ictalurus punctatus) after Oral Dosing', J. of Agri and Food Chem, 56: 5.

Cifuentes, A. 2012. 'Food Analysis: Present, Future, and Foodomics', ISRN Anal Chem, 2012: 1-16.

Cox, Robin A. 2012. 'Revised Mechanisms for Simple Organic Reactions.' in, Advances in Phys Org Chem Volume 46.

De Luca, M., S. Mas, G. Ioele, F. Oliverio, G. Ragno, and R. Tauler. 2010. 'Kinetic studies of nitrofurazone photodegradation by multivariate curve resolution applied to UV-spectral data', Int J Pharm, 386: 99-107.

European Commission. 1993. "Council Regulation (EEC) No 2901/93 amending Annexes I, II, III and IV to Regulation (EEC) No 2377/90 laying down

a Community procedure for the establishment of maximum residue limits of

veterinary medicinal products in foodstuffs of animal origin." In, edited by European Commission.

Government of Canada. 2017. 'List of Banned Drugs'. https://www.canada.ca/en/health-canada/services/drugs-health-products/veterinary-drugs/list-banned-drugs.html.

Hoenicke, K., R. Gatermann, L. Hartig, M. Mandix, and S. Otte. 2004. 'Formation of semicarbazide (SEM) in food by hypochlorite treatment: is SEM a specific marker for nitrofurazone abuse?', Food Addit Contam, 21: 526-37.

Hoogenboom, L. A., and T. H. Polman. 1993. "Simultaneous detection of protein bound residues of nitrofuran drugs furazolidone, furaltadone, nitrofurazone and nitrofurantoin." In EuroResidue II, 6. Veldhoven, Netherlands.

Hu, C., and Xu,G. 2013. 'Mass-spectrometry-based metabolomics analysis for foodomics', TrAC Trends in Analytical Chemistry, 52: 36-46.

Knolhoff, A. M., and T. R. Croley. 2016. 'Non-targeted screening approaches for contaminants and adulterants in food using liquid chromatography hyphenated to high resolution mass spectrometry', J Chromatogr A, 1428: 86-96.

Laali, K. K., and V. J. Gettwert. 2001. 'Electrophilic Nitration of Aromatics in Ionic Liquid Solvents', J. Org. Chem., 66: 6.

Love, D. C., S. Rodman, R. A. Neff, and K. E. Nachman. 2011. 'Veterinary drug residues in seafood inspected by the European Union, United States, Canada, and Japan from 2000 to 2009', Environ Sci Technol, 45: 7232-40.

Marziano, N. C., P. G. Traverso, and G. G. Cimino. 1980. 'Thermodynamic Nitration Rates of Aromatic Compounds. Part 1. The Nitration of Benzene and Some Benzene Derivatives in Aqueous Sulphuric and Perchloric Acids. A Comparison of the Results referred to Water as Standard State', J.C.S Perkin II: 5.

McCalla, D.R. 1983. 'Mutagenicity of Nitorfuran Derivatives: Review', Environ Mutagen, 5: 21.

McCracken, R., B. Hanna, D. Ennis, L. Cantley, D. Faulkner, and D. G. Kennedy. 2013. 'The occurrence of semicarbazide in the meat and shell of Bangladeshi fresh-water shrimp', Food Chem, 136: 1562-7.

Olive, P.L., and D.R. McCalla. 1977. 'Cytotoxicity and DNA Damage to Mammalian Cells by Nitrofurans', Chem-Biol Interactions, 16: 11.

Patti, G. J., O. Yanes, and G. Siuzdak. 2012. 'Innovation: Metabolomics: the apogee of the omics trilogy', Nat Rev Mol Cell Biol, 13: 263-9.

Pereira, A. S., J. L. Donato, and G. De Nucci. 2004. 'Implications of the use of semicarbazide as a metabolic target of nitrofurazone contamination in coated products', Food Addit Contam, 21: 63-9.

Quilliam, M. A., McCarry, B. E., H. Hoo, K., McCalla, D. R., and Vaitekunas, S.. 1987. 'Identification of the photolysis products of nitrofurazone irradiated with laboratory illumination', Canadian J. of Chem, 65: 1128-32.

Saari, L., and K. Peltonen. 2004. 'Novel source of semicarbazide: levels of semicarbazide in cooked crayfish samples determined by LC/MS/MS', Food Addit Contam, 21: 825-32.

Stadler, R. H., L. Verzegnassi, W. Seefelder, and L. Racault. 2015. 'Why semicarbazide (SEM) is not an appropriate marker for the usage of nitrofurazone on agricultural animals', Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 32: 1842-50.

Tian, L., and S. Bayen. 2018. 'Thermal degradation of chloramphenicol in model solutions, spiked tissues and incurred samples', Food Chem, 248: 230-37.

Van Poucke, C., C. Detavernier, M. Wille, J. Kwakman, P. Sorgeloos, and C. Van Peteghem. 2011. 'Investigation into the possible natural occurence of semicarbazide in Macrobrachium rosenbergii prawns', J Agric Food Chem, 59: 2107-12.

Von Eyken, A., and S. Bayen. 2019. 'Optimization of the Data Treatment Steps of a Non-targeted LC-MS-Based Workflow for the Identification of Trace Chemical Residues in Honey', J Am Soc Mass Spectrom.

Von Eyken, A., D. Furlong, S. Arooni, F. Butterworth, J.-F. Roy, J. Zweigenbaum, and S. Bayen. 2019. 'Direct injection high performance liquid chromatography coupled to data independent acquisition mass spectrometry for the screening of antibiotics in honey', J. of Food and Drug Anal.

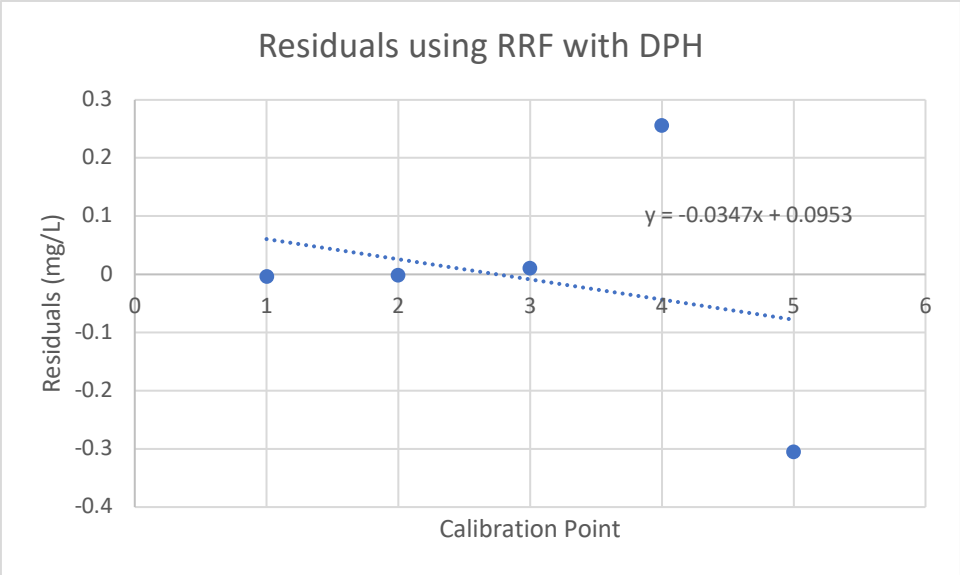
2.6 Supplementary Figures and Tables:

Water Experiment				
Concentration in Vial (ppm)	Boiled Time (min)	NFZ Area	Diphenyhydramine Area	Calculated NFZ Concentration (mg/L)
5	0	1209093	495861	4.16
5	0	1355065	562516	4.11
5	0	1192884	509578	3.99
5	30	1132389	482286	4.00
5	30	1190017	535365	3.79
5	30	1104371	482587	3.90
5	60	1152064	495684	3.96
5	60	599837	227237	4.50
5	60	1233307	558166	3.77
5	120	1112155	578143	3.28
5	120	1053329	455789	3.94
5	120	701825	334064	3.58
5	240	839948	588705	2.43
5	240	983525	571823	2.93
5	240	800597	492428	2.77
Acid Experiment				
Concentration in Vial (ppm)	Boiled Time (min)	NFZ Area	Diphenyhydramine Area	Calculated NFZ Concentration (mg/L)
5	0	952888	562722	2.89
5	0	1055084	669291	2.69
5	0	518103	350849	2.52
5	30	982023	589052	2.84
5	30	947818	562221	2.87
5	30	967126	544780	3.03
5	60	1008878	615360	2.80
5	60	987854	544729	3.09
5	60	1098530	599454	3.12
5	120	871724	576857	2.58
5	120	1136769	667293	2.90
5	120	840897	536852	2.67
5	240	825448	628956	2.24
5	240	799786	561959	2.43
5	240	934289	588892	2.70

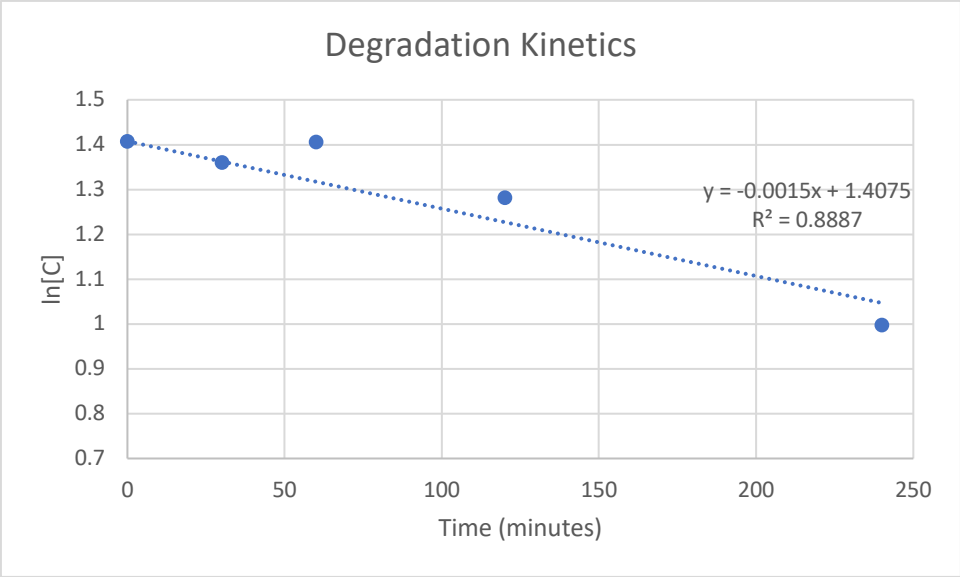
Supplementary Table 2.1: Calculated NFZ concentrations

Water Experiment				
Cooking Time (min)	Average Concentration (mg/L)	Standard Deviation	RSD	p-value
0	4.09	0.09	2.1	
30	3.90	0.11	2.7	0.055
60	4.08	0.38	9.3	0.485
120	3.60	0.33	9.2	0.073
240	2.71	0.25	9.4	0.008
Acid Experiment				
Cooking Time (min)	Average Concentration (mg/L)	Standard Deviation	RSD	p-value
0	2.70	0.18	6.9	
30	2.91	0.10	3.4	0.154
60	3.00	0.18	6.0	0.138
120	2.72	0.17	6.2	0.458
240	2.46	0.24	9.6	0.211

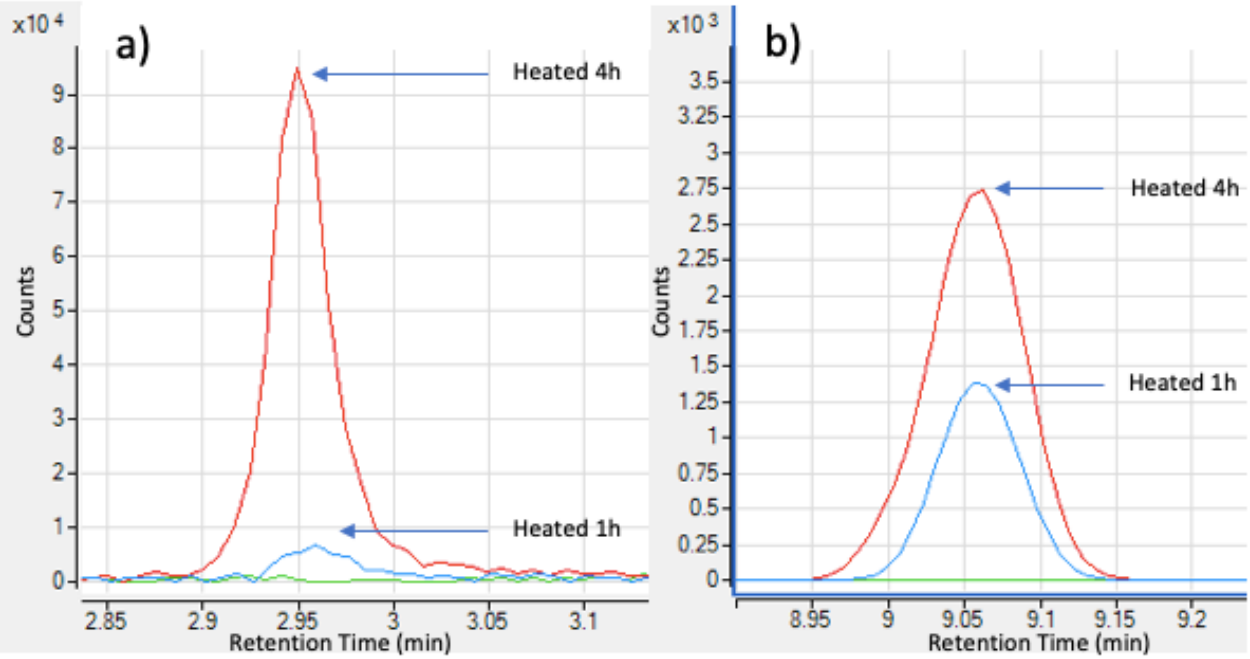
Supplementary Table 2.2: T-test results for significance of degradation



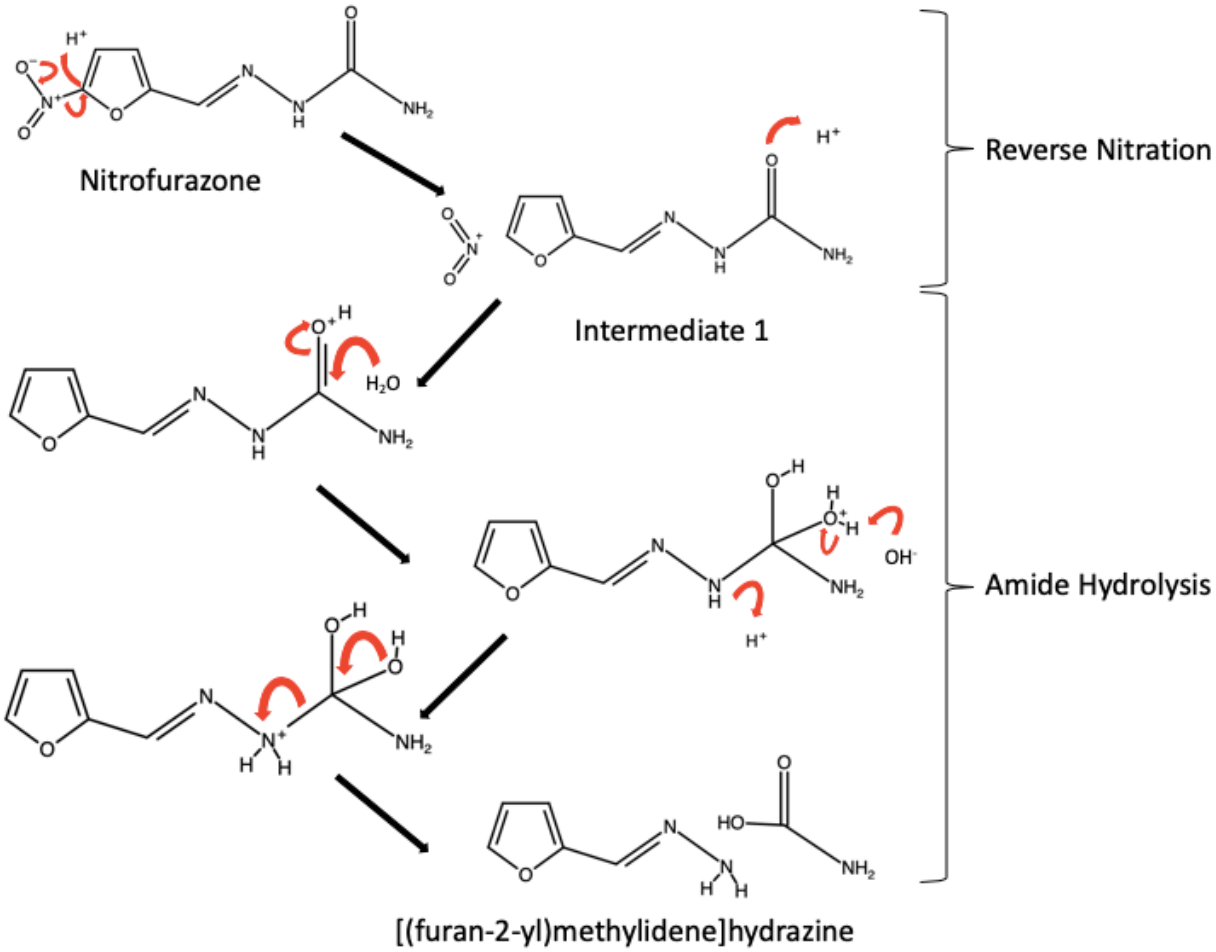
Supplementary Figure 2.1: Residuals of calibration curve using RRF with DPH



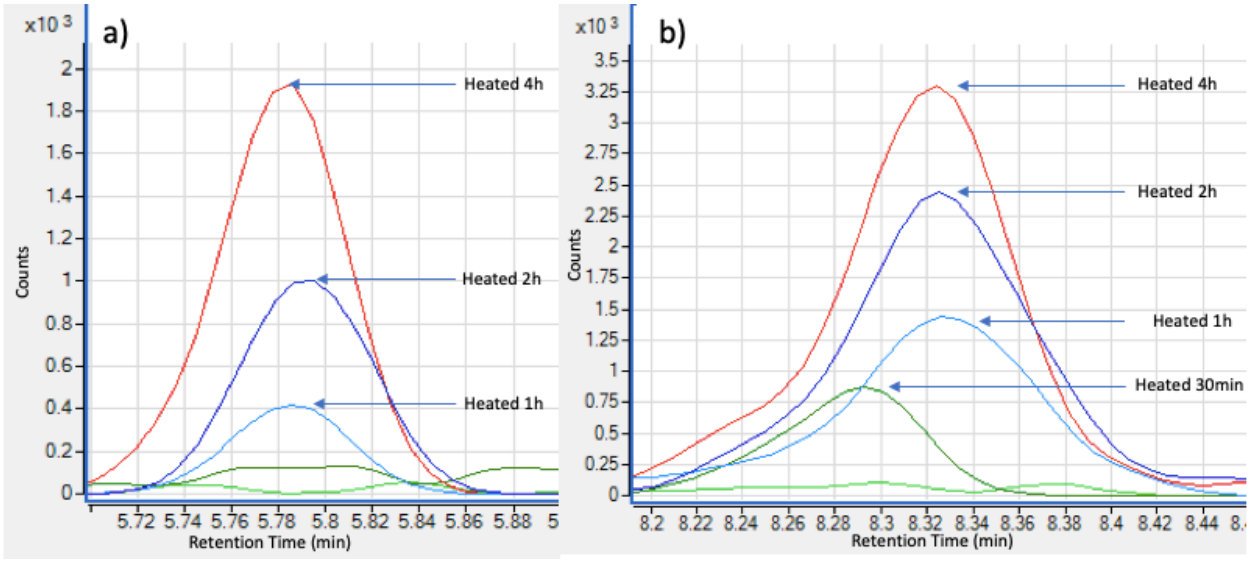
Supplementary Figure 2.2: Plot of ln[C] vs Time (minutes) used to calculate the k value of the degradation



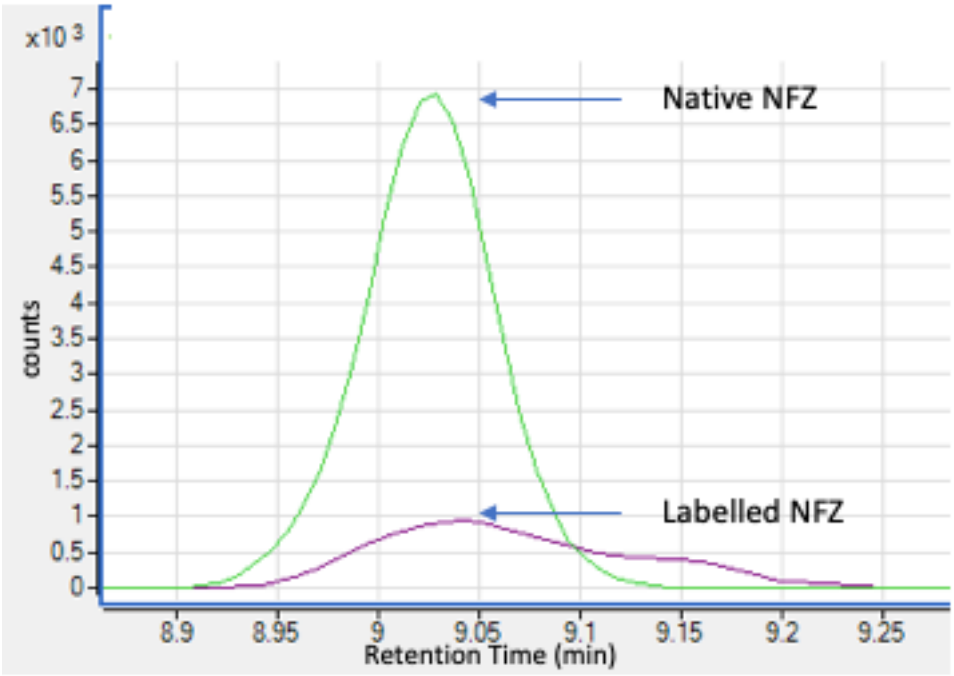
Supplementary Figure 2.3: Chromatograms showing increasing peak size for CPD2 (a) and CPD10 (b)



Supplementary Figure 2.4: Proposed mechanism for degradation of CPD 2.



Supplementary Figure 2.5: EIC for Intermediate 1 (a) with $[M+H]^+$ 154.0616 *m/z* and Intermediate 2 (b) with $[M+H]^+$ 156.0409 *m/z.* A slight shift in retention time in the heated sample after 30 mins in (b) is observed and could be due to changes beginning to happen in the matrix as the sample begins to heat which later stabilize with further heating.



Supplementary Figure 2.6: Comparison of native and labelled standard degradation of CPD10

Connecting Statement II

Chapter 2 provided evidence of the thermal degradation of NFZ in pH 1.3 and 7 solutions. A non-targeted approach provided information on several entities whose signal is increasing during thermal treatment of the pure standard. Although NFZ metabolizes quickly in shrimp, these entities are a good starting point in the analysis of the data of the following chapter.

Chapter 3 will introduce the application of non-targeted analysis of NFZ treated shrimp in the aim to detect a novel metabolite that could be used for the monitoring of NFZ by regulatory agencies. It will also discuss the development of statistical models for the differentiation between sample types using the large data banks generated by this type of analysis.

Chapter 3: Application of Non-Targeted Analysis in the Discrimination Between Nitrofurazone Treated and Control Pacific White Shrimp: Searching for a Replacement for Semicarbazide

Abstract: The validity of semicarbazide (SEM) as a metabolite for monitoring nitrofurazone (NFZ) abuse in farming has been brought to question in the recent years. Its occurrence from sources other than NFZ treatment make it questionable in its application in regulatory agencies. A comparison of four non-targeted extraction procedures was done to determine which one could give the most useful information in detecting a new metabolite for NFZ abuse monitoring. The chosen extraction seemed to show the most differences between treated and control samples but once more samples were analyzed and the data further treated, it was still unable to find a metabolite that could replace SEM. Despite this, significant differences in the profiles of entities detected were used to develop a statistical model based on Partial Least Squares Discriminant Analysis (PLS-DA) that could differentiate between treated and control shrimp. The model passed the internal cross-validation using the "leave one third out" method and showed an accuracy of up to 98%. However, once the model was applied to an external sample set it was unable to differentiate between treated and control shrimp.

3.1 Introduction:

Nitrofurazone (5-nitro-2-furaldehyde semicarbazone) is a synthetic broad-spectrum antibiotic of the nitrofuran family that has been banned by most regulatory agencies since the early 1990's due to increasing concerns on its carcinogenicity (Olive and McCalla 1977). The European Commission (EC) issued a warning with regards to nitrofurans in 1990, and finally banned them completely from use in food producing animals in 1993 (European Commission 1990, 1993). A similar ban was observed in Canada in 1994 for 5 nitrofuran compounds in food producing animals of which nitrofurazone was part (Canada 2017). Despite this, nitrofuran drugs are still used by farmers in some countries due to their efficacy and affordability, and therefore monitoring efforts by regulatory agencies became imperative in ensuring clean imported products (Vass, Hruska, and Franek 2008). Nitrofuran parent drugs are depleted quickly after

treatment, but tissue bound metabolites have been found to remain detectable for over 50 days in animal tissue and are often the best way to monitor the abuse of these antibiotics (Chu et al. 2008).

Semicarbazide (SEM) is currently the metabolite used to monitor the abuse of nitrofurazone (Figure 3.1) by regulatory agencies since the development of the analytical method by Hoogenboom et al. (1991) to detect furazolidone via its metabolite 3-amino-2-oxazolidinone (AOZ), and its later application to other nitrofuran drugs (Hoogenboom and Polman 1993). However, there has been growing evidence that SEM detection is not unique to NFZ abuse.

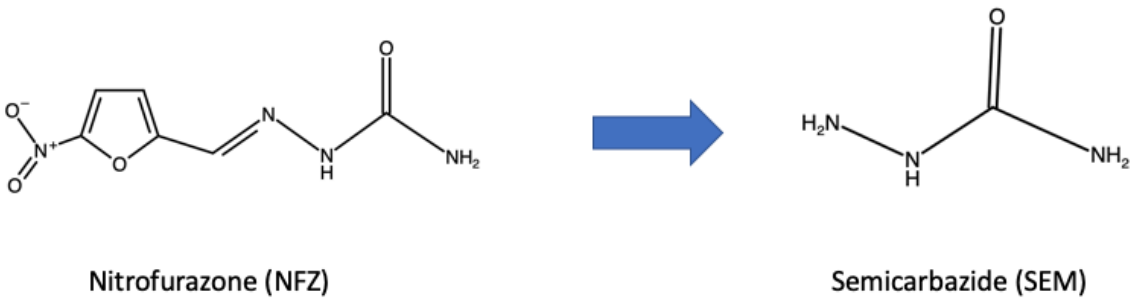


Figure 3.1: Structure of Nitrofurazone and its metabolite Semicarbazide.

Saari and Peltonen (2004) first detected SEM in crayfish that were caught from the wild at levels higher than the Minimum Required Performance Limit (MRPL) of 1 µg/kg and should not have been exposed to NFZ. Contemporarily, reports began to show that SEM was a side-product in the degradation of Azodicarbonamide, a flour bleaching and maturing additive (Pereira, Donato, and De Nucci 2004; Stadler et al. 2015) and this could cause problems for analysis of any shrimp samples coated with flour. Hoenicke et al. (2004) also showed that amino acid degradation due to hypochlorite treatment (as can sometimes be used in tank decontamination during shrimp production) can lead to SEM. Later studies also confirmed the suspicions of Saari and Peltnonen (2004) that SEM might be occurring naturally in some crustaceans (Van Poucke et al. 2011; McCracken et al. 2013; Hui, Zhang, and Yu 2015), particularly focusing on shrimp. Finally, Tian et al. (2016) studied the distribution of SEM as an environmental contaminant in Laizhou

Bay in China and discuss the implications that this might have in the detection of SEM in fish and shrimp products in the area. Figure 3.2 below is a summary of these studies. SEM is likely not the best way to monitor NFZ abuse since it may lead to significant product loss due to false positives.

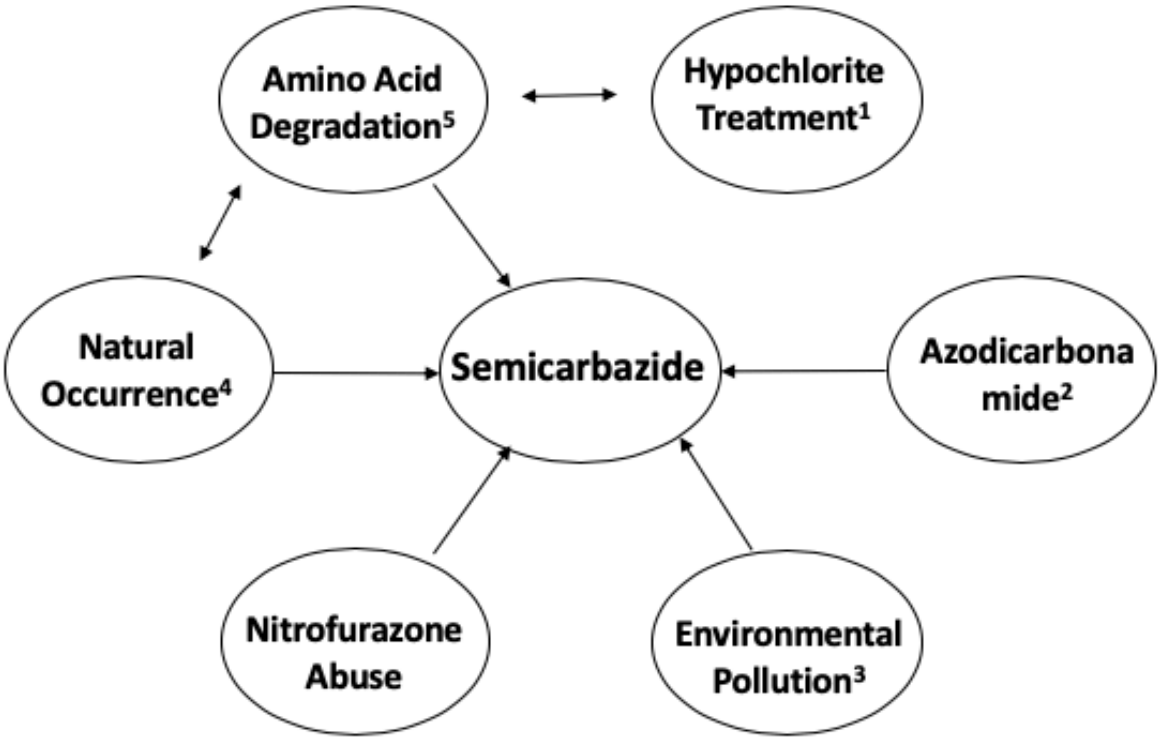


Figure 3.2: Summary of Studies Showing Different Sources of SEM. 1 and 5 (Hoenicke et al. 2004), 2 (Pereira, Donato, and De Nucci 2004; Stadler et al. 2015; Vass, Hruska, and Franek 2008), 3 (Tian et al. 2016), and 4 (Saari and Peltonen 2004; McCracken et al. 2013; Van Poucke et al. 2011; Hui, Zhang, and Yu 2015)

Efforts have been made in recent years to detect alternative metabolites to SEM that could be used to monitor NFZ abuse. Mulder, Beumer, and Van Rhijn (2007) suggest monitoring for biurea in samples analyzed for SEM, as it is another degradation product of azodicarbonamide (ADC) and would also be present in the sample if SEM presence is due to ADC contamination. This is an interesting method but does not account for any of the other possible sources of SEM described in Figure 3.2. Wang et al. (2010) found the presence of a cyano-metabolite in NFZ

treated catfish equivalent to the cyano-metabolite observed by Vroomen et al. (1987) for furazolidone. They discuss it as a possible biomarker for NFZ abuse in catfish, but this has never been detected in shrimp. Finally, Zhang et al. (2015) found that 5-nitro-2-furaldehyde (NF) could be detected in case of nitrofurazone abuse. The problem with this is that it remains a confirmation analysis since NF would theoretically be present in the case of treatment with any nitrofuran drug, and SEM analysis would still be necessary to confirm that this is indeed from nitrofurazone. Furthermore, no studies have been done to show whether any of these proposed metabolites might occur from sources other than NFZ abuse.

Advances in High Resolution Mass Spectrometry (HRMS) instrumentation and bioinformatics have made possible a whole new approach to new entity discovery using non-targeted analysis. A non-targeted approach utilises a broad extraction with minimal sample clean-up in order to see the widest range of molecules present in a sample (Patti, Yanes, and Siuzdak 2012). The sample is then often analyzed with liquid chromatography in tandem with HRMS (or sometimes Nuclear Magnetic Resonance (NMR)) (Antignac et al. 2011). The main problem with this workflow is that there is no standardization of extractions or instrumental analysis, and extractions need to be optimized for the matrix and application desired, particularly since it is recognized that no single method can detect all metabolites in a sample due to the high complexity of metabolites mixtures in most matrices (Cifuentes 2012; Knolhoff and Croley 2016). Von Eyken et al. (2019) study the use of an “All Ions MS/MS” analysis in which 4 collision energies are analyzed simultaneously in order to obtain even more information about the injected samples.

Data analysis is considered the rate limiting step in non-targeted analysis as the broad extraction and high-resolution analysis result in hundreds of thousands of peaks in the Total Ion Chromatograms (TIC). Different software is available for the alignment of chromatographic data and several authors have discussed the impact of choice of software and parameters in the final results observed (Von Eyken and Bayen 2019; Bletsou et al. 2015; Cajka and Fiehn 2016). Statistical analysis is then performed on the aligned data to obtain the answers to the desired questions. The aim of the presented project was to use this type of workflow to analyze NFZ

treated and control shrimp in hope to determine a new metabolite that could be used to monitor NFZ abuse.

Depending on the desired application, the large amount of data produced can be used to develop statistical models which can differentiate and class different groups of samples. This has been observed in food analysis in several studies. Righetti et al. (2016) use Partial Least Squares Discriminant Analysis (PLS-DA) to differentiate between different families of ancient grains. Martinez Bueno et al. (2018) and Novotna et al. (2012) discuss the use of Linear Discriminant Analysis (LDA) and PLS-DA respectively for the differentiation between organic and non-organic tomatoes. (Mi et al. 2019) have used PLS-DA models to differentiate between types of pork meat with lipidomic analysis and report up to 91.5% accuracy using over 100 variables. (Bondia-Pons et al. 2014) also mention the use of PLS-DA models to differentiate the origins of different Goji berry extracts. However, Rubingh et al. (2006) have discussed the risk of overfitting when using megavariate data. For this reason, cross-validation of models as well as external validation is often done to minimize the risk. A secondary aim of the proposed project was to use the data set generated by the non-targeted extraction to develop a model that could differentiate between control shrimp and shrimp treated by nitrofurazone.

3.2 Materials and Methodology:

3.2.1 Chemicals:

HPLC grade water, HPLC grade acetonitrile, HPLC grade methanol, HPLC grade EtOAc, 1N hydrochloric acid, Optima LC/MS formic acid (FA), ammonium acetate, and acetic acid were all purchased from Fischer Scientific (Hampton, NH, USA). Magnesium sulphate, sodium chloride, nitrofurazone, ^{13}C-^{15}N-labelled nitrofurazone, semicarbazide hydrochloride, sodium hydroxide, D3-acetylmorphine, D3-diphenylhydramine, dipotassium phosphate, and 2-nitrobenzaldehyde were all purchased from Sigma Aldrich (St. Louis, MO, USA)

Primary secondary amine (PSA) was purchased from Agilent Technologies (Santa Clara, CA, USA), sea salt free of nitrate and phosphate was purchased from Instant Ocean (Blacksburg, VA, USA), and BIOS Bacterial Mix was purchased from Aquaforest (Brzesco, Poland).

3.2.2 Shrimp Treatment:

The shrimp treatment procedure was derived from the procedure proposed by Douny et al. (2013) and from guidance with the shrimp farming experts at Planet Shrimp (Aylmer, ON, Canada). Three Seapora 60L tanks equipped with a Resun DC Air Pump, a Marina 100W Submersible Aquarium Heater, and a Tidal55 Aquarium Power Filter were filled with deionized water 3 weeks prior to receiving the shrimps. The salinity of each tank was then adjusted to 16 g/L by adding 960g of sea salt (Instant Ocean, Blacksburg, VA, USA). During this time the water was treated with 2 drops of BIOS bacterial mix (Aquaforest, Aylesbury, UK) daily and kept at a temperature of 29°C. Nitrate, nitrite and dissolved oxygen (DO) levels were monitored throughout the preparation using Hach Nitrite and Ammonia test kits (London, ON, Canada) and a DO meter (Traceable, Webster, TX, USA). An account of the monitoring data is presented in Supplementary Figure 3.1.

Pacific white shrimp (*L. Vannamei*) with an average weight of 10 g were driven from the facilities of Planet Shrimp (Aylmer, ON, Canada) in two 80 L coolers equipped with an oxygen tank and water at 20°C to reduce the metabolic rate and hence the release of nitrates. The shrimps were starved for one night prior to transport for the same reason. Once in the laboratory, each tank was filled with 50 shrimps. Multiple studies have used similar tank densities when performing antibiotic treatment experiments (Chu and Lopez 2005; Wang et al. 2004; Mohney et al. 1997). The first tank was designated the control tank (C) and the other two as treated tank 1 (T1) and treated tank 2 (T2). Shrimp that died during transport were immediately stored at -80°C upon arrival to the lab and were used to test the extraction procedures.

The shrimps were acclimated to the new tank conditions for 2 days prior to NFZ treatment. They were fed 5 g/tank of control feed (pellets coated with 3% (w/w) cod liver oil) (WN Pharmaceuticals, Coquitlam, BC, Canada) approximately every 7 hours, representing half their satiation rate of 3.8% body weight per day. After the acclimation period, T1 and T2 were fed 15g of treated feed (0.4% (w/w) NFZ coated with 3 % (w/w) cod liver oil) over 12 hours, while C was

fed 15g of control feed over the same time. The feed preparation was done following the procedure used by Douny et al. (2013).

The shrimps were then sacrificed by removing them from the water and placing them on ice beds 12 hours after the last NFZ treatment. According to Chu et al. (2008) this was the time period after which the parent drug was fully metabolized and could no longer be detected by their analysis. They were then stored at -80°C until being analyzed. Upon thawing, the shrimps were peeled carefully and sliced in half lengthwise. Half the shrimp was used for the raw extraction and the other half for the cooked extraction. The shells were used to validate the consumption of the NFZ treated feed by using the methodology currently used by regulatory agencies for their analysis (Chu, 2005).

3.2.3 Sample Treatment:

Raw Extraction Comparison:

Given the lack of standardization in non-targeted analysis, there is no golden standard when it comes to the choice of extraction for a given matrix. The closest thing to a standardized method is known as the QuEChERS extraction, which stands for Quick Easy Cheap Effective Rugged and Safe and has been tested for several matrices. There are no reports of non-targeted extractions of shrimp for the study of nitrofuran drugs in the literature, and therefore the first step was to validate and compare extraction procedures used in similar matrices or for similar analytes. Four extractions were chosen due to their use of different solvents and/or extract clean-up procedures, including one QuEChERS extraction (Nacher-Mestre et al. 2013; Villar-Pulido et al. 2011; Dasenaki and Thomaidis 2015; Chu and Lopez 2005). Each extraction test was comprised of 5 T1 shrimp, 5 T2 shrimp, 5 shrimps dead during transport, 5 procedural blanks, and 9 shrimps dead during transport spiked with NFZ for recovery tests at 60 ng/g, 600 ng/g and 6 µg/g in triplicate. All solvents used for these extractions were HPLC grade. Calibration samples of NFZ were prepared using a stock solution of 603.5 mg/L NFZ to final concentrations 0.02412 mg/L, 0.04824 mg/L, 0.4824 mg/L, 0.9648 mg/L, and 2.412 mg/L. They all contained a final labelled NFZ concentration of 1.0 mg/L, and concentration of injection internal standards (IS) D3-

acetylmorphine and D3-diphenylhydramine of 0.02 mg/L. All samples contained the same injection IS concentrations added before injection.

Nacher-Mestre et al. (2013) described a non-targeted screening method in fish and fish feed using a water-acetonitrile (80:20, v/v) solvent mix for their extraction. A variation of their methodology was **Extraction 1 (E1)** in this study. Briefly, 1 g of shrimp muscle was homogenized with a mortar and pestle, at this point all samples were spiked with 10 µL of 0.1 µg/mL ^{13}C-^{15}N-labelled NFZ, and recovery samples were spiked with the corresponding NFZ concentrations (60 ng/g, 600 ng/g and 6 µg/g). The tissue samples were then further homogenized with 10 mL of acetonitrile/water (80:20, v/v) with 0.1% FA using a vortex mixer (Fischer Scientific, Hampton, NH, USA) for 1 hour and then sonicated for 15 minutes (3510 Branson, ITM Instruments, Montreal, QC, Canada). The sample was then centrifuged at 4000 rpm (5702 Centrifuge, Eppendorf, Einfield, CT, USA) for 10 minutes. 6 mL of the supernatant were frozen at -20°C for 14-16 h for fat and protein precipitation. Finally, the extract was centrifuged again at 4000 rpm for 10 minutes and the supernatant was filtered using 0.22 µm filters then diluted 1/10 with water prior to injection.

Dasenaki and Thomaidis (2015) described another procedure which used a mix of water, methanol, and acetonitrile in the extraction of milk powder, fish, butter, and eggs. A variation of their methodology was extraction was **Extraction 2 (E2)** in this study. The first step was the same as in E1, where 1g of shrimp tissue was homogenized with a mortar and pestle and spiked with labelled NFZ, and with NFZ for the recovery samples. The shrimp tissue was then vortexed with 2 mL of water (0.1% FA) for 30 seconds, followed by addition of 2 ml of acetonitrile and 30 again vortexed for 30 seconds. Finally, 2 mL of methanol were added, the samples were again vortexed for 30 seconds and sonicated for 20 minutes. The homogenized samples were centrifuged for 10 minutes at 4000 rpm and 6 mL of the supernatant were frozen at -20°C for 14-16 h. The extract was centrifuged again at 4000 rpm for 10 minutes and 4 mL of supernatant were evaporated to dryness under N_2 flow at 40°C and reconstituted in 1 mL of water (0.1% FA). Finally, the extracts were filtered through 0.22 µm filters and diluted 1/10 with water prior to injection.

The solvent of choice when analyzing nitrofuran antibiotics in targeted analysis is often ethyl acetate. No non-targeted methodology using ethyl acetate as a solvent was found in the

literature, therefore a variation of E1-E2 using it was developed for this study and called **Extraction 3 (E3**). The first step was the same as before, where 1 g of shrimp tissue was homogenized and spiked with labelled NFZ, and with NFZ for recovery samples. They were then vortexed with 10 mL of ethyl acetate for 1 hour, sonicated for 15 minutes, and centrifuged at 4000 rpm for 10 minutes. The supernatant was frozen for 14-16 h and centrifuged again at 4000 rpm for 10 minutes. 4 mL of the supernatant were evaporated to dryness under N_2 flow at 40°C and reconstituted in 1 mL of water (0.1% FA). Finally, the extracts were filtered through 0.22 µm filters and diluted 1/10 with water prior to injection.

Finally, the last extraction tested was a variation of the procedure presented by Villar-Pulido et al. (2011), who used acetonitrile as a solvent and cleaned up extracts with different salts. This is called **Extraction 4 (E4)** in this study and is the example of a QuEChERS extraction. As for E1-E3, the first step was the homogenization of 1 g of shrimp and spiking with labelled NFZ, and NFZ for recovery samples. The tissue was then vortexed with 10 mL of acetonitrile (1% acetic acid) for 1 minute. 4 g of $MgSO_4$ and 1.75 g of NaCl were added to the samples and then vortexed again for 1 minute. The samples were then centrifuged at 4000 rpm for 3 minutes. Five milliliters of the supernatant were added to a tube containing 250 mg of PSA and 750 mg of $MgSO_4$, vortexed for 1 minute, and centrifuged again at 4000 rpm for 3 minutes. Finally, 4 ml of supernatant were evaporated to near dryness and reconstituted in 1 mL of water (0.1% FA). Finally, the extracts were filtered through 0.22 µm filters and diluted 1/10 in water prior to injection.

Extraction 1 was chosen for the comparison between shrimp from tank C and from tanks T1 and T2. For the final procedure, 10 shrimps from each C, T1, and T2 were extracted, as well as 5 procedural blanks. The samples size was deemed acceptable when compared to the sampling done by other non-targeted experiments. Bondia-Pons et al. (2014) use a sample size of three for each type of goji berry analyzed, and Martinez-Bueno et al. (2018) use a sample size of 7 per type of sample both in the development of statistical models with PLD-DA.

Cooked Extraction Variation:

For the 30 shrimps selected for the final extraction with E1, 1g of the second half of the shrimp was homogenized with a mortar and pestle and cooked for 30 minutes inside an amber vial in a thermostatic boiling water bath whose temperature was measured to remain stable at 100 °C (Thermo Scientific, Waltham, MA, USA). The solid was then transferred into 50 mL centrifuge tubes and the inside of the amber vial was rinsed with the 10 mL of acetonitrile/water (80:20, v/v) with 0.1% FA and added to the centrifuge tube for vortex mixing. The rest of the procedure was identical to that of E1 for the raw shrimps.

Original SEM Analysis:

In order to validate the treatment of the shrimp, the methodology used by Chu and Lopez (2005) was used to analyze the shells of the shrimp analyzed with the chosen extraction from 2.3.1. Briefly, around 2 g of shell were homogenized with a mortar and pestle. The samples were washed sequentially with 5 mL of methanol, ethanol, and ethyl acetate, centrifuged and the supernatant discarded to remove any non-tissue-bound SEM. At this point, a standard calibration curve was built by spiking shrimp dead during transport with a SEM stock solution at five concentrations: 0, 25.47, 50.9, 203.8, 407.6 µg/L. The pellets were then re-suspended in 10 mL of 0.125 M HCl to which 400 µL of 50 mM 2-nitrobenzaldehyde (2-NBA) in DMSO were added for derivatization and were then incubated for 14-16 hours in a water bath set to 37°C. Samples were then neutralized with K_2HPO_4 and NaOH and centrifuged. The supernatant was filtered through 0.22 µm filters and the pellets sequentially washed with 3 mL of HPLC grade water. The washes were filtered back into their respective samples. The filtrates were washed with 10 mL of hexanes. The aqueous layer was then partitioned 3 times with 4 ml of ethyl acetate, and the extract was evaporated to dryness under nitrogen at 40°C. Samples were then re-suspended in 200 µL of HPLC grade water with 0.1% FA and analyzed using HPLC-QTOF system.

3.2.4 Instrumental Analysis:

Samples were analyzed using a 1290 Series LC System from Agilent Technologies (Santa Clara, CA, USA) equipped with an InfinityLab Poroshell 120 EC-C18 column (3.0 X 150 mm, 2.7 µm) fitted with an Ecplise Plus C18 guard column (2.1 X 5mm, 1.8 µm) both from Agilent Technologies. The mobile phases were a mixture of HPLC grade water with 0.1% formic acid (Mobile Phase A) and HPLC grade Acetonitrile with 0.1% formic acid (Mobile Phase B) with a flow rate of 0.3 mL/min. The solvent gradient in the 25-minute run was as follows: 0 min (95%A, 5%B), 1.00 min (95%A, 5%B), 15.00 min (0%A, 100%B), 20.00 min (0%A, 100%B), and 20.10 min (95%A, 5%B). The LC system was coupled to a 6545 series Quadrupole Time of Flight Mass Spectrometer (Q-TOF-MS) from Agilent Technologies, equipped with a Dual AJS ESI ion source operating in positive ionization mode. The ESI was operating with a gas temperature of 325°C, and a drying gas flow of 5 L/min, a pressure of 20psig at the nebulizer and a sheath gas temperature of 275°C and flow rate of 12L/min. The capillary voltage was set to 4000 V and the nozzle voltage to 2000 V. Finally, the MS TOF fragmentor was set to 175 V, and the skimmer at 65 V. The data for the chosen extraction was obtained in All Ion acquisition mode, which is achieved by obtaining data simultaneously for all masses with the fragmentor set to 0, 10, 20 and 40eV (Von Eyken et al. 2019). The experiments were repeated for all samples with the ESI ion source operating in negative mode as well. For the tandem MS experiments, all parameters were set as in the original analysis, and selected peaks were set to fragment at 30 V using targeted MS/MS acquisition. Polarity of the ESI was selected according to the peak of interest.

3.2.5 Data Treatment:

Using Agilent Masshunter Workstation software Quantitative Analysis B.07.01, the peaks for nitrofurazone, labelled nitrofurazone, and injection internal standards were monitored in all the recovery samples. Using the relative response factor of nitrofurazone versus labelled nitrofurazone in the calibration standards as calculated by Equation 1 below, the concentration of recovered nitrofurazone for each extraction could be calculated.

$$RRF = \frac{\frac{Area\ NFZ}{Area\ IS}}{\frac{[NFZ]}{[IS]}}$$

Equation 1: Relative Response Factor

The treatment of non-targeted data can be separated into two main sections, data deconvolution and subsequent data treatment. The initial deconvolution step is of utmost importance as it provides the information for any following statistical analyses. Von Eyken and Bayen (2019) discuss the optimization of the data deconvolution step for their direct injection analysis of honey samples. Their guidelines were useful for the initial comparison of the different extractions. The Q-TOF data was aligned with Agilent Masshunter Profinder software (version B.08.00). Briefly, the peaks with counts above 200 were selected for molecular feature extraction, setting gain or loss of protons as the only source of ions, and looking for any common organic molecules in the isotopic patterns. The retention time error was set to 0.00% +0.3 minutes, and the mass error to 20.0 ppm +2 mDa. The aligned peak data was then analyzed using Agilent Masshunter Mass Profiler Professional (MPP) software (version B.14.8) using the same alignment parameters as Profinder. The Unique Entity Analysis tool was used to compare the number of entities in each extraction, and the Principle Component Analysis tool was used to observe grouping of samples in each extraction. Volcano plots were also used to observe the number of statistically significant differences between treated shrimp and shrimp dead during transport for each extraction.

The alignment of the data for the final E1 extraction of raw and cooked shrimp was optimized after observation of errors when comparing final results in MPP to the raw data. The importance of optimizing the data alignment for each matrix analyzed is also discussed by Von Eyken and Bayen (2019). Following recommendations by Agilent Technologies to reduce the data size prior to statistical analysis which could lead to discrepancies between raw and treated data, two main data treatment pathways were followed. For both pathways it was found that a slightly larger margin of error provided more consistent results, and therefore the final parameters were mostly the same as for the extraction comparison, except for a retention time error of 0.00% + 0.4 minutes, and a mass error of 50 ppm +2mDa.

Molecular Feature Extraction with Upper Limit:

The first way to reduce the size of the dataset could be simply to focus on the top "X" entities observed by Profinder. This value can be defined in the last step of the Profinder parameter selection. This data treatment pathway operates under the assumption that our molecules of interest fall within these entities. The value of "X" has to be maximized as to include the largest number of entities while keeping the software from crashing. In the case of the present study, the maximum X that could be used while running our 40 samples simultaneously was found to be 10000. From here on this pathway will be referred to as the 10000 Max Entities data treatment. It is important to note that this pathway is not applicable to all data types. In the case of the present samples, molecular differences between treated shrimp and control shrimp were expected to fall within this maximum as the samples had been treated with a significant amount of nitrofurazone. However, this might not be applicable when looking for trace contaminants in environmental or food samples.

The entity lists generated by this workflow were then imported into MPP for statistical analysis. Primarily, a volcano plot analysis with a p-value cut-off of 0.05 and a fold change cut off of 1 were used to generate entity lists (named Reduced Entity Lists from here on). These entities were then quantified in the raw data using the Masshunter Workstation Quantitative Analysis software. The results were analyzed with Microsoft Excel (Version 16.11.1) and were subject to a one tailed t-test with p-value cut-off of 0.05, and visual analysis using box and whisker plots. Entities who passed the t-test cut-off and whose treated sample median was higher than the 3rd quartile of the control, and whose treated median was above 35000 counts were selected for MS/MS analysis.

The MS/MS data was analyzed using Agilent Masshunter Workstation Qualitative Analysis (version B07.01) to generate formulae for the fragmentation patterns, and then with the Agilent MassHunter Molecular Structure Correlator (version B.07.00) (MSC) software to attempt to generate structural matches.

Targeted Batch Extraction:

The second suggestion which was explored was to investigate metabolites that would be present in the treated samples but not necessarily in the control. Knowing this, the treated samples could be aligned separately with no upper limit (given the smaller sample number the software did not crash). Once aligned, the entity list was imported into MPP in order to reduce the entity list to entities that were present in 80% of all the treated samples. This new entity list was exported using the "export for recursion" function of MPP and used to run a targeted batch extraction (TBE) in Profinder for the whole batch of samples. This was done with slightly broader retention to account for inter-sample variability.

The data extraction was performed using a CEF file type to import the entity list generated by MPP and setting the mass and retention time to "required" for a match, with a maximum possible number of matches set to 1. A gain or loss of protons was set as the only possible source of ions, and the isotope model was set to "common organic molecules". The formula matching mass tolerance was set to $\pm$25 ppm and RT tolerance to $\pm$0.4 minutes, with a symmetric mass expansion of $\pm$20 ppm. The peak filter was set to absolute height of 200 counts, and the peak mass spectrum was taken at the apex of peak. Finally, the compound group filters were set to an absolute height of 1000 counts, and the fragment confirmation option was removed.

The generated entity list for all the samples was then imported back into MPP for statistical analysis. From here on the data treatment was the same as for the 10000 Max Entities treatment. This pathway will be discussed as the TBE treatment from here on.

Model Building:

Partial least squares (PLS) prediction models were created for the different data treatments and polarities using the class prediction tool of MPP. PLS was selected as it seemed to differentiate the best between treated and control samples when compared to the other types of models in this tool (Support Vector Machine, Naïve Bayes, Decision Tree, Neural Network). This type of model has also been observed in the past for analysis of food samples (Righetti et al. 2016; Bondia-Pons et al. 2014; Mi et al. 2019). The PLS parameters were set to 4 components

with Pareto scaling, N-fold of 3, and 10 repeats after testing different parameters to maximize the accuracy of the model, as recommended by the MPP manual. All 4 components were used for the validation in step 3. These models were created for the "All Entities" entity list, the "Reduced Entity List", and the "Volcano Plot Entity List" for each of positive and negative modes, and for each of 10000 Max Entities data treatment and TBE data treatment.

The models were validated following guidelines by Rubingh et al. (2006) and Righetti et al. (2016) for the "leave one third out" validation method. Briefly, the 30 samples (10 control and 20 treated) were randomized and separated into three testing groups each of 4 controls and 7 treated samples to maintain same ratio of control to treated. The model was developed using the remaining 2/3 of the data for each set, and then tested on the last third. This was repeated until all three testing groups were testes with the model.

3.3 Results and Discussion:

3.3.1 Method Validation:

The instrument calibration curve for nitrofurazone corrected with the labelled nitrofurazone standard area resulted in excellent linearity (R^2 = 0.9959) from 24 ppb to 2.4 ppm of nitrofurazone. In order to ensure quality of data, the guidelines proposed by Cajka and Fiehn (2016) were used. Quality control (QC) samples were generated by aliquoting 10 µL of each sample. Samples were randomized during the acquisition run, and every 10 samples a QC sample, the third calibration standard, and a blank were run to ensure stability of the intensity values and repeatability. NFZ recovery samples were run for each extraction, and again repeated for the chosen E1 extraction, the result of the latter was between 105-112%.

Moreover, in order to ensure that the shrimps had consumed the feed with NFZ, the standard method for SEM analysis was run on the shells of the 30 shrimps used for the E1 extraction. SEM was observed in all 20 treated samples between 9-50 µg/kg and was observed in one control sample at 4.76 µg/kg. This comes to show two things, that the shrimp did indeed consume NFZ with the feed, and that SEM can be detected in the shell of non-treated shrimp as mentioned by several authors in the past (Van Poucke et al. 2011; McCracken et al. 2013). The levels of SEM

measured are similar to those observed by Van Poucke et al. (2011) of around 70 µg/kg for treated shrimp shells, but they also reported a higher concentration of up to 14.5 µg/kg in control shrimp shells.

3.3.2 Comparison of Extractions:

As mentioned before, the lack of standardization in the literature about non-targeted extractions means that there is no extraction that is useful for all matrices and applications. The aim of this first step was to determine which extraction procedure could provide the most information about the shrimp samples to investigate nitrofurazone metabolism. The choice of the right extraction for this purpose is key given that in a complex matrix such as shrimp muscle different solvents might extract different metabolites and affect the overall metabolites observed during analysis (Cajka and Fiehn 2015).

The four tested extractions were compared using the statistical analysis tools available within MPP. The unique entities between the different extractions were calculated, E1 had the highest number at 686, and E4 the lowest at 474. The unique entities to the treated shrimp for each extraction were looked at in both positive and negative mode were calculated and E2 had the highest in positive mode with 83, and E4 in negative mode with 109. The total number of entities was highest in E2 for both positive and negative mode. Knolhoff, Kneapler, and Croley (2019) discuss looking at total entities and unique entities as a way to discriminate between different extractions in non-targeted analysis. The number of entities with significant differences obtained with the volcano plot tool in both positive and negative mode was highest for E1. The separation with Principle Component Analysis (PCA) built with the entity list from the volcano plot analysis was compared visually by assessing whether the different sample groups separated appropriately. E1 and E3 seemed to separate the best in both positive and negative mode. E2 separated appropriately but showed some overlap within sample groups, and E4 did not separate at all in negative mode whist showing some overlap between sample types in positive mode. The recovery of nitrofurazone was also calculated for all extractions in positive mode, and it fell within the acceptable range of 80-120% for E1, E3, and E4. These results are summarized in Table 3.1 below. In particular for its significantly higher number of volcano plot entities in both positive

and negative mode, E1 was chosen for all subsequent analyses with treated and control shrimp. The reason that importance was given to the number of entities from the volcano plot was that this signified that there were the highest number of statistically significant differences between treated and control shrimp with this extraction. This would give the highest chance to find a new metabolite for NFZ abuse monitoring within this list.

Extraction	E1	E2	E3	E4
Unique Entities	686	541	522	474
Treated Unique Entities +	69	83	48	69
Treated Unique Entities -	106	90	95	109
Total Entities +	2032	2075	2022	2006
Total Entities -	2093	2118	2090	2051
PCA Separation +	Good	OK	Good	OK
PCA Separation -	Good	OK	Good	Bad
Volcano Plot Entities +	369	186	145	75
Volcano Plot Entities -	228	197	154	55
Recovery	119	77	111	86

Table 3.1: Summary of Extraction Comparison in green is the highest for each group and in red is the lowest.

3.3.3 Results for Raw and Cooked Shrimp:

The masses for the possible metabolites mentioned in the literature were monitored in both the MPP entity lists and by extracting the masses directly from the raw data. The secondary metabolite, NF, discussed by Zhang et al. (2015) nor the open chain cyano derivative discussed by Wang et al. (2010) were found in the raw data. Previous unpublished data about possible thermal degradation products of NFZ in water and acid provided several masses to look for but none were found in the shrimp extracts. This was expected given that NFZ metabolized quickly

in the shrimp, and it was unlikely that the thermal degradation would occur by the same mechanism than the metabolism of NFZ by the shrimp.

The initial data treatment with MPP resulted in large entity lists that were too large to verify manually. For this reason, a method was developed using the Masshunter Workstation Quantitative Analysis software to quantify these in all samples. In order to do so, the entity lists obtained from both the 10000 Max Entities treatment and the TBE treatment from MPP were combined in order to obtain a single list to quantify in the raw data for each polarity.

The area for all these entities in the samples was used to perform a t-test with Microsoft Excel in order to verify the data generated by MPP. A cut-off p-value of 0.05 was employed since it was the same parameter used to generate the lists with the volcano plots in MPP. Theoretically all entities quantified should have passed this test, but the list was significantly reduced. This further points out the need to verify results given by MPP in the raw data to ensure there is no misinterpretation or loss of data during the Profinder pre-treatment. The cut-off of the box and whisker plots further reduced the entity list to a manageable size for running MS/MS experiments to attempt to elucidate structures for the observed masses. The sample with the maximum amount for a given compound was chosen for the MS/MS analysis. Table 3.2 below shows the changes in size of the entity lists as they move through the data treatment

	Raw Shrimp Positive	Raw Shrimp Negative	Cooked Shrimp Positive	Cooked Shrimp Negative
MPP Entity List Entities	256	59	26	33
T-Test Cut-Off Entities	106	21	21	24
Box and Whisker Plot Cut-Off Entities	27	6	13	7

Table 3.2: Reduction of Entity Lists Throughout Data Treatment

Samples were chosen to represent the median for control and treated samples for each entity of interest using the box and whisker plots. The entities were extracted from the raw data of their respective samples for the cooked and raw shrimp to compare their presence in each type of sample. This helped to compare entities that were present in both the raw and the cooked extracts. An example of these comparison graphs is given below in Figure 3.3 for a compound

from the cooked shrimp entity list in positive mode. The monoisotopic mass used for extraction was 341.2680 *m/z*, and the molecular monoisotopic mass was 340.2613 *m/z*. Here it is clear that this compound appears both in the raw and the cooked shrimp extracts, and that in both cases it is significantly higher in the treated versus the control samples.

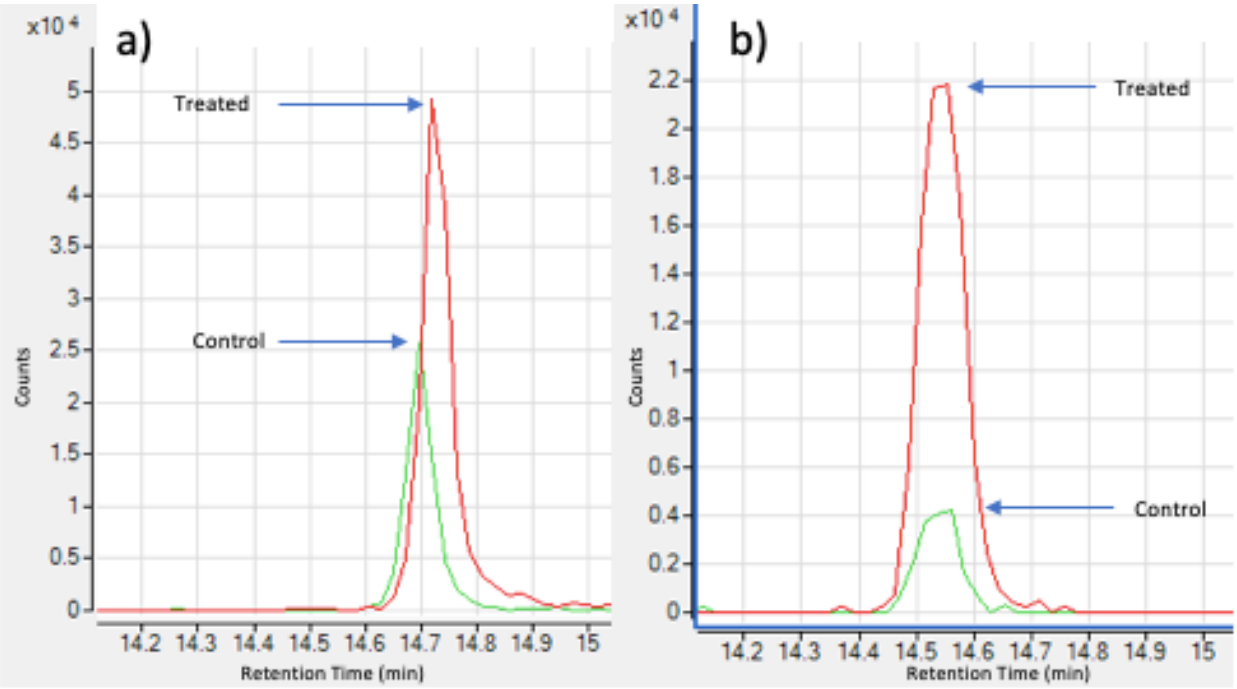


Figure 3.3: Extraction of Compound 7 from Cooked Shrimp Positive Polarity in a) raw shrimp and b) cooked shrimp samples

Despite finding several entities that were significantly higher in the treated samples, no entity was completely absent from all control samples whilst being present in at least 80% of the treated samples. Moreover, the masses obtained from previous thermal degradation studies of NFZ in pH 1.3 and 7 were not found in either the control or the treated shrimp. For this reason, it can be concluded that using this extraction and data treatment, no replacement for SEM could be identified as a way to monitor NFZ abuse in shrimp farming. It is important to re-acknowledge that this conclusion is merely based on the chosen extraction and data treatment and does not mean that no replacement for SEM exists. Other studies have been successful in identifying

metabolites of exposure using similar methodologies, like Sri Harsha et al. (2018) who identified multiple human urinary biomarkers linked to the consumption of peas. This suggests that other extractions might be successful in detecting and identifying a novel metabolite which could replace SEM.

However, the data obtained still showed many entities with significant differences between the treated and control shrimp, and these differences were exploited in an attempt to build a statistical model that could differentiate between the different sample types.

3.3.4 Example of Structure Determination:

The entities run with targeted MS/MS data acquisition were then analyzed using the Agilent Masshunter Molecular Structure Correlator (MSC). Of the 53 entities analyzed in this way, only 11 had structural matches. One of them was chosen since it was apparent in both positive and negative mode in the raw shrimp and was higher in treated versus control in both raw and cooked shrimp. This entity was taken as a proof of concept in the identification capabilities of this type of workflow.

The entity in question was identified by the MSC to have a formula $C_6H_5NO_2$, and the MS/MS fragmentation pattern matched with nicotinic acid (or niacin) among others. Xia et al. (2015) have shown that niacin is a necessary nutrient in shrimp development and is often added to feed during shrimp development. This gave enough reason to purchase a standard and compare the retention times and fragmentation patterns of the standard and the entity observed in the samples. The comparison is shown below in Figure 3.4. This confirmed that the entity observed is in fact niacin and that niacin appears to be present in higher levels in the treated shrimp when compared to the control shrimp. The mechanism of why this would be remains unknown.

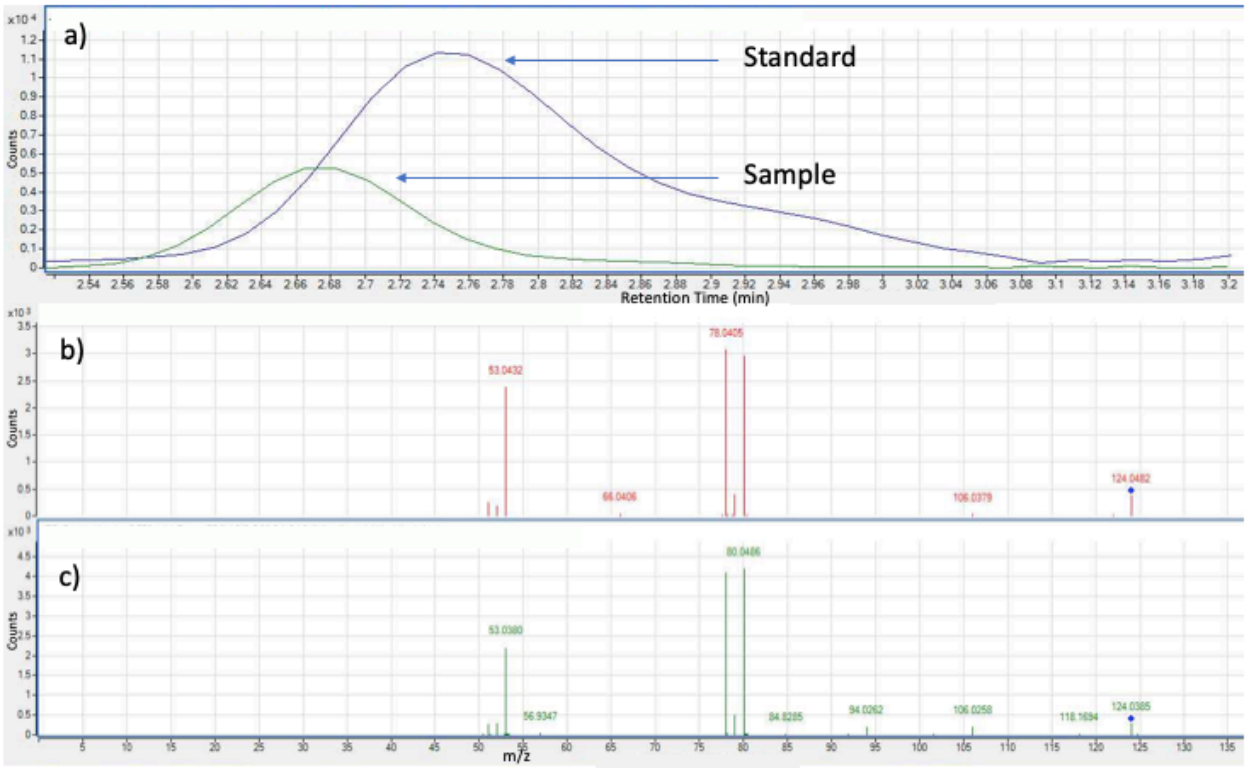


Figure 3.4: Comparison of retention time (a) between niacin standard and peak in the sample, and mass spectra for raw treated shrimp (b) and niacin standard (c) showing the same fragmentation patterns.

3.3.5 Model Validation:

The class prediction tool of MPP was used to build a partial least squares (PLS) model that could exploit the differences between treated and control shrimp and attribute a class to random samples. The results for the "leave one third out" cross validation as proposed by Righetti et al. (2016) can be observed in Table 3.3 below. This approach is a way to reduce the over-fitting of multivariate models and to have an internal test to the validity of the model for its applied purpose. The validation was performed on both cooked and raw shrimp models, for each of the two data treatment pathways and polarities.

The volcano plot entity list gave the highest accuracy in all cases, which was to be expected given that it accounts for entities higher in control vs treated as well as those higher in treated vs control, as opposed to the reduced entity list which only accounts for entities higher in the

treated vs control. The all entities list was considered to prove that the data treatment was indeed filtering out entities that were not useful for our purpose. Overall, negative ionization mode seemed to provide better predictive capabilities with a maximum of 98% accuracy for the Raw Negative 10000 Max Entities sample set tested with the Volcano Entity list model. Higher accuracy was also observed in negative mode had been observed by Novotna et al. (2012) in their model to differentiate between organic and conventional farming of tomatoes and peppers. The reason for this is likely that for the purpose of these experiments there are more metabolic differences in the case of molecules ionizing more efficiently in negative mode when compared to positive mode.

Raw Shrimp			
10000 Max Entities Positive			
	Accuracy Control	Accuracy Treated	Total Accuracy
All Entities (9388 entities)	75	62	68
Reduced Entity List (216 entities)	92	71	82
Volcano Entity List (477 entities)	100	86	93
Targeted Batch Extraction Positive			
	Accuracy Control	Accuracy Treated	Total Accuracy
All Entities (4153 entities)	75	66	71
Reduced Entity List (186 entities)	58	76	67
Volcano Entity List (304 entities)	92	81	86
10000 Max Entities Negative			
	Accuracy Control	Accuracy Treated	Total Accuracy
All Entities (10228 entities)	25	76	51
Reduced Entity List (66 entities)	100	81	91
Volcano Entity List (400 entities)	100	95	98
Targeted Batch Extraction Negative			
	Accuracy Control	Accuracy Treated	Total Accuracy
All Entities (1739 entities)	33	48	40
Reduced Entity List (21 entities)	92	76	84
Volcano Entity list (75 entities)	92	95	93

Cooked Shrimp			
10000 Max Entities Positive			
	Accuracy Control	Accuracy Treated	Total Accuracy
All Entities (9656 entities)	58	76	67
Reduced Entity List (20 entities)	92	95	93
Targeted Batch Extraction Positive			
	Accuracy Control	Accuracy Treated	Total Accuracy
All Entities (2255 entities)	75	76	76
Reduced Entity List (42 entities)	83	95	89
10000 Max Entities Negative			
	Accuracy Control	Accuracy Treated	Total Accuracy
All Entities (4157 entities)	75	71	73
Reduced Entity List (27 entities)	83	81	82
Targeted Batch Extraction Negative			
	Accuracy Control	Accuracy Treated	Total Accuracy
All Entities (901 entities)	75	81	78
Reduced Entity List (23 entities)	92	86	89

Table 3.3: Results for Cross Validation of Different Models

3.3.6 Model External Testing Results:

The final models created by using all 30 samples in the training were then tested with an external sample lot. This external sample lot consisted of 5 control shrimps, 5 nitrofurazone treated shrimps from each tank, and 5 malachite green treated shrimps that came from the same batch of purchased shrimp but were treated by another researcher with malachite green for a separate study. This sample set was extracted following the procedure for E1. The aim of this was to go one step further and show if the models that had proven efficient during the cross validation were able to differentiate between treated and control shrimp in an external batch. Further still, if it would be possible to differentiate between shrimp treated with nitrofurazone and those treated with malachite green.

In order to avoid inter-day variability of the detector as a variable in the application of the model, the original raw shrimp extracts obtained with E1 were re-run the same day as the new batch. The mass and retention time variability were confirmed by looking at the injection internal standards and nicotinic acid (observed in all samples) to confirm that this would not affect the testing results. Mass measurement error was calculated to be between 0.8-7 ppm for all three peaks, and retention time error was ranged from 0.005 to 0.02 minutes for all peaks. This is within the parameters chosen for Profinder and MPP since the mass error was set to 50 ppm and the retention time error to 0.4 minutes. This means that molecules within this mass and retention time error should have been grouped together and given that the results fall within these error guidelines they should not have an impact on the application of the model. Other authors use monitoring of compound of known mass to ensure the mass error is within the parameters of the data treatment procedures. Villar-Pulido et al. (2011) discussed that a mass error of under 2 ppm was observed in most molecules studied, and Nacher-Mestre et al. (2013) discussed the use of leucine enkephalin for mass calibration and error checks and specify that their masses showed a deviation of 5 ppm.

Some of the entities from the reduced entity list at a range of masses and retention times were chosen to verify whether the data from the initial extraction could be applicable to the new extraction. The chromatograms showing that there seemed to be enough similarities to attempt applying the model to the new extraction data can be found in Supplementary Figure 3.2. Having

done this initial verification, the data was aligned with Profinder as it had been done with the original extraction and three models were built per ionization polarity as had been done in the previous section (all entities, reduced entities, and volcano plot entities). However, when the model was applied to the new extraction data it grouped all the samples together. In positive mode all three models tested identified all samples as controls, and in negative mode all three models identified all samples as treated. This raised several questions on what could lead to the discrepancy between the internal and external validations.

For further understanding, a comparison between the entities in the initial run of the original extracts (B1.1) and the second run of the same extracts (B1.2) was done. Furthermore, the entities in B1.2 were compared with the external validation set (B2). This comparison, done with the Unique Entity Analysis tool of MPP, can be seen in Figure 3.5 below. It became clear that even if it was the exact same extracts run twice in the HPLC-QTOF, there were significant differences between the entities observed after sample treatment. There were only 3912 entities in common between the two runs of the same extracts after the same sample treatment. This number was similar to the 3554 entities in common between B1.2 and B2 as shown in Figure 3.5(b). The lack of repeatability when analyzing the same extracts can mean that the algorithm used by Profinder to align the data was determining different entities despite them being the same extracts. Cajka and Fiehn (2015) discussed that not enough studies have been run comparing different data alignment software. Coble and Fraga (2014) ran a comparison between four different software and found that only 2 out of 14 metabolites found for their samples were in common between the four software. Indeed, it was observed in the present study that the data alignment software can even detect different entities depending on which samples are run together during this step. This suggests that the data treatment might be the reason why the model was not successful in identifying samples correctly.

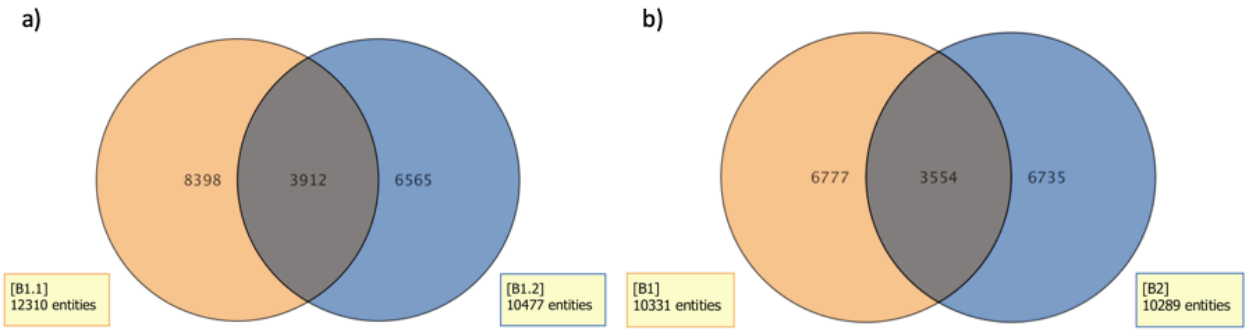


Figure 3.5: Unique Entity Comparison of the different runs of the initial extracts (a) and the initial and second sample sets (b) in positive mode.

Principal component analysis (PCA) is usually used as a way to ensure the quality of data in non-targeted analysis studies by confirming grouping of pooled QC samples as Cajka and Fiehn (2015) proposed. However, PCA was done here on the B2 using the entity lists obtained from the Volcano Plot analysis of both B1.2 and of B2 to see if there was still grouping occurring in B2. This would show if the entities used to build the model are useful to separate the samples by type in B2. This is shown in Figure 3.6 below, and with this it became clear that the entities that showed significant differences in B1.2 and managed to group samples by type with PCA (Supplementary Figure 3.3) in this batch did not separate samples by type in B2 (Figure 3.6(a)). However, when using the entities showing significant differences between the samples in B2 to build a PCA, samples grouped perfectly by type (Figure 3.6(b)). This suggests that the entities showing differences between treated and control samples in B1.2 vs B2 are not the same, and that the extraction was not as repeatable as first thought by looking at the recoveries of NFZ on different days. This would be the major cause for the model misidentifying samples in B2 when using a model built with B1.2 and explains why it was unable to group samples of the external validation set.

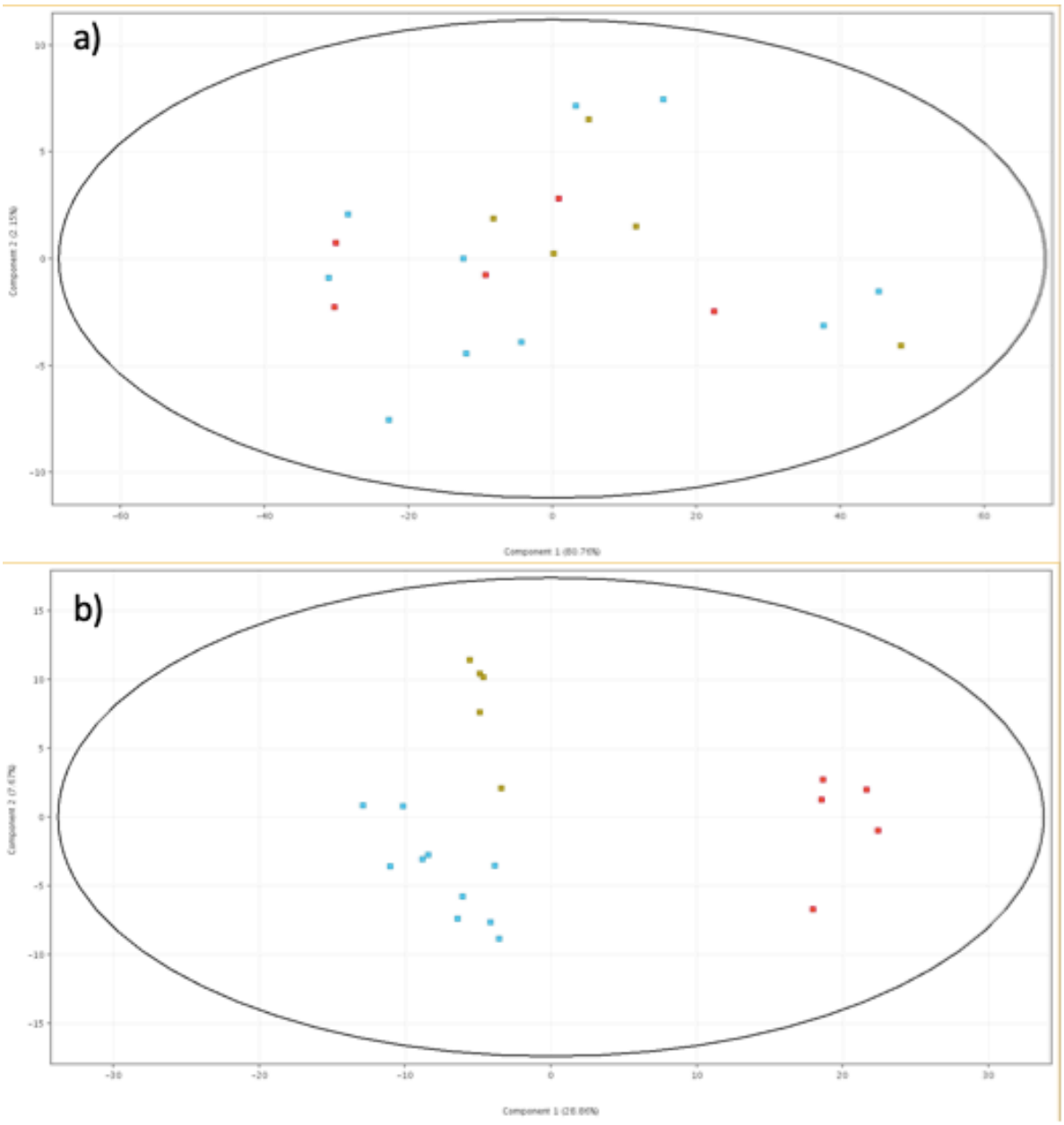


Figure 3.6: PCA of B2 with the Volcano Plot entity list from B1.2 (a) and the volcano plot entity list from B2 (b). In blue are the NFZ treated samples, in yellow the MG treated samples, and in red the control samples.

Rubingh et al. (2006) suggested that these external validations should be necessary when proving the applicability of a model, and our results concur with this statement although some studies in the literature sometimes fail to go this extra step to validate their models. Despite the initial cross validation implying that the model was successful in differentiating between the

different types of samples, an external application of the model still showed that there are significant errors in its application. This comes to show that the variability of the entities used to build the model goes beyond the distinction of treated or control sample, and that a larger sample group would be needed to have higher statistical relevance in this differentiation.

3.4 Conclusions:

Non-targeted analysis can be a powerful tool in new molecule discovery and identification, but the study of complex matrices results in the lack of a golden standard when it comes for extraction procedures. The extraction selected for this experiment (E1) seemed to provide the most information when compared to the other extractions tested but was still unable to detect a metabolite that could replace SEM in the monitoring of NFZ abuse. This suggests that the extraction chosen was not the most appropriate for the detection of a unique metabolite of NFZ and other extractions should be tested.

Despite this, significant differences between treated and control samples were observed, and were exploited to develop a model that with internal cross-validation seemed to be able to differentiate with up to 98% accuracy between treated and control shrimp. One of such entities was confirmed to be Nicotinic Acid (or Vitamin B3). However, once the model was applied to an external validation the model was not able to differentiate between treated and control samples. This is probably due to differences in the extraction efficiency for some molecules between different days, but also due to differences in the entities observed after data treatment with the software used as seen with analysis of the same extracts twice.

Further tests should be done increasing the sample size in the model building sample set to ensure that variability due to the extraction can be minimized and that a more statistically significant entity list is used to build a model. A more powerful computer would be necessary to handle larger sample sets during the peak alignment step without having to run multiple batches of samples. This could potentially differentiate between treated and control shrimp with a smaller risk of false positive assignment than the current SEM analysis. As many authors have described, the importance of continuing to develop and optimize non-targeted extractions will

provide useful knowledge in the search to unify current regulatory analysis with the discovery of novel antibiotics and environmental contaminants.

3.5 Acknowledgments:

The authors would like to acknowledge Anca Baesu for her Malachite Green treated shrimp and Richard Zhu for his invaluable work in validating the consumption of NFZ by analyzing the shells of the selected shrimps. Moreover, the invaluable advice provided by the experts from Planet Shrimp and their willingness to participate in this research by providing the live shrimps used for these experiments.

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3.6 References:

Antignac, J. P., F. Courant, G. Pinel, E. Bichon, F. Monteau, C. Elliott, and B. Le Bizec. 2011. 'Mass spectrometry-based metabolomics applied to the chemical safety of food', TrAC Trends in Analytical Chemistry, 30: 292-301.

Bletsou, A. A., Jeon, J., Hollender, J., Archontaki, E., and Thomaidis, N. S. 2015. 'Targeted and non-targeted liquid chromatography-mass spectrometric workflows for identification of transformation products of emerging pollutants in the aquatic environment', TrAC Trends in Analytical Chemistry, 66: 32-44.

Bondia-Pons, I., O. Savolainen, R. Törrönen, J. A. Martinez, K. Poutanen, and K. Hanhineva. 2014. 'Metabolic profiling of Goji berry extracts for discrimination of geographical origin by non-targeted liquid chromatography coupled to quadrupole time-of-flight mass spectrometry', Food Research International, 63: 132-38.

Cajka, T., and O. Fiehn. 2016. 'Toward Merging Untargeted and Targeted Methods in Mass Spectrometry-Based Metabolomics and Lipidomics', Anal Chem, 88: 524-45.

Chu, P.S., and M.I. Lopez. 2005. 'Liquid Chromatography-Tandem Mass Spectrometry for the Determination of Protein-Bound Residues in Shrimp Dosed with Nitrofurans', J. of Agric and Food Chem, 53: 6.

Chu, P.S., M.I. Lopez, A. Ann, K.R. El Said, and S.M. Plakas. 2008. 'Residue Depletion of Nitrofuran Drugs and Their Tissue-Bound Metabolites in Channel Catfish (Ictalurus punctatus) after Oral Dosing', J. of Agric and Food Chem, 56: 5.

Cifuentes, A. 2012. 'Food Analysis: Present, Future, and Foodomics', ISRN Anal Chem, 2012: 1-16.

Coble, J. B., and C. G. Fraga. 2014. 'Comparative evaluation of preprocessing freeware on chromatography/mass spectrometry data for signature discovery', J Chromatogr A, 1358: 155-64.

Dasenaki, M. E., and N. S. Thomaidis. 2015. 'Multi-residue determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs using liquid chromatography-tandem mass spectrometry', Anal Chim Acta, 880: 103-21.

Douny, C., J. Widart, E. De Pauw, F. Silvestre, P. Kestemont, H. Thi Tu, N. Thanh Phuong, G. Maghuin-Rogister, and M.L. Scippo. 2013. 'Development of an analytical method to detect metabolites of nitrofurans', Aquaculture, 376-379: 54-58.

European Commission. 1990. "Council Regulation (EEC) No 2377:90 Laying down a Community procedure for the establishment of maximum residue limits of

veterinary medicinal products in foodstuffs of animal origin." In, edited by European Commission.

European Commission. 1993. "Council Regulation (EEC) No 2901/93 amending Annexes I, II, III and IV to Regulation (EEC) No 2377/90 laying down

a Community procedure for the establishment of maximum residue limits of

veterinary medicinal products in foodstuffs of animal origin." In, edited by European Commission.

Government of Canada 2017. 'List of Banned Drugs'. https://www.canada.ca/en/health-canada/services/drugs-health-products/veterinary-drugs/list-banned-drugs.html.

Hoenicke, K., R. Gatermann, L. Hartig, M. Mandix, and S. Otte. 2004. 'Formation of semicarbazide (SEM) in food by hypochlorite treatment: is SEM a specific marker for nitrofurazone abuse?', Food Addit Contam, 21: 526-37.

Hoogenboom, L. A., and T. H. Polman. 1993. "Simultaneous detection of protein bound residues of nitrofuran drugs furazolidone, furaltadone, nitrofurazone and nitrofurantoin." In EuroResidue II, 6. Veldhoven, Netherlands.

Hoogenboom, L. A., M. Van Kammen, M. C. Berghmans, J. H. Koeman, and H. A. Kuiper. 1991. 'The Use of Pig Hepatocytes to Study the Nature of Protein-Bound Metabolites of Furazolidone: A New Analytical Metho for Their Detection', Food and Chemical Toxicology, 29: 8.

Hui, Y., X. Zhang, and H. J. Yu. 2015. "Investigation on the Natural Occurrence of Semicarbazide in Macrobrachium Nipponese prawns." In International Symposium on Material, Energy and Environ Eng. Changsha, China.

Knolhoff, A. M., and T. R. Croley. 2016. 'Non-targeted screening approaches for contaminants and adulterants in food using liquid chromatography hyphenated to high resolution mass spectrometry', J Chromatogr A, 1428: 86-96.

Knolhoff, A. M., C. N. Kneapler, and T. R. Croley. 2019. 'Optimized chemical coverage and data quality for non-targeted

screening applications using liquid chromatography/high-resolution

mass spectrometry', Anal Chim Acta, 1066: 9.

Martinez Bueno, M. J., F. J. Diaz-Galiano, L. Rajski, V. Cutillas, and A. R. Fernandez-Alba. 2018. 'A non-targeted metabolomic approach to identify food markers to support discrimination between organic and conventional tomato crops', J Chromatogr A, 1546: 66-76.

McCracken, R., B. Hanna, D. Ennis, L. Cantley, D. Faulkner, and D. G. Kennedy. 2013. 'The occurrence of semicarbazide in the meat and shell of Bangladeshi fresh-water shrimp', Food Chem, 136: 1562-7.

Mi, S., K Shang, X. Li, C. H. Zhang, J. Q. Liu, and D. Q. Huang. 2019. 'Characterization and discrimination of selected China's domestic pork using

an LC-MS-based lipidomics approach', Food Control, 100: 10.

Mohney, L. L., R. R. Williams, T. A. Bell, and D. V. Lightner. 1997. 'Residues of oxytetracycline in cultured juvenile blue shrimp, Penaeus stylirostris (Crustacea: Decapod) , fed medicated feed for 14 days', Aquaculture, 149: 10.

Mulder, P. P., B. Beumer, and J. A. Van Rhijn. 2007. 'The determination of biurea: a novel method to discriminate between nitrofurazone and azodicarbonamide use in food products', Anal Chim Acta, 586: 366-73.

Nacher-Mestre, J., M. Ibanez, R. Serrano, J. Perez-Sanchez, and F. Hernandez. 2013. 'Qualitative screening of undesirable compounds from feeds to fish by liquid chromatography coupled to mass spectrometry', J Agric Food Chem, 61: 2077-87.

Novotna, H., O. Kmiecik, M. Galazka, V. Krtkova, A. Hurajova, V. Schulzova, E. Hallmann, E. Rembialkowska, and J. Hajslova. 2012. 'Metabolomic fingerprinting employing DART-TOFMS for authentication of tomatoes and peppers from organic and conventional farming', Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 29: 1335-46.

Olive, P.L., and D.R. McCalla. 1977. 'Cytotoxicity and DNA Damage to Mammalian Cells by Nitrofurans', Chem-Biol Interactions, 16: 11.

Patti, G. J., O. Yanes, and G. Siuzdak. 2012. 'Innovation: Metabolomics: the apogee of the omics trilogy', Nat Rev Mol Cell Biol, 13: 263-9.

Pereira, A. S., J. L. Donato, and G. De Nucci. 2004. 'Implications of the use of semicarbazide as a metabolic target of nitrofurazone contamination in coated products', Food Addit Contam, 21: 63-9.

Righetti, L., J. Rubert, G. Galaverna, S. Folloni, R. Ranieri, M. Stranska-Zachariasova, J. Hajslova, and C. Dall'Asta. 2016. 'Characterization and Discrimination of Ancient Grains: A Metabolomics Approach', Int J Mol Sci, 17.

Rubingh, C. M., S. Bijlsma, E. P. Derks, I. Bobeldijk, E. R. Verheij, S. Kochhar, and A. K. Smilde. 2006. 'Assessing the performance of statistical validation tools for megavariate metabolomics data', Metabolomics, 2: 53-61.

Saari, L., and K. Peltonen. 2004. 'Novel source of semicarbazide: levels of semicarbazide in cooked crayfish samples determined by LC/MS/MS', Food Addit Contam, 21: 825-32.

Sri Harsha, P. S. C., R. Abdul Wahab, C. Cuparencu, L. O. Dragsted, and L. Brennan. 2018. 'A Metabolomics Approach to the Identification of Urinary Biomarkers of Pea Intake', Nutrients, 10.

Stadler, R. H., L. Verzegnassi, W. Seefelder, and L. Racault. 2015. 'Why semicarbazide (SEM) is not an appropriate marker for the usage of nitrofurazone on agricultural animals', Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 32: 1842-50.

Tian, X., Y. Xu, X. Song, X. Gong, Y. Liu, Q. Zhou, Z. Wang, and C. Xia. 2016. 'Temporal and spatial distribution of semicarbazide in western Laizhou Bay', Mar Pollut Bull, 112: 393-98.

Van Poucke, C., C. Detavernier, M. Wille, J. Kwakman, P. Sorgeloos, and C. Van Peteghem. 2011. 'Investigation into the possible natural occurence of semicarbazide in Macrobrachium rosenbergii prawns', J Agric Food Chem, 59: 2107-12.

Vass, M., K. Hruska, and M. Franek. 2008. 'Nitrofuran antibiotics: a review on the application, prohibition and residual analysis', Veterinarni Medicina, 53: 32.

Villar-Pulido, M., B. Gilbert-Lopez, J. F. Garcia-Reyes, N. R. Martos, and A. Molina-Diaz. 2011. 'Multiclass detection and quantitation of antibiotics and veterinary drugs in shrimps by fast liquid chromatography time-of-flight mass spectrometry', Talanta, 85: 1419-27.

Von Eyken, A., and S. Bayen. 2019. 'Optimization of the Data Treatment Steps of a Non-targeted LC-MS-Based Workflow for the Identification of Trace Chemical Residues in Honey', J Am Soc Mass Spectrom.

Von Eyken, Annie, Daniel Furlong, Samareh Arooni, Fred Butterworth, Jean-François Roy, Jerry Zweigenbaum, and Stéphane Bayen. 2019. 'Direct injection high performance liquid chromatography coupled to data independent acquisition mass spectrometry for the screening of antibiotics in honey', J. of Food and Drug Anal.

Vroomen, L. H., M. C. Berghmans, P. Hekman, L. A. Hoogenboom, and H. A. Kuiper. 1987. 'The elimination of furazolidone and its open-chain cyano-derivative from adult swine', Xenobiotica, 17: 1427-35.

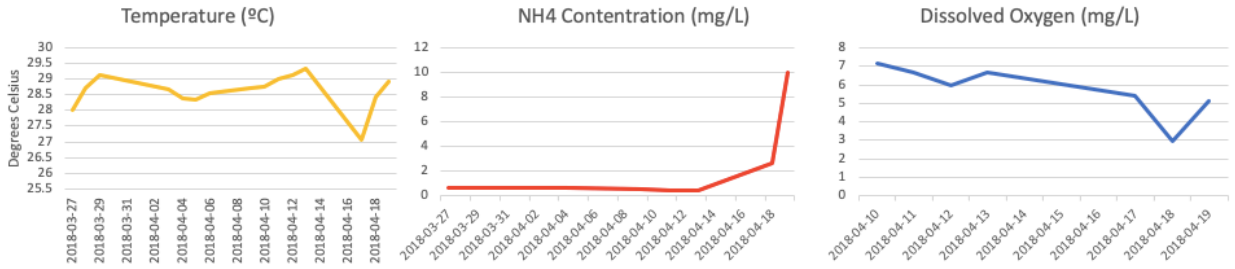
Wang, W., H. Lin, C. Xue, and J. Khalid. 2004. 'Elimination of chloramphenicol, sulphamethoxazole and oxytetracycline in shrimp, Penaeus chinensis following medicated-feed treatment', Environ Int, 30: 367-73.

Wang, Y., E. L. Jester, K. R. El Said, A. Abraham, J. Hooe-Rollman, and S. M. Plakas. 2010. 'Cyano metabolite as a biomarker of nitrofurazone in channel catfish', J Agric Food Chem, 58: 313-6.

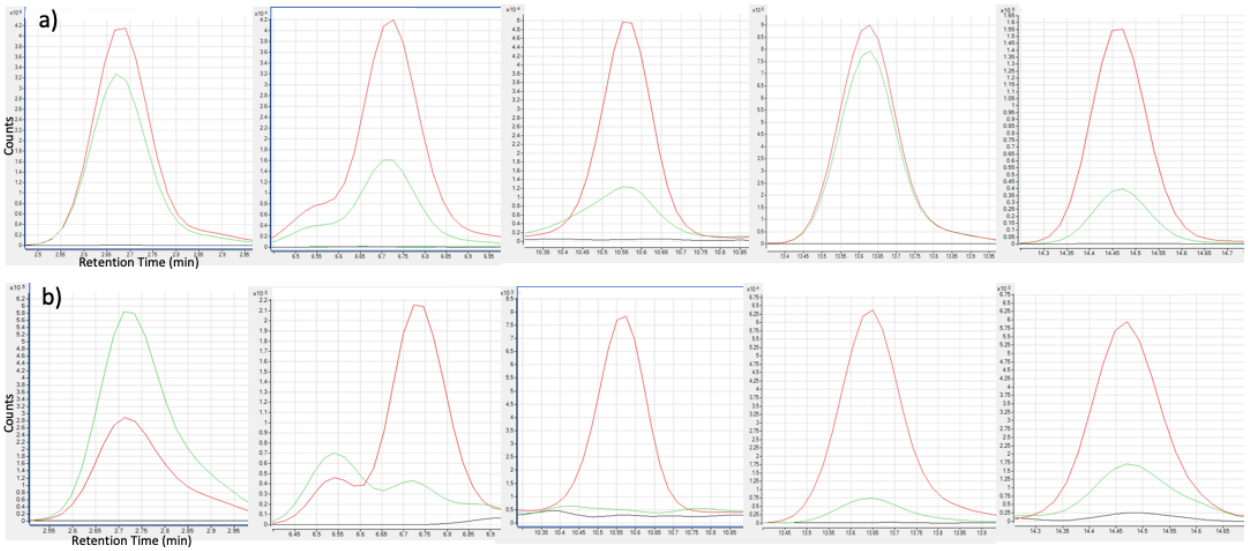
Xia, M. H., X. L. Huang, H. L. Wang, M. Jin, M. Li, and Q. C. Zhou. 2015. 'Dietary niacin levels in practical diets for Litopenaeus vannamei
to support maximum growth', Aquaculture Nutrition, 21: 853-60.

Zhang, S., Y. Guo, Z. Yan, X. Sun, and X. Zhang. 2015. 'A selective biomarker for confirming nitrofurazone residues in crab and shrimp using ultra-performance liquid chromatography-tandem mass spectrometry', Anal Bioanal Chem, 407: 8971-7.

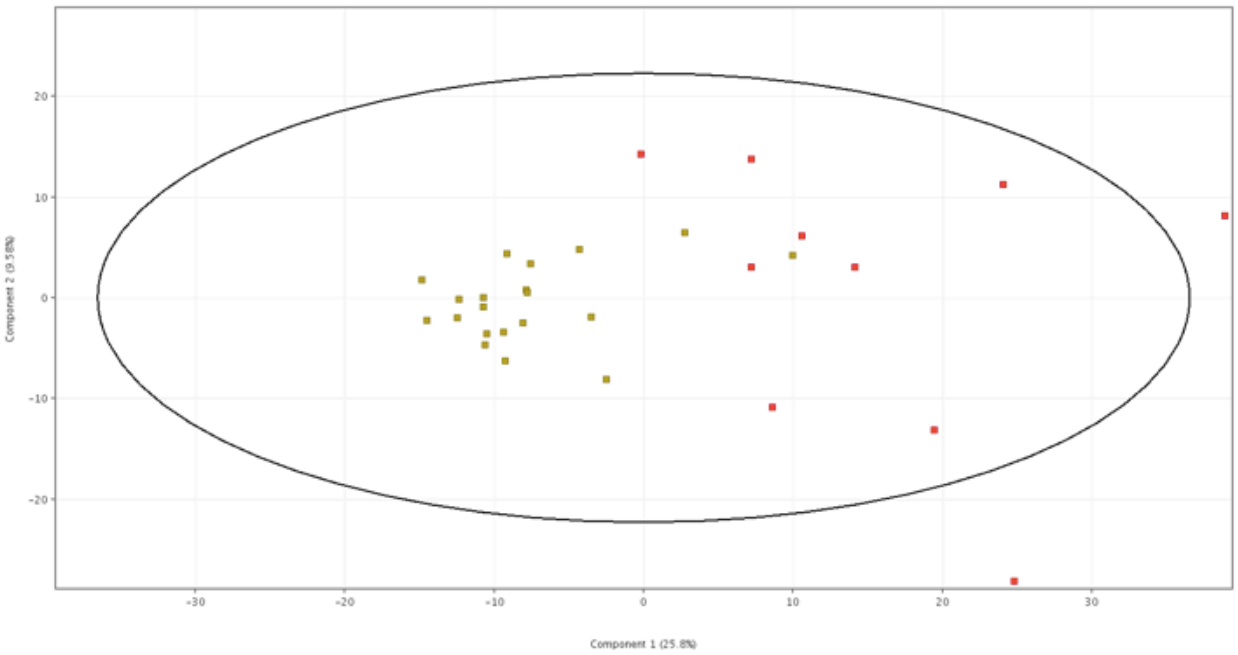
3.7 Supplementary Material:



Supplementary Figure 3.1: Water conditions during the weeks prior to shrimp arrival and once the shrimp had been added to the tanks. This is plotted as an average of the three tanks.



Supplementary Figure 3.2 EIC showing similar patterns in treated (red) versus control (green) shrimp in the initial extracts (a) and the external validation set (b). From left to right, the extraction masses were 124.0398 *m/z*, 1065.8439 *m/z*, 340.3486 *m/z*, 398.3278 *m/z*, and 341.2678 *m/z*.



Supplementary Figure 3.3: PCA showing grouping of treated (yellow) and control (red) shrimp samples using the entity list from the volcano plot in B1.2

Summary and Conclusions

Nitrofurazone (NFZ) is broad spectrum synthetic antimicrobial agent of the nitrofuran family that has been banned since the early 90's for food producing animals by most regulatory agencies (European Commission 1993; Canada 2017) due to increasing evidence of carcinogenicity (Olive and McCalla 1977). Nitrofuran drugs metabolize rapidly in animals and the parent drug is not detectable 12 hours after treatment (Chu et al. 2008). For this reason, methods have been developed to detect side-chain metabolites of the main nitrofuran drugs which are detectable up to 54 days after treatment (Chu et al. 2008). In the case of NFZ, the metabolite currently used for this purpose is SEM. However, it has been found that SEM can be detected even in cases where NFZ was not used, particularly in shrimp farming (McCracken et al. 2013; Van Poucke et al. 2011). This questions the validity of SEM as a metabolite to monitor the abuse of NFZ. In this project a non-targeted analytical approach was employed to explore the thermal stability of NFZ, as well as metabolic differences between shrimp treated with NFZ and control shrimp, in hope of finding a new metabolite capable of acting as a marker for NFZ abuse which could reduce the rate of false positives in imported products and avoid unnecessary product loss.

Chapter 2 showed that despite the lack of literature on the subject, NFZ degrades thermally at 100°C. A "dilute and shoot" methodology revealed several entities that increase with cooking time. Although the identity of none of the entities was ultimately confirmed with a standard, the accurate mass, retention time, proposed formulas, and MS/MS fragmentation pattern of 10 molecules was obtained. The Agilent Molecular Structure Correlator (MSC) software gave structural matches for six out of the ten molecules, but a high number of possibilities coupled to a lack of previous literature on the stability of NFZ made it hard to narrow

down the selection and unaffordable to test all the standards. One of the molecules was tentatively identified as [(furan-2-yl)methylidene]hydrazine given the high structural similarity with NFZ and logical reaction mechanism that could be possible within the experimental conditions. Despite this, once a standard was purchased there was a significant difference in retention time and in their fragmentation patterns.

Given that NFZ metabolizes rapidly in animals, it was unlikely that these masses would be observed in the shrimp from a controlled exposure experiment described in the following chapter, but it still provided important information with regards to the overall stability of different bonds within the structure.

Chapter 3 explored the metabolic differences between NFZ treated shrimp and control obtained and treated in-lab. Given the lack of standardization when it comes to non-targeted extraction procedures, four different extractions were tested which had been used either for similar purposes or for similar matrices. A variation of the extraction proposed by Nacher-Mestre et al. (2013) was ultimately chosen for the comparison between treated and control shrimp. The data generated by injecting these extracts into the HPLC-QTOF system in both positive and negative ionization modes provided thousands of entities which were used for statistical analyses. With this extraction and data treatment, no molecule was found to be absent in control samples while being present in over 80% of the treated samples, and therefore act as a replacement for SEM in routine analysis.

Despite this, hundreds of entities were found to be present at significantly different levels between the treated and control shrimp, one of which was identified to be nicotinic acid (or vitamin B3). These differences were exploited and used to develop statistical models with a

Partial Least Squares (PLS) algorithm that could potentially differentiate between treated and control shrimp. Internal cross-validation of the model using the "leave one third out" method as described by Rubingh et al. (2006) concluded that the models had up to 98% accuracy when using entity lists obtained with the Volcano Plot tool of Agilent Masshunter Mass Profiler Professional software. However, once the model was tested on another set of external samples, it was unable to differentiate between treated and control shrimp. A comparison between the sample sets seemed to show that this was due to variability in the extraction despite the recovery of NFZ remaining quite similar throughout. A model built with a significantly larger sample set might be able to account for this variability and result in a more robust model.

It is important to note that the lack of a replacement for SEM with this extraction and data treatment does not signify that this replacement does not exist. Future studies should focus on other extraction procedures that could potentially extract molecules that were not extracted with the water/acetonitrile mix used by the chosen extraction. Acid hydrolysis treatment of the shrimp tissue might also be helpful in solubilizing tissue bound metabolites which might not have been accessible in the simple extraction procedure chosen. It is important however to keep in mind that sample clean-up should be minimized when performing a non-targeted approach in order to lose as little information as possible during the extraction. Given its good recovery, and the fact that ethyl acetate is often used in nitrofuran analysis, a variation of the Chu and Lopez (2005) extraction modified to minimize clean-up could be a good next step in the search for an alternative to SEM.

References

Antignac, J. P., F. Courant, G. Pinel, E. Bichon, F. Monteau, C. Elliott, and B. Le Bizec. 2011. 'Mass spectrometry-based metabolomics applied to the chemical safety of food', *TrAC Trends in Analytical Chemistry*, 30: 292-301.

Baduel, C., J. F. Mueller, H. Tsai, and M. J. Gomez Ramos. 2015. 'Development of sample extraction and clean-up strategies for target and non-target analysis of environmental contaminants in biological matrices', *J Chromatogr A*, 1426: 33-47.

Bletsou, A. A., J. Jeon, J. Hollender, E. Archontaki, and N. S. Thomaidis. 2015. 'Targeted and non-targeted liquid chromatography-mass spectrometric workflows for identification of transformation products of emerging pollutants in the aquatic environment', *TrAC Trends in Analytical Chemistry*, 66: 32-44.

Cajka, T., and O. Fiehn. 2016. 'Toward Merging Untargeted and Targeted Methods in Mass Spectrometry-Based Metabolomics and Lipidomics', *Anal Chem*, 88: 524-45.

Chu, P.S., and M.I. Lopez. 2005. 'Liquid Chromatography-Tandem Mass Spectrometry for the Determination of Protein-Bound Residues in Shrimp Dosed with Nitrofurans', *Journal of Agricultural and Food Chemistry*, 53: 6.

Chu, P.S., M.I. Lopez, A. Ann, K.R. El Said, and S.M. Plakas. 2008. 'Residue Depletion of Nitrofuran Drugs and Their Tissue-Bound Metabolites in Channel Catfish (Ictalurus punctatus) after Oral Dosing', *J. of Agric and Food Chem*, 56: 5.

Cifuentes, A. 2012. 'Food Analysis: Present, Future, and Foodomics', *ISRN Anal Chem*, 2012: 1-16.

Coble, J. B., and C. G. Fraga. 2014. 'Comparative evaluation of preprocessing freeware on chromatography/mass spectrometry data for signature discovery', *J Chromatogr A*, 1358: 155-64.

Cooper, K. M., and D. G. Kennedy. 2007. 'Stability studies of the metabolites of nitrofuran antibiotics during storage and cooking', *Food Addit Contam*, 24: 935-42.

Cox, Robin A. 2012. 'Revised Mechanisms for Simple Organic Reactions.' in, *Advances in Phys Org Chem Volume 46*.

Dasenaki, M. E., and N. S. Thomaidis. 2015. 'Multi-residue determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs using liquid chromatography-tandem mass spectrometry', *Anal Chim Acta*, 880: 103-21.

De Luca, M., S. Mas, G. Ioele, F. Oliverio, G. Ragno, and R. Tauler. 2010. 'Kinetic studies of nitrofurazone photodegradation by multivariate curve resolution applied to UV-spectral data', *Int J Pharm*, 386: 99-107.

Douny, C., J. Widart, E. De Pauw, F. Silvestre, P. Kestemont, H. T. Tu, N. T. Phuong, G. Maghuin-Rogister, and Marie-Louise Scippo. 2013. 'Development of an analytical method to detect metabolites of nitrofurans', *Aquaculture*, 376-379: 54-58.

European Commission. 1990. "Council Regulation (EEC) No 2377:90 Laying down a Community procedure for the establishment of maximum residue limits of
veterinary medicinal products in foodstuffs of animal origin." In, edited by European Commission.

European Commission. 1993. "Council Regulation (EEC) No 2901/93 amending Annexes I, II, III and IV to Regulation (EEC) No 2377/90 laying down
a Community procedure for the establishment of maximum residue limits of
veterinary medicinal products in foodstuffs of animal origin." In, edited by European Commission.
European Commission. 2003. "Commission Decision amending Decision 2002/657/EC as regards the setting of minimum required performance limits
Government of Canada. 2017. 'List of Banned Drugs'. https://www.canada.ca/en/health-canada/services/drugs-health-products/veterinary-drugs/list-banned-drugs.html.
(MRPLs) for certain residues in food of animal origin." In, edited by European Commission.
Hirakawa, K., K. Midorikawa, S. Oikawa, and S. Kawanishi. 2003. 'Carcinogenic semicarbazide induces sequence-specific DNA damage through the generation of reactive oxygen species and the derived organic radicals', *Mutation Research/Gen Toxico and Environ Mutagen*, 536: 91-101.
Hoenicke, K., R. Gatermann, L. Hartig, M. Mandix, and S. Otte. 2004. 'Formation of semicarbazide (SEM) in food by hypochlorite treatment: is SEM a specific marker for nitrofurazone abuse?', *Food Addit Contam*, 21: 526-37.
Hoogenboom, L. A., and T. H. Polman. 1993. "Simultaneous detection of protein bound residues of nitrofuran drugs furazolidone, furaltadone, nitrofurazone and nitrofurantoin." In *EuroResidue II*, 6. Veldhoven, Netherlands.
Hoogenboom, L. A., M. Van Kammen, M. C. Berghmans, J. H. Koeman, and H. A. Kuiper. 1991. 'The Use of Pig Hepatocytes to Study the Nature of Protein-Bound Metabolites of Furazolidone: A New Analytical Metho for Their Detection', *Food and Chem Toxico*, 29: 8.
HSDB. 2003. 'Nitrofurazone'. https://toxnet.nlm.nih.gov/cgi-bin/sis/search2/f?./temp/~hMrNAU:3.
HSDB. 2005. 'Semicarbazide'. https://toxnet.nlm.nih.gov/cgi-bin/sis/search2/f?./temp/~AnKoyS:1.
Hu, C., and G. Xu. 2013. 'Mass-spectrometry-based metabolomics analysis for foodomics', *TrAC Trends in Analytical Chemistry*, 52: 36-46.
Hui, Y. H., X. Zhang, and H. J. Yu. 2015. "Investigation on the Natural Occurrence of Semicarbazide in Macrobrachium Nipponese prawns." In *International Symposium on Material, Energy and Environ Eng*. Changsha, China.
IARC. 1997. 'Nitrofural (Nitrofurazone)'. http://www.inchem.org/documents/iarc/vol50/09-nitrofural.html.
IARC. 1998. 'Semicarbazide Hydrochloride'. http://www.inchem.org/documents/iarc/vol12/semicarbazidehydrochloride.html.
Jia, W., X. Chu, J. Chang, P. G. Wang, Y. Chen, and F. Zhang. 2017. 'High-throughput untargeted screening of veterinary drug residues and metabolites in tilapia using high resolution orbitrap mass spectrometry', *Anal Chim Acta*, 957: 29-39.
Joshua, K., A. Sujatha, L.V. Ramana, E. Carolin, V. Supraba, and H. Dinesh Kumar. 2002. "Use of various chemicals in shrimp hatcheries and its sustainability in hatchery Technology." In *National Workshop on Aquaculture Drugs*. Cochin University of Science and Technology.

Knolhoff, A. M., and T. R. Croley. 2016. 'Non-targeted screening approaches for contaminants and adulterants in food using liquid chromatography hyphenated to high resolution mass spectrometry', *J Chromatogr A*, 1428: 86-96.

Laali, K. K., and V. J. Gettwert. 2001. 'Electrophilic Nitration of Aromatics in Ionic Liquid Solvents', *J. Org. Chem.*, 66: 6.

Love, D. C., S. Rodman, R. A. Neff, and K. E. Nachman. 2011. 'Veterinary drug residues in seafood inspected by the European Union, United States, Canada, and Japan from 2000 to 2009', *Environ Sci Technol*, 45: 7232-40.

Martinez Bueno, M. J., F. J. Diaz-Galiano, L. Rajski, V. Cutillas, and A. R. Fernandez-Alba. 2018. 'A non-targeted metabolomic approach to identify food markers to support discrimination between organic and conventional tomato crops', *J Chromatogr A*, 1546: 66-76.

Marziano, N. C., P. G. Traverso, and G. G. Cimino. 1980. 'Thermodynamic Nitration Rates of Aromatic Compounds. Part 1. The Nitration of Benzene and Some Benzene Derivatives in Aqueous Sulphuric and Perchloric Acids. A Comparison of the Results referred to Water as Standard State', *J.C.S Perkin II*: 5.

McCalla, D.R. 1983. 'Mutagenicity of Nitorfuran Derivatives: Review', *Environ Mutagen*, 5: 21.

McCracken, R., B. Hanna, D. Ennis, L. Cantley, D. Faulkner, and D. G. Kennedy. 2013. 'The occurrence of semicarbazide in the meat and shell of Bangladeshi fresh-water shrimp', *Food Chem*, 136: 1562-7.

Mohney, L. L., R. R. Williams, T. A. Bell, and D. V. Lightner. 1997. 'Residues of oxytetracycline in cultured juvenile blue shrimp, Penaeus stylirostris (Crustacea: Decapod) , fed medicated feed for 14 days', *Aquaculture*, 149: 10.

Mulder, P. P., B. Beumer, and J. A. Van Rhijn. 2007. 'The determination of biurea: a novel method to discriminate between nitrofurazone and azodicarbonamide use in food products', *Anal Chim Acta*, 586: 366-73.

Nacher-Mestre, J., M. Ibanez, R. Serrano, J. Perez-Sanchez, and F. Hernandez. 2013. 'Qualitative screening of undesirable compounds from feeds to fish by liquid chromatography coupled to mass spectrometry', *J Agric Food Chem*, 61: 2077-87.

Novotna, H., O. Kmiecik, M. Galazka, V. Krtkova, A. Hurajova, V. Schulzova, E. Hallmann, E. Rembialkowska, and J. Hajslova. 2012. 'Metabolomic fingerprinting employing DART-TOFMS for authentication of tomatoes and peppers from organic and conventional farming', *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, 29: 1335-46.

Olive, P.L., and D.R. McCalla. 1977. 'Cytotoxicity and DNA Damage to Mammalian Cells by Nitrofurans', *Chem-Biol Interactions*, 16: 11.

Patti, G. J., O. Yanes, and G. Siuzdak. 2012. 'Innovation: Metabolomics: the apogee of the omics trilogy', *Nat Rev Mol Cell Biol*, 13: 263-9.

Pereira, A. S., J. L. Donato, and G. De Nucci. 2004. 'Implications of the use of semicarbazide as a metabolic target of nitrofurazone contamination in coated products', *Food Addit Contam*, 21: 63-9.

Quilliam, M. A., B. E. McCarry, K. H. Hoo, D. R. McCalla, and S. Vaitekunas. 1987. 'Identification of the photolysis products of nitrofurazone irradiated with laboratory illumination', *Canadian J. of Chem*, 65: 1128-32.

Radovnikovic, A., E. R. Conroy, M. Gibney, J. O'Mahony, and M. Danaher. 2013. 'Residue analyses and exposure assessment of the Irish population to nitrofuran metabolites

from different food commodities in 2009-2010', *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, 30: 1858-69.
Righetti, L., J. Rubert, G. Galaverna, S. Folloni, R. Ranieri, M. Stranska-Zachariasova, J. Hajslova, and C. Dall'Asta. 2016. 'Characterization and Discrimination of Ancient Grains: A Metabolomics Approach', *Int J Mol Sci*, 17.
Rubingh, C. M., S. Bijlsma, E. P. Derks, I. Bobeldijk, E. R. Verheij, S. Kochhar, and A. K. Smilde. 2006. 'Assessing the performance of statistical validation tools for megavariate metabolomics data', *Metabolomics*, 2: 53-61.
Saari, L., and K. Peltonen. 2004. 'Novel source of semicarbazide: levels of semicarbazide in cooked crayfish samples determined by LC/MS/MS', *Food Addit Contam*, 21: 825-32.
Samanidou, V. F., and E. N. Evaggelopoulou. 2007. 'Analytical strategies to determine antibiotic residues in fish', *J Sep Sci*, 30: 2549-69.
Stadler, R. H., L. Verzegnassi, W. Seefelder, and L. Racault. 2015. 'Why semicarbazide (SEM) is not an appropriate marker for the usage of nitrofurazone on agricultural animals', *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, 32: 1842-50.
Tengstrand, E., J. Rosen, K. E. Hellenas, and K. M. Aberg. 2013. 'A concept study on non-targeted screening for chemical contaminants in food using liquid chromatography-mass spectrometry in combination with a metabolomics approach', *Anal Bioanal Chem*, 405: 1237-43.
Tian, L., and S. Bayen. 2018. 'Thermal degradation of chloramphenicol in model solutions, spiked tissues and incurred samples', *Food Chem*, 248: 230-37.
Tian, W. R., Y. X. Sang, and X. H. Wang. 2014. 'Semicarbazide - from state-of-the-art analytical methods and exposure to toxicity: a review', *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, 31: 1850-60.
Tian, X., Y. Xu, X. Song, X. Gong, Y. Liu, Q. Zhou, Z. Wang, and C. Xia. 2016. 'Temporal and spatial distribution of semicarbazide in western Laizhou Bay', *Mar Pollut Bull*, 112: 393-98.
Turnipseed, S. B., J. J. Lohne, and J. O. Boison. 2015. 'Review: Application of High Resolution Mass Spectrometry to Monitor Veterinary Drug Residues in Aquacultured Products', *J AOAC Int*, 98: 550-8.
Valera-Tarifa, N. M., P. Plaza-Bolaños, R. Romero-González, J. L. Martínez-Vidal, and A. Garrido-Frenich. 2013. 'Determination of nitrofuran metabolites in seafood by ultra high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry', *J. of Food Comp and Anal*, 30: 8.
Van Poucke, C., C. Detavernier, M. Wille, J. Kwakman, P. Sorgeloos, and C. Van Peteghem. 2011. 'Investigation into the possible natural occurence of semicarbazide in Macrobrachium rosenbergii prawns', *J Agric Food Chem*, 59: 2107-12.
Vass, M., K. Hruska, and M. Franek. 2008. 'Nitrofuran antibiotics: a review on the application, prohibition and residual analysis', *Veterinarni Medicina*, 53: 32.
Villar-Pulido, M., B. Gilbert-Lopez, J. F. Garcia-Reyes, N. R. Martos, and A. Molina-Diaz. 2011. 'Multiclass detection and quantitation of antibiotics and veterinary drugs in shrimps by fast liquid chromatography time-of-flight mass spectrometry', *Talanta*, 85: 1419-27.
Von Eyken, A., and S. Bayen. 2019. 'Optimization of the Data Treatment Steps of a Non-targeted LC-MS-Based Workflow for the Identification of Trace Chemical Residues in Honey', *J Am Soc Mass Spectrom*.

Von Eyken, Annie, D. Furlong, S. Arooni, F. Butterworth, J. F. Roy, J. Zweigenbaum, and S. Bayen. 2019. 'Direct injection high performance liquid chromatography coupled to data independent acquisition mass spectrometry for the screening of antibiotics in honey', *J. of Food and Drug Anal*.

Vroomen, L. H., M. C. Berghmans, P. Hekman, L. A. Hoogenboom, and H. A. Kuiper. 1987. 'The elimination of furazolidone and its open-chain cyano-derivative from adult swine', *Xenobiotica*, 17: 1427-35.

Wang, W., H. Lin, C. Xue, and J. Khalid. 2004. 'Elimination of chloramphenicol, sulphamethoxazole and oxytetracycline in shrimp, Penaeus chinensis following medicated-feed treatment', *Environ Int*, 30: 367-73.

Wang, Y., E. L. Jester, K. R. El Said, A. Abraham, J. Hooe-Rollman, and S. M. Plakas. 2010. 'Cyano metabolite as a biomarker of nitrofurazone in channel catfish', *J Agric Food Chem*, 58: 313-6.

Xia, M. H., X. L. Huang, H. L. Wang, M. Jin, M. Li, and Q. C. Zhou. 2015. 'Dietary niacin levels in practical diets for Litopenaeus vannamei
to support maximum growth', *Aquaculture Nutrition*, 21: 853-60.

Zhang, S., Y. Guo, Z. Yan, X. Sun, and X. Zhang. 2015. 'A selective biomarker for confirming nitrofurazone residues in crab and shrimp using ultra-performance liquid chromatography-tandem mass spectrometry', *Anal Bioanal Chem*, 407: 8971-7.

Zhang, S., P. Li, Z. Yan, J. Long, and X. Zhang. 2017. 'Identification and quantification of nitrofurazone metabolites by ultraperformance liquid chromatography-quadrupole time-of-flight high-resolution mass spectrometry with precolumn derivatization', *Anal Bioanal Chem*, 409: 2255-60.