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Monitoring Gulf Coast Fish Quality

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By

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Globalization and the rise of fish importation has led to an increase in mislabeling. To combat this problem, analytical and molecular methods have been employed. First, nitrofuran metabolites were extracted, hydrolyzed, and derivatized in channel catfish, swai, and tilapia. Utilizing high performance liquid chromatography coupled with triple quadrupole mass spectrometry, derivatized metabolites were detected at levels of 1 ng/mL with coefficients of determination greater than 0.998. Recoveries greater than 90% and relative standard deviation less than 17% indicate that the method is successful. Secondly, chip based electrophoresis coupled with restriction fragment length polymorphism was used for the species differentiation. By analyzing restriction digestion products, fragmentation patterns from fin-clip and muscle could consistently differentiate different species requiring two or fewer endonucleases for positive identification. This method of screening reduces the expertise, time, and expense required to reduce fish mislabeling. In tandem, these methodologies could significantly reduce the dangers of fish mislabeling.

DEDICATION

I dedicate this work to mom and dad.

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Mislabeling

In recent years, authentication of seafood has become increasingly important due to global growth. Not only has the world market supply increased from 54 million tons in 1964 to 154 million tons as of 2011 but approximately half of total fish supplies are dedicated to international trade with 16.6% accounting for consumption of animal protein [39, 50]. In recent years, mass mislabeling of fish and other seafood has become much more prevalent. Seafood fraud is not limited to grocery stores, restaurants, or sushi bars and can usually be sourced back directly to importers [36]. Numerous studies on the mislabeling of seafood products have been conducted within the past decade. The National Seafood Laboratory found that the 37% of fish and 13% of other seafood products analyzed were mislabeled [71]. In a study conducted by Oceana, a non-profit organization, approximately 1200 samples from over 600 different businesses were analyzed using FDA bar-coding protocols. The study found that 33 percent of the samples were mislabeled with substitutions among red snapper being the highest at 89 percent. This is supported by a study conducted by Marko et al, which showed that approximately three-quarters of all red snapper sold were often mislabeled or substituted with rock-fish or other types of snapper. Other common substitutions include basa (*Pangasius bocourti*) or swai (*Pangasiandon hypophtalmus*) instead of channel catfish

(*Ictalurus punctatus*) [33], and escolar (*Lepidocybium flavobrunneum*) in the place of albacore tuna (*Thunnus alalunga*) [51]. As shown by these studies, mislabeling of seafood products represents pertinent risks to consumers such as financial fraud, health issues resulting from allergies or adulterants, and hampering conservation efforts. To combat the problem of mislabeling, government legislation has been signed into law requiring seafood products such as catfish, bonito, crab, and oysters to be marketed under a statement of identity, which may only be used to describe those specific species. Under these guidelines, only channel catfish may be sold under the label of catfish. [4]. For all other seafood, the U.S. Food and Drug Administration provides guidelines that help consumers and producers better understand what constitutes an acceptable market name. Other than statements of identity and scientific nomenclature, acceptable market names include common names as long as they are not misleading, contain geographic descriptors, or vernacular [9]. Mislabeling or substitution of a fish species for another represents a form of economic deception. For instance, "red-fish" which is significantly cheaper than red snapper is often substituted for the purpose of economic gain [70]. White fish is substituted for albacore tuna for similar reasons but also has ill desired side effects. Often, the substituted fish is actually escolar or tilapia. In the case of the escolar, also referred to as butter fish or snake mackerel, the substitution poses a potential health risk because ingestion of minimal amounts of escolar can result in gastrointestinal issues. Escolar diets consist primarily of food sources that are high in wax esters which are stored in the fatty tissue after consumption. Human beings lack the digestive enzymes necessary to break down these esters which results in a condition referred to as keriorrhea where the orange colored esters uncomfortably pass through the digestive system [69].

Another scenario that often arises is simply the misidentification or incorrect labeling of certain fish species. Atlantic halibut can often be labeled as Pacific halibut and vice versa. Another example is the labeling of farm-raised salmon as wild salmon to earn more revenue. Common names and vernacular also cause issues with proper naming. In one study, a sample labeled as king-fish was actually *Scomberomorus cavalla*. The label of king-fish is also commonly used to describe *Scomberomorus regalis*. Despite the vernacular used, *S. cavalla* actually describes a fish with the common name king mackerel and the market name Spanish mackerel. Above all, mislabeling of seafood represents an annulment of contract between consumer and producer [70, 35].

In order to protect consumers, new and existing methodologies have been developed or adapted to improve seafood identification. Correct identification of processed seafood products and fish fillets at points of origin can reduce the risks associated with mislabeling. Described in the following sections are common protocols that have been developed to improve the identification or description of animal species.

DNA Methodology

Differentiation by morphological means requires some training in taxonomy to differentiate species. Typically, taxonomists use morphological features Figure 1.1 supplemented with geographical, behavioral, and genetic information when available; however, due to the variation in fish life cycles and introduction of hybrid species, positive identification using morphology has become increasingly difficult [68].

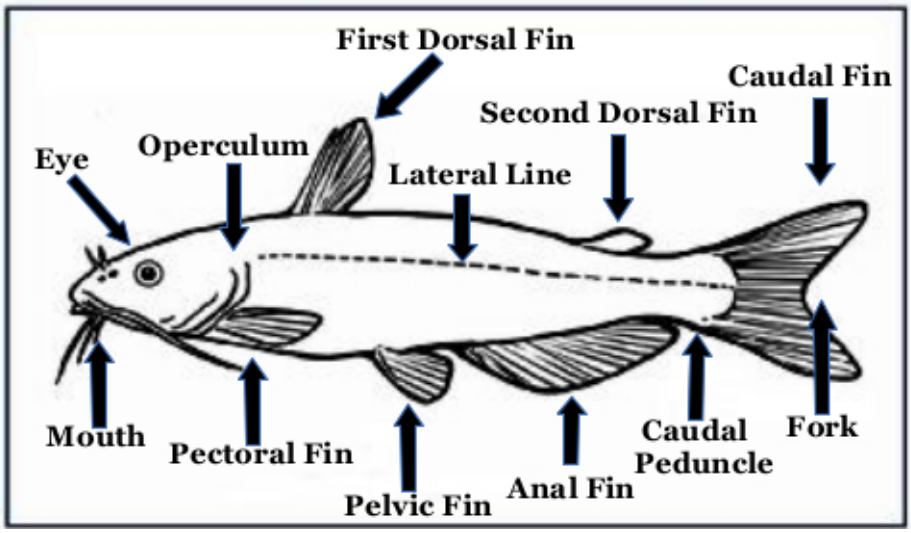

Figure 1 Diagram of morphological features

Diagram of morphological features of channel catfish illustrating the difficulties inherent in identifying fish fillets lacking the very features required to identify them.

Taxonomic identification is limited in its scope, requiring individuals who are highly trained which can be time consuming. Often, batches of fish require identification for the purpose of conservation and are very large which increases costs exponentially. Twenty-one percent of fish sold in 2006 consisted of whole or gutted fish while the rest consisted of fish that had been processed in some way: filleting, canning, or cooking [52]. Large-scale identification by hand is nearly impossible and it's because of this that morphological differentiation of species as the sole method of identification is no longer sufficient.

New and rapid methodology is necessary to curb fraud before products reach the consumer. Many methods exist for the differentiation of fish species. Those commonly used include: forensically informative nucleotide sequencing (FINS), restriction fragment length polymorphism (RFLP), single-stranded conformational polymorphism (SSCP),

amplified fragment length polymorphism AFLP, Random Amplified Polymorphic DNA RAPD and DNA bar-coding [51].

Analysis of DNA over protein for the identification of species has grown more common due to the fact that very little source material is required. Additionally, mitochondrial DNA (mtDNA) has replaced nucleic DNA due to the ring structure being more stable and thus more resistant to denaturation during processing which can alter the structure due to adjustment of pH, temperature, and hydrolysis through the addition of water [36]. These attributes are more conducive towards the analysis of cooked or canned products, fillets, fin-clips, eggs, or larvae. For most fish, this offers species level specificity of identification from egg to shelf. These methodologies are advantageous to the fields of food security and conservation because most species, including hybrids, can be identified during any part of the life cycle. The use of mtDNA was first described in 1992 with the development of forensically informative nucleotide sequencing (FINS) for the identification of four different (thunnus) species by sequencing mitochonrdial cytochrome B gene [5]. FINS works by amplifying nucleotide sequences from cytochrome b, coenzyme oxidase subunit I (COI), or 16S RNA [22]. Similar to phylogenetic methods, amplicons are compared to a reference and sequences with nucleotide substitutions the lowest genetic distance away are considered to be in the same species group. These protocols are useful for population genetics and phylogenetic studies, but due to higher costs and time requirements, FINS isn't suitable for large scale differentiation. Also, FINS is unable to handle samples of mixed species [21, 46, 6].

RFLP has also been used in conjunction with polymerase chain reaction (PCR) to differentiate species based on digested fragment polymorphisms [3]. Similar to FINS, a

gene region is selected, extracted, and amplified. Diverging at this point, RFLP protocols then call for the use of different restriction endonucleases which cleave the gene region of interest resulting in fragments particular to that species. Unlike FINS, RFLP is much more cost efficient and is often used as a screening method [21]. RFLP can be used to fingerprint cooked and mixed samples, but quality of the source DNA is critical for successful differentiation [46, 47, 10]. Analysis of multiple samples is required to build a reliable fingerprint and some samples lacking unique fragmentation patterns benefit from the use of multiple restriction sites [43, 60]. An alternative to FINS and RFLP is single-stranded conformational polymorphism (SSCP) which also relies on polymorphisms for differentiation. SSCP amplifies DNA genes such as mitochondrial cytochrome B before denaturing the amplicon into single strands which are then separated by PAGE electrophoresis [47, 55]. Intra-species variation is even lower than RFLP with differences as minute as one nucleotide detectable. This allows for differentiation of fragments of 100 bp evenwith mixed samples. This specificity comes with the cost of requiring the reference sample being run on the same gel as unknowns [10, 56]. This makes SSCP unsuitable for fingerprinting and more useful for population studies.

AFLP, similar to RFLP, utilizes restriction enzymes, typically MseI and EcoRI, to digest whole DNA [67, 45]. With one enzyme making short frequent cuts and the other making longer less frequent cuts, an adapter is then linked to the product before amplification with PCR. The resulting amplicons, about 100 fragments, are then amplified again with only 1/256 of the original DNA having been amplified [18, 16]. Using radioactive labels, the fragments are then separated by gel electrophoresis which allows for a very specific fingerprint [20]. Quick and cheap like RFLP, AFLP is far more

specific than RAPD and lacks the requirement of reference samples like SSCP. Unfortunately, unlike RFLP, AFLP is a very time consuming process and requires high quality DNA so would not handle cooked or mixed samples [45, 20, 16]. Species differentiation often requires some knowledge of DNA sequences for the development of primers for analysis. RAPD analysis bypasses this requirement by using randomly selected primers for the amplification of target DNA sequences. Although random, each amplicon produced should be unique to each species when analyzed with electrophoresis and compared to previously identified samples [72, 8].

RAPD is both cheap and quick requiring little source material for analysis making it an attractive tool for differentiation when compared to RFLP and AFLP. Unfortunately, relying on randomly amplified DNA has drawbacks such as decline in reproducibility in cooked samples and the possibility of incorrect species matching due to DNA regions from different species producing the same fragments [3].

Early sequencing methodologies were not efficient because techniques varied from lab to lab depending on the instrumentation and capabilities of that lab. Also, research labs and regulatory agencies had different goals, which led to different groups publishing research on a wide variety of methodologies using the same fish species [68, 52]. To combat this problem, the initial protocols outlining DNA bar-coding were developed to compensate for these inefficiencies. Fish Bar-coding protocols are a set of rules outlining a single gene of interest to be used for identification. Bar-coding is used for a wide variety of services including: conservation, tree of life, ecosystem and behavior analysis, and food safety projects. Because of this, bar-coding methodologies were chosen as the foundation for the Bar-code of Life initiative [53, 68]. The Fish Bar-

code of Life (Fish-BOL) initiative, which was implemented in 2005 represents a world-wide collaboration with the goal of developing a standard reference bar-code library of different species. This library contains sequences of a 648 base pair region of the mitochondrial COI gene in addition to taxonomic data. The COI sequence was chosen due to the lack of intra-species variation and the presence of inter-species variation among most species in addition to the availability of primers. As of 2010, approximately 25% of all known fish species have been processed with at least one species from 89% of all families sequenced and identified. Using this standardized system, only three percent of sequences observed have been unusable for differentiation when at least 2 specimens are sequenced [29].

Antibiotics

Antibiotics are drugs used to kill or inhibit gram-negative and positive bacteria, which are differentiated via staining to determine intracellular structure. Gram-negative bacteria such as *Eromonas*, *Pseudomonas*, and *Vibrio* cause most bacterial infections in fish. Diseases resulting from infection can cause fin rot, gill disease, and tumors. In aquaculture, antibiotics are used as a prophylactic to prevent the spread of diseases. Modes of introduction include feed and medicinal bath with the goal of preventing development of bacterial cell walls, damaging of membranes, and the disabling of key protein and nucleic acid synthesis. These methods are both cheap and effective which contributes to their continued use despite being banned in most countries. When absorbed, these antibiotic residues are persistent in tissues and can remain behind causing a variety of health concerns. Heavy use of antibiotics results in an increase in resistance, development of human allergies upon consumption, and production of toxic effects.

Monitoring aquaculture quality can serve to reduce the need for new antibiotics and improve food quality [59].

In seafood, commonly used adulterants include antibiotics such as quinolones, amphenicols, and nitrofurans and dyes such as malachite green and crystal violet. These residues are used as antimicrobials to combat a variety of diseases in farm raised fish [59]. Quinolones are categorized into four generations separated by different chemical modifications to improve performance. The second generations of quinolones are commonly referred to as flouroquinolones due to the addition of a fluorine group to the C-6 group of a quinolone [41]. Flouroquinolones work by inhibiting the DNA gyrase in order to prevent duplication of the bacterial cells [59]. Third and fourth generation quinolones (flouroquinolones) were modified to increase effectiveness against gram-negative bacteria and improve gram-positive and anaerobic coverage. Quinolones are often used because they are very effective at preventing urinary and digestive tract infections. Unfortunately, continued use can result in increased sensitivity and arthralgia [40]. Amphenicols are a synthetic group of antibiotics with a wide range of effectiveness, which include thiamphenicol, florenicol, and chloramphenicol [59].Chloramphenicol was the first large scale synthetic whose mode of action is to prevent mitochondrial protein synthesis by binding to the 16S ribosomal subunit. Chloramphenicol is frequently used due to its low manufacturing cost and effectiveness against both gram-positive and gram-negative bacteria. Increased presence of tissue bound residues can result in bone marrow depression which can cause fatal anemia [59]. Lastly nitrofurans are another synthetic compound that have been used on a variety of farm raised animals including cattle, poultry, and fish. Nitrofurans are easily absorbed through the skin where the parent

compounds quickly break down resulting in several metabolites that then bind to muscle tissue. Frequently applied topically or through bath treatments, nitrofurans are carcinogenic and mutagenic [38]. Dyes such as malachite green and crystal violet are often used as antimicrobials and fungicides. Part of the triphenylmethane family, these cheap and effective dyes are quickly absorbed into fish tissue where they are converted to leuco-malachite green and leuco-crystal violet. Similar to nitrofurans, these dyes are carcinogenic and mutagenic and are thus prohibited [12].

Many countries have imposed bans on the use of these adulterants (Table 1) but due to the high level of importation many still find their way into the food supply. Consumer safety is ensured by government regulatory bodies such as the Food and Drug Administration of the United States and the European Union. The European Union enforces food safety by setting maximum residue limits (MRLs) and minimum performance limits (MPRL). MRLs are defined as maximum legal levels contained in food allowed to reach consumers while MPRL's are the minimum capabilities of analytical methods [30, 49, 48]. In some cases, no such level exists and is determined by the capabilities of current screening methods. The FDA has similar standards and has banned the use of fluroquinolones, amphenicols, and nitrofurans for extra-label use with the exception of nitrofurans for topical use. The EU has placed MRL's for malachite green and crystal violet at 2 ng/g [61, 12, 17].

Table 1 Adulterant Regulations

Adulterant	Type	Organization	MRL	MPRL	Regulation	Year
Chloramphenicol	Antibiotic	FDA	Banned	--	21 CFR 522.390	1992
		EU	Banned	0.3 ug/kg	Commission Decision 2003/181	2003
		CFIA	Banned	--	C.01.610.1	1994
Nitrofuran	Antibiotic	FDA	Banned	--	21 CFR 510.551	1991
		EU	Banned	1 ng/kg	EEC 2309/93; 1442/95	1993/1995
		CFIA	Banned	–	C.01.610.1	1994
Fluruoroquinolone	Antibiotic	FDA	Banned	- -	21 CFR 530.41	1997/2005
		EU	- -	- -	N/a	- -
		CFIA	Banned	1 ng / g	B.01.048	2003
Malachite Green	Dye	FDA	Banned	- -	21 C.F.R. section 814.9.	1983
		EU	2 mg/kg	1 mg/kg	Commission Decision 2004/25	2004
		CFIA	1 ppb	0.5 ng/g	C.01.610.1	1994

Most adulterants are commonly screened with high performance liquid chromatography (HPLC) coupled to a variety of detectors. In the past, ultraviolet-visible (UV-VIS), fluorescence, electrochemical, and mass spectrometry detectors have all been used for residue detection [59]. In the past, UV-VIS and fluorescence detection were primarily used for the detection of nitrofurans, chloramphenicol, and fluoroquinolones, but mass spectrometry has become more common due to the specificity provided [59, 11]. Unfortunately, no catch-all methods exist for the detection of both antibiotics and dyes that are frequently used as adulterants. Multi-residue methods do exist but are limited in scope. Nitrofurans have been extracted from fish tissue in conjunction with chloraphenicol, fluoroquinolones, and sulpha drugs using liquid chromatography coupled with UV-VIS [57, 31]. Multiresidue methods for the detection of dyes in fish tissue also exist using liquid chromatography coupled with mass spectrometry [11, 65, 63].

Due to increasing globalization and the rise of importation, monitoring of fish species has become quite important. High influx of seafood poses risks to health, security, economy, and conservation. These concerns must be dealt with to ensure not

only the safety of a nation's constituents but states' interests as well. To combat overfishing, mislabeling, and adulteration, regulatory bodies require the most efficient and rapid protocols and current databases. While one definitive protocol doesn't yet exist to both identify aquatic species and detect adulterants, fast methods have been examined in this work to accommodate both tasks in a twenty-four hour period. Generating sequence data is quite expensive and time consuming and requires an up-to-date central database for comparison. Until new methodologies such as mini-bar-coding which relies on shorter sequence fragments and next generation sequencing are explored further, identification of mixed species with DNA bar-coding will remain problematic [36].

For the identification of fish species, RFLP and chip-based electrophoresis have been proposed to construct a database of species that have some economic or conservational importance. Extraction and fragmentation are relatively quick making PCR-RFLP a reliable method for building a database. Databases composed of fragments are easily searchable compared to lengthy sequences which require more specialized skills to acquire and analyze.

Nitrofurans are a common family of carcinogenic and mutagenic adulterant used for the prophylaxis of farm raised fish. Although rapidly depleted in tissue, the metabolites left behind are easily analyzed using liquid chromatography coupled with triple quadrupole mass spectrometry once derivatized. A novel method for the identification of nitrofuran metabolites in commonly farm raised fish species is detailed below.

CHAPTER II

DETECTION OF NITROFURAN METABOLITES IN FISH FILLETS QUADRUPOLE MASS SPECTROMETRY

Abstract

An analytical method has been developed for the detection of nitrofuran metabolites in channel catfish, swai, and tilapia fillets utilizing high performance liquid chromatography coupled with electrospray ionization triple quadrupole mass spectrometry. Derivatization and hydrolysis under acidic conditions with 2-Nitrobenzaldehyde followed by a solid phase extraction cleanup prepared the metabolites for analysis. Compounds were detected as low as 1 ng/mL with coefficients of determination greater than 0.998. Samples were spiked with 5 ng/mL solutions of nitrofuran metabolites with recoveries of 90-130% and relative standard deviations less than 17 percent. Application of the method to real samples resulted in the detection of semicarbazide in some samples.

Introduction

Furazolidone, furaltidone, nitrofurazone, and nitrofurantoin are members of a group of synthetic antibiotics commonly referred to as nitofurans. Frequently used for treatment of gastrointestinal disorders in humans and farm animals such as cattle, poultry, fish, and shrimp; Nitrofurans are widely applied due to cost, effectiveness, and ease of

application such as: introduction in feed, water baths, and topical ointments [67, 59]. Due to mutagenic and cargcenogic properties, nitrofurans have been banned by the U.S. Food and Drug Administration (FDA), European Union (EU), and other regulatory bodies. Since 1995, the EU has banned use of all nitrofurans on animals, which are destined for food production [25]. For nitrofurans, no MRL exists so in 2003, the EU set the minimum performance residue limit (MPRL) at 1 mg/kg [26].

Naturally, in order to meet these performance limits, quick and reliable methods of detection must exist. Nitrofurans present an interesting conundrum due to the fact that the compounds are rapidly metabolized once absorbed. The compounds 1-aminohydantoin(AHD), 3-amino-2-oxazolidinone(AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), and semicarbazide (SEM) are the corresponding metabolites for the parent residues Figure 2.

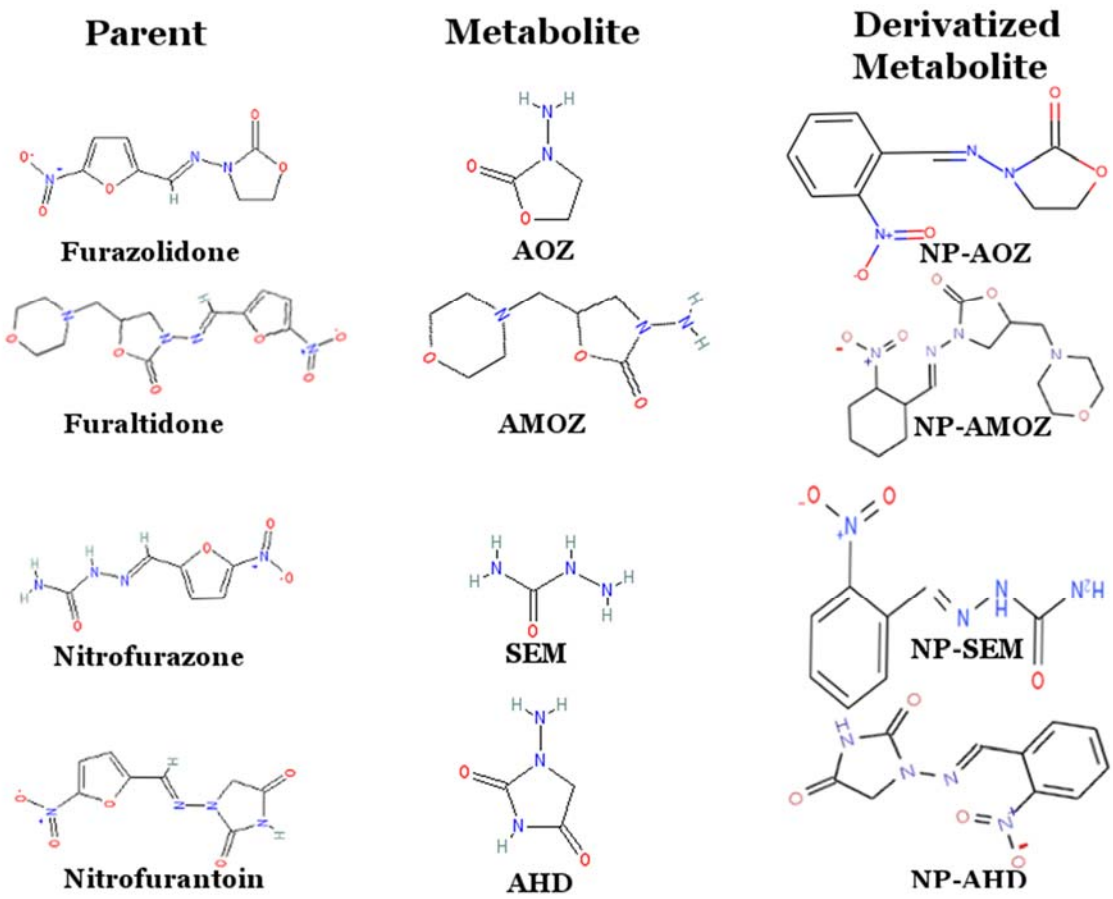

Figure 2 Parent nitrofurans, metabolites, and nitrophenyl derivatives.

Historically, liquid chromatography coupled with UV-VIS or diode array has been used to detect these compounds. [14]. Enzyme-linked immunosorbent assay (ELISA) based methods are in development [44], but currently liquid chromatography coupled with mass spectrometry represents the most efficient detection method. Due to matrix effects and such low molecular weights (75-201 g/Mol) of the metabolites, derivatization and Solid Phase Extraction (SPE) are required to make these polar compounds better suited for reverse-phase chromatographic separation and analysis.

Previous studies have detected nitrofuran metabolites in poultry [13, 19, 49, 32], pigs [42, 49, 1], fish [13, 62, 64, 24], and shrimp [64, 30, 23, 19]. Due to rapid absorption and stability combined with freezing and canning of seafood products, the production process provides a suitable environment for the preservation of these compounds. Because of increasing global demand for seafood, the instances of nitrofuran usage have also increased [7] creating a scenario in which processed fish of immediately unknown origins enters the food supply. Processing such as filleting, canning, or cooking along with the growing problem of fish mislabeling, regardless of intent, serves to further mask the identity of imported fish species. Because of the inherent difficulty in identifying fish fillets and the increase of farm-raised fish production as a global entity, rapid detection of nitrofuran metabolites in a variety of tissues is vitally important.

Mississippi is the top producer of farm-raised catfish in the United States resulting in 175 million dollars of revenue per year [73]. Due to the increase in global importation and demand, the Americas have seen an increase in fish species from European and Asian countries in American markets. Because of differing regulations, fish species such as basa and swai entering US markets may contain antibiotics such as nitrofurans as adulterants.

Antibiotics of concern have been detected in imported fish above 1 ng/mL [7]. Because most fish entering the country have been processed in some way, fillets of catfish and basa or swa can be indistinguishable from one another. Therefore to maintain financial and health security, it would be advantageous to not only detect adulterants in fish tissue, but to also have a set of protocols allowing for the detection of nitrofurans regardless of the species origin. In this study, a method has been developed to detect

nitrofuran metabolites in the muscle tissue of channel catfish, swai, and tilapia fillets using liquid chromatography coupled with Electrospray Ionization (ESI) triple quadrupole mass spectrometry.

Materials and Methods

Nitrofuran standards of AOZ, AMOZ, AHD-HCl, and SEM and internal standards AOZ-d4 and AMOZ--5 were all purchased from Sigma-Aldrich. Analytical standards with purity greater than 99% were used. Standard solutions of 100 ng/mL were prepared by diluting 1 mg/ml solutions of each standard with 10 mL of methanol (MeOH). Internal standard solutions were prepared in the same way. Solutions were stored in a dark location and used within 6 months. Ethyl Acetate (EAc), n-hexane, HPLC grade water, Sodium Hydroxide, and 2-Nitrobenzaldehyde were also purchased from Sigma-Aldrich. Chromatographic analysis was performed with a 1260 Infinity High Performance Liquid Chromatograph equipped with a Zorbax Eclipse XDB C8 column (150mm x 4.6mm ID, 5 micron particle size) from Agilent Technologies (Santa Clara, California). Mass analysis occurred in an Agilent 6460 Triple Quadrupole Mass Spectrometer outfitted with a Jet Stream nitrogen source operating in positive electrospray ionization mode (ESI+). Mobile phase conditions for chromatographic analysis consisted of MeOH (A) and 10 mM ammonium formate in aqueous solution (B). Ten μL of sample was injected on column at a flow rate of 0.8 mL/minute with a column temperature of 30 C for the entire duration of the run.

Gradient elution began at 20% of eluent B increasing to 95% over a period of six minutes. Eluent B was increased to 100% for two minutes before returning to starting conditions in a 3 minute post run period for a total run time of 11 minutes per sample.

Mass Spectrometer analysis occurred under the following source conditions: sheath gas temperature, 400° C; drying gas, 325° C; sheath gas flow, 12 Liters/minute; drying gas flow, 10 Liters/minute; nebulizer pressure, 25 psi; and capillary voltage, 4000 volts. Analysis of nitrofuran metabolites and internal standards was conducted in Multiple Reaction Monitoring (MRM) mode during a 1.5 minute observation window with a dwell time of 50 ms. Method development, acquisition, and quantitation were performed using Agilent's Masshunter software packages. SPE cleanup was performed utilizing Chromabond C_6H_5 cartridges.

2.5 grams of homogenized fish fillet was weighed into a 50-ml polypropylene tube before addition of 0.2 M HCl and 100 µL of 0.1 M 2-NBA freshly prepared in a solution of methanol. As previously described cite extraction, hydrolysis, and dervitization occurred sequestered from light for 16-20 hours in a water bath at 37° C. Samples were acclimated to room temperature before neutralization at pH 7.1-7.5 with 600-800 µL of 2.5 M NaOH dependent upon matrix along with 1.5 mL of 0.1 M di-sodium hydrogen phosphate solution. Samples were vortexed and centrifuged at 3500 RPMs (x G) before addition of 10 mL hexane. The aqueous layer was transferred to a new 50 mL polypropylene tube before undergoing SPE clean-up using X Chromabond C_6H_5 3 mL cartridges. The samples were conditioned with 6 mLs of HPLC grade ethyl acetate, methanol, and water before loading of the sample. The cartridge was first washed with 6 mL of water then 6 mL of 30% methanol. The metabolites of interest were eluted into a 15 mL falcon tube with 8 mL of ethyl acetate followed by evaporation to dryness under a steady stream of nitrogen using a Turbovap. Samples were brought up in 1 mL of mobile phase (80% H20 w/ 10 mM Ammonium Formate) 20% MeoH v/v). Lastly, the

samples were filtered through a 0.45 micron PTFE filter into auto-sampler vials. Samples were stored at 0 C and analyzed within 1 week.

For this study, Mississippi farm-raised catfish fillets and imported swai and tilapia fillets were purchased from the local Kroger super market (Starkville, MS). Samples were homogenized in a Magic Bullet blender then stored in a freezer at 0 C before derivitization and analysis. Samples devoid of the analyte of interest were used as blank material for preparation of calibration standards and for the calculation of extraction efficiency and relative standard deviation (RSD).

Results and Discussion

Tilapia, channel catfish, and swai fish were chosen because all samples were relatively easy to acquire, could be used across multiple studies, and all fish are farm-raised in their respective countries of origin and thus subject to adulteration. Imported samples prone to contamination needed to be tested for adulteration. Extraction, hydrolysis, and derivitization procedures were identical for each species. Mass Spectrometer parameters were adjusted using the Masshunter Optimizer software. This software automates the process of selecting the appropriate fragmentor voltage, collision energy, and product ions by performing several injections for each individual analyte at a concentration of 1 mg/mL given the molecular weight of the parent ion. Analysis of the calibration and internal standards was performed without chromatographic separation with mobile phase conditions for eluent A and B set at 50 percent. As further described in Table 2, the two transitions with the highest abundance were selected for verification of analyte identity. For the two internal standards, the transition with the highest abundance

was selected. Source conditions and solvent selection were based off of previous studies of nitrofuran metabolites using similar instrumentation and methods [14, 66].

Table 2 Mass Spectrometer source conditions

Compound	Molecular Weight (grams / mole)	Transition	Fragmentation Voltage (Volts)	Collision Energy (Volts)	Dwell Time (ms)	Retention Time (minutes)	R Squared	Recovery %	RSD %
AOZ	235	236 → 134	117	9	50	5.36	0.999868	110.74	14.95%
		236 → 104	117	21	50	5.36			
AMOZ	334	335 → 291.1	103	5	50	5.78	0.998695	104.62	11.93%
		335 → 100	103	37	50	5.78			
SEM	208	209 → 192.1	83	5	50	5.49	0.998326	120.47	16.50%
		209 → 91.1	83	29	50	5.49			
AHD	248	249 → 134	126	9	50	5.73	N/A	N/A	N/A
		249 → 104	126	17	50	5.73			

Initially a C18 reverse phase column was chosen for chromatographic separation but total analysis time and quality of separation were less than ideal. An Eclipse XDB C8 column was selected for the experiment due to a higher affinity for the metabolites, which resulted in adequate separation and a shorter run time. A flow rate of 0.8 mL/minute was chosen to achieve a relatively short run time while also maintaining adequate chromatographic separation and column pressure within appropriate operating conditions. Intermediate solutions of calibration standards were injected at volumes of 5, 10, 20, and 30 µL and were examined qualitatively, with 10 µL being chosen to minimize the total volume required for each injection while still maintaining chromatographic separation and instrument sensitivity. Once optimum conditions were reached for gradient, flow rate, and injection volume, an injection program was created to inject 1 µL of an intermediate internal standard solution (20 ng/mL solution of d4-AMOZ and d5-AOZ) along with 9 µL of sample or calibrant. Chromatograms of nitrofuran metabolites at 1.25 ng/mL can be seen in Figure 3 below.

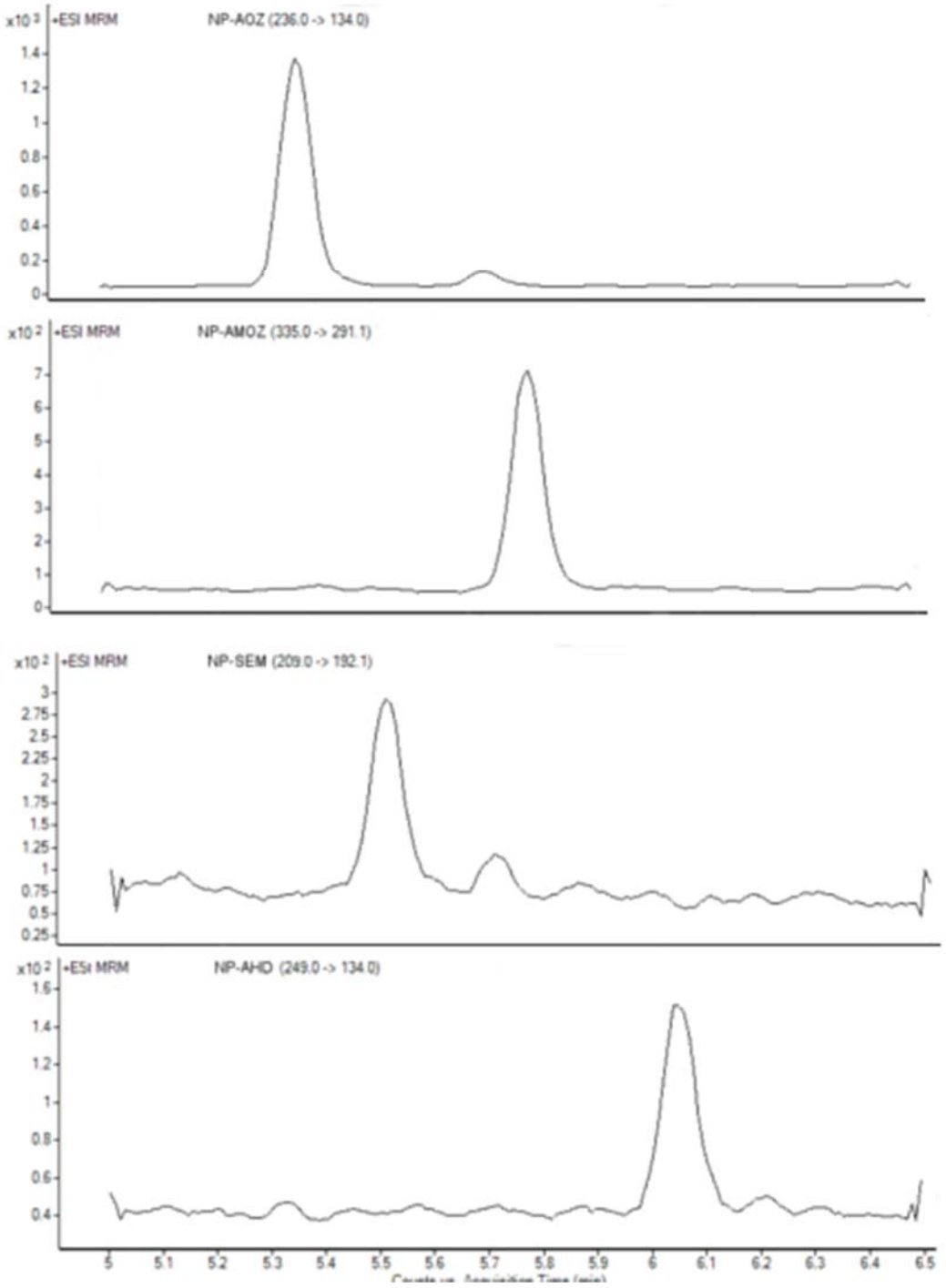

Figure 3 Nitrofuran metabolite chromatograms

LC-MS-MS chromatograms of derivatized nitrofuran metabolites (NP-AOZ, NP-AMOZ, NP-SEM, NP-AHD) at concentrations of 1.25 ng/mL.

The metabolite AHD was not retained through SPE cleanup most likely due to the 30% MeOH wash step along with a lack of affinity to the C_6H_5 cartridges. This lead to poor detection of the standard during analysis resulting in a poor calibration curve. AOZ, AMOZ, and SEM were retained through sample cleanup producing calibration curves (Figure 4, 5, and 6) with r-squared values greater than 0.998 which were considered adequate for further calculations. The lower limit of detection was 0.625 ng/mL.

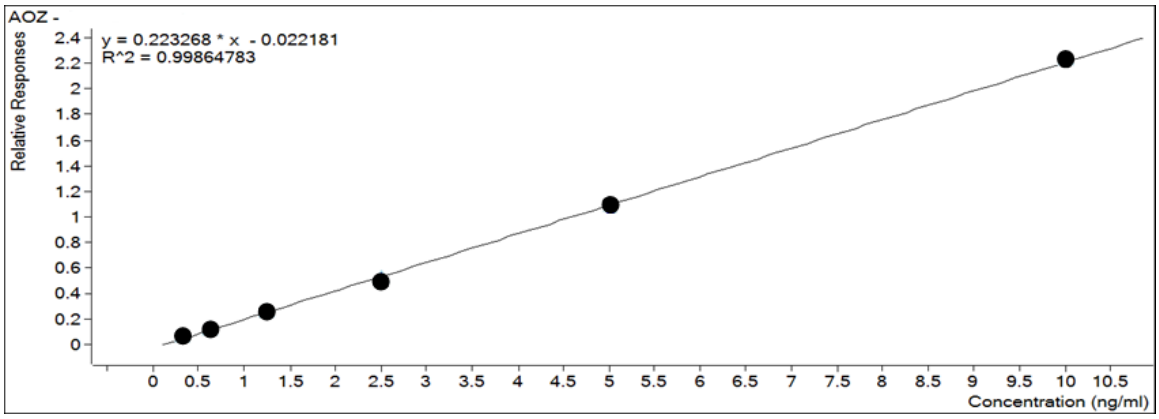

Figure 4 AOZ calibration curve

Calibration cuve of AOZ standard spiked into swai at 0.3125, 0.625, 1.25, 2.5, 5, and 10 ng/mL.

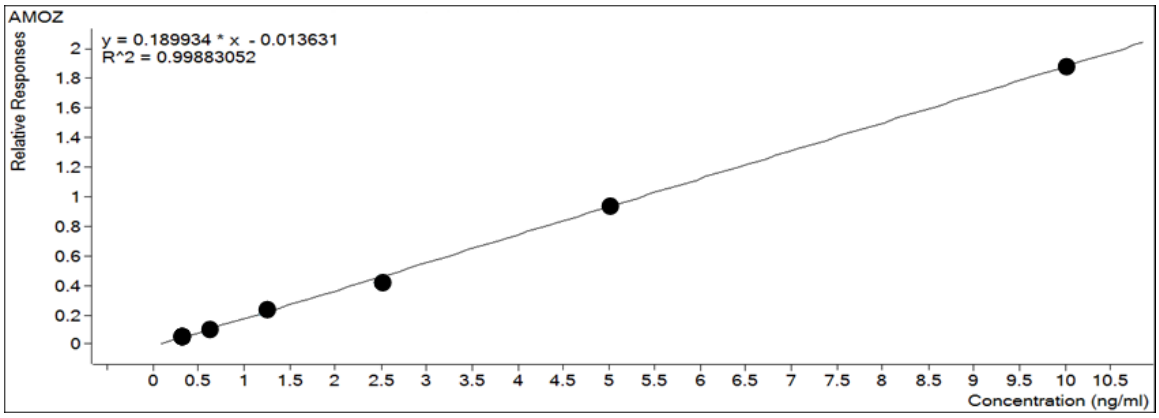

Figure 5 AMOZ calibration curve

Calibration curve of AMOZ standard spiked into swai fillet at 0.3125, 0.626, 1.25, 2.5, and 10 ng/mL

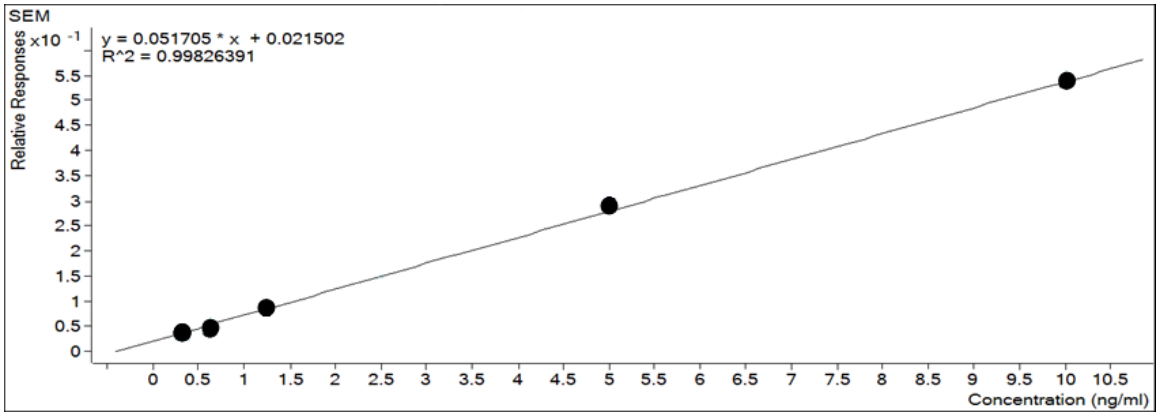

Figure 6 SEM calibration curve

Calibration curve of SEM standard spiked into swai fillet at 0.3125, 0.625, 1.25, 2.5, and 10 ng/mL

Derivatized standard solutions (1000 ng/mL) were spiked into each matrix with 4 replicates at a final expected concentration of 5 ng/mL. Underivatized standard solution (1000 ng/mL) was also spiked into each matrix with 3 replicates at a final concentration of 5 ng/mL. The starting derivatized standard did not undergo SPE cleanup or analysis, making the efficiency of derivatization an unknown. Despite this, the resulting data (not shown) indicated that the efficiency of derivatization is less than 100% despite presence of 2-NBA in greater excess. Recoveries, shown in Figure 7, indicate exceptional extraction and derivatization of underivatized standards. RSD was determined to be less than 17 percent which is comparable to results observed by [66, 2].

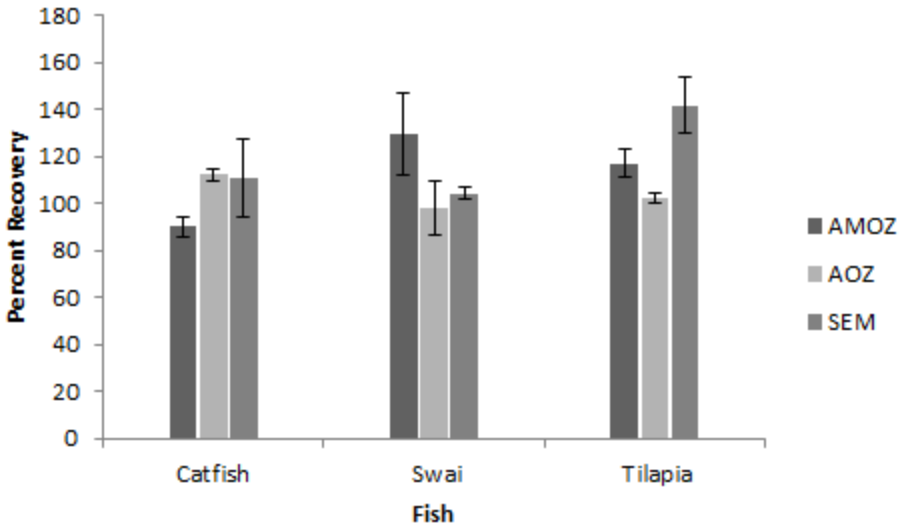

Figure 7 Extraction efficiency

Chart illustrating extraction efficiency of AMOZ, AOZ, and SEM when spiked at 5 ng/mL in catfish, swai, and tilapia matrices. Recoveries range from 90-130 percent.

Blank samples (n=4) of catfish and swai along with (n=3) tilapia fillets were analyzed for the presences of nitrofuran metabolites. AOZ and AMOZ were not detected in any of the 11 samples analyzed. SEM was detected in all three matrices and this is attributed to the fact that semicarbabzide is a poor marker for nitrofurazone and is frequently found in soft plastic packaging and flour [15]. Blank samples were compared to reagent blanks, which underwent the entire experiment without the presence of tissue or standard solutions.

Conclusion

A short, robust method was developed for the analysis of nitrofuran metabolites using liquid chromatography coupled with triple quardupole mass spectrometry. Because of the lengthy derivatization process, cleanup and detection methods were optimized to reduce the time spent on analysis. Use of a phenyl column resulted in cleaner extracts for

analysis. Chromatographic analysis occured in fewer than 12 minutes with detection of concentrations as low as 1 ng/mL. Alteration of the cleanup process is necessary to achieve better analysis of the AHD metabolite. Despite this, the recoveries and repeatability of the experiment are adequate. The method was tested and proved accurate in the determination of nitrofuran metabolites in real world samples.

CHAPTER III

DIFFERENTIATION OF FISH SPECIES WITH CHIP BASED ELECTROPHORESIS

Abstract

Chip-based electrophoresis was used in conjunction with polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) for the differentiation of catfish, swai, and other economically important species. By analyzing the restriction digestion products of several endonucleases (DdeI, HaeIII, NlaIII) using Agilent's 2100 bioanalyzer, unique fragmentation patterns were recorded using only muscle tissue or fin-clips as source material. Multiple sample runs produced consistent results indicating that as few as one restriction enzyme was required for positive identification. This method of screening reduces the expertise, time, and expense required to reduce the mass mislabeling of imported fish. With further database development, PCR-RFLP could become the standard screening method.

Introduction

Farmers, fisherman, and consumers are dependent on what is now a global fish market. Increases in worldwide demand and production have created new challenges and exacerbated old ones. Mislabeling of fish species, regardless of intent, poses risks to consumer health and security, state economies, and conservation efforts. Global entities such as the FAO along with the European Union (EU), the United States Food and Drug

Administration (FDA), and the Canadian Food Investigative Agency (CFIA) have all set forth regulations to combat this growing problem [4, 34, 25, 26, 27, 28, 54]. Similar to FAO and EU regulations, congressmen in the US have passed laws which describe how seafood products in the United States should be labeled [4]. The current problem is not a lack of regulation but a lack of enforcement of these regulations.

The FDA lays out pretty straightforward guidelines for the proper labeling and description of seafood products. In addition to common and scientific names, congress has produced a list of acceptable market names that explicitly state products such as catfish, bonito, and crab must be labeled as such. The guidelines only state that vernacular and geographic descriptions are generally not acceptable. Due to a lack of enforcement, nonprofit groups such as Oceana have found mislabeling rates higher than 50% in the United States [69].

Mislabeling which can occur at multiple points of contact such as super markets, sushi bars, restaurants, and even at the port or distributor level which represents a large security risk. Currently, the FDA identifies about 2 percent of incoming shipments [28]. Increased enforcement of existing regulations is necessary to prevent sickness, fraud, and allow for emerging markets to grow. Rapid growth of importation and processing of seafood products has resulted in incoming shipments of fish fillets that cannot be immediately identified. Many modern methodologies exist for the analysis of DNA for the identification of fish fillets such as DNA bar-coding and PCR-RFLP. DNA bar-coding requires the sequencing of a specific gene sequence from mitochondrial DNA, usually cytochrome C oxidase subunit I. While extremely reliable, gene sequencing is often quite expensive and time consuming [36]. PCR-RFLP is a cheap alternative that

uses enzyme restriction digestion coupled with PCR amplification to examine gene sequences. Mitochondrial DNA is also a common target of PCR-RFLP. Studies have shown that mitochondrial cytochrome b can be used for comparison of salmon, eels, and hakes at various stages of processing [58, 37].

The southern states of Mississippi, Alabama, Arkansas, and Tennessee are mass producers of farm-raised catfish with Mississippi earning 175 million dollars in revenue as of last year [73]. With revenue slowly declining each year, dilution of the market with imported fish species poses financial and health risks. Pangasius species such as Pangasius hypothalmus and *Pangasius bocourti* originating from Vietnam and Taiwan have in the past contained banned antibiotic adulterants such as nitrofurans, amphenicols, and quinolones [7]. These residues are both mutagenic and carcinogenic and remain bound to tissue long after processing [59]. Because processing often alters the overall morphology of the fish, it often becomes difficult for an individual, taxonomists included, to differentiate one fillet from another [68]. A database containing restriction digestion fragments resulting from PCR-RFLP would be a suitable means of quickly screening incoming fish species.

This study sought to accomplish two goals. The first was to utilize the bioanalyzer to differentiate multiple American catfish species from Asian Pangasiid using both fish fillets and fin clips as source material. Once this was completed, the database was used to identify basa fillets sourced from an internet supplier. The second goal of this project was to fill the aforementioned database with a variety of fish species that had some economic or environmental importance. Bonito, red snapper, king mackerel, wahoo, and many other fish species were sourced from local super markets or donated by the FDA's

Dauphin Island lab for identification. Because red snapper and king mackerel were in greater supply, these samples were combined at different ratios to test the bioanalyzer's ability to detect multiple fragment patterns in a single sample.

Materials and Methods

Sample Collection

Samples for this study were collected from several different locations. First, Mississippi farm-raised catfish, swai, tilapia, and flounder fillets were purchased from the local Kroger supermarket. With the exception of the farm-raised catfish, all other fish were imported from other locales. Secondly, a variety of fish samples were collected from the FDA marine research lab in Dauphin Island, Alabama. Collected during Alabama's annual fishing rodeo, samples of muscle tissue were collected from blue runner, bonito, flounder, king mackerel, red drum, red snapper, Spanish mackerel, and tripletail. These fish samples were positively identified on site using morphological features and all were frozen at 0° C. Lastly, fin clips were obtained from wild-caught channel catfish and blue catfish in the Mississippi River in Memphis Tennessee and the Pascagoula River in Pascagoula, Mississippi and stored in ethanol. Whenever possible, three to four samples were collected for each species for analysis to verify intra-species consistency. Also, samples of red snapper and king mackerel DNA extract were combined in ratios of 95, 90, 85, and 80 percent for analysis.

DNA Extraction Protocol

Upon receipt of samples with the exception of fin clips, all fish were homogenized using a Magic Bullet blender and stored in falcon tubes at 0° C until

analysis. To prepare for analysis, samples were thawed and 150 mg (+/- 50mg) were weighed out and stored at 40° C until extraction. Genomic DNA was extracted utilizing the reagents and protocols supplied in Agilent Technologies DNA 1000 kit. Salt wash buffer and 80% ethanol solution were prepared using nuclease free sterile water and 100% ethanol (Sigma Aldrich) and all reactions were scaled to meet the needs of the current sample set. Extracts were stored at 0° C or immediately amplified using polymerase chain reaction if time permitted.

PCR Amplification

Extractions yielded DNA with concentrations ranging from 5 ng/ µL to 500 ng/ µL which is suitable for further analysis. While spectrophotometric analysis of genomic DNA or analysis of PCR products to verify quality yield can be performed, for screening purposes these optional steps are unnecessary. A positive control of salmon DNA with an approximate concentration of 50 ng/µL l along with a negative control of sterile water was used to verify successful amplification and digestion. All preparations for the PCR reaction were scaled to fit the needs of the sample set plus one excess and were performed on ice. To amplify the region of interest, a short section of mitochondrial cytochrome B, one microliter of genomic DNA extract was combined with 24 µL of a PCR reaction mixture containing sterile water, 2 x Master mix, and the universal primers: L14735 (5- AAA AAC CAC CGT TGT TAT TCA ACT A-3) and H15149ad (5-GCI CCT CAR AAT GAY ATT TGT CCT CA-3). The PCR reaction was carried out in an Eppendorf (Hamburg, Germany) thermo cycler under the following conditions: One cycle of 5 minutes at 95° C followed by 40 cycles of 30 seconds at 95° C, 30 seconds at

50° C, and 30 seconds at 72° C with a final cycle of 7 minutes at 72° C. PCR products were stored at -20° C or immediately digested if time permitted.

Restriction Fragment Length Polymorphism and Bioanalyzer Analysis

PCR amplification products underwent digestion in three individual PCR tubes containing the restriction endonucleases: Dde I, Hae III, and Nla III. The fragments were digested in a volume of 5 μL containing sterile water, a buffer solution, and the corresponding enzyme for a minimum 2 hours at 37° Cbut often extended overnight. The digestion process was halted with 60mM EDTA following a modified incubation period of 20 minutes at 80° C to ensure total cessation of the reaction. Digestion products were analyzed using Agilent's Bioanalyzer lab-on-a-chip electrophoresis. Chips were prepared according to the protocols provided by Agilent. In brief, chips are loaded with gel containing a dye followed by addition of DNA markers. Then, each digested sample and ladder is loaded in the appropriate well before immediate analysis. Absence of 12 samples on a chip requires the addition of sterile water to ensure proper analysis. Sample analysis is essentially automated occurring during a 30 minute window with results viewable after that time.

Results and Discussion

The goal of this study is to ascertain the capabilities of Agilent's 2100 bionalyzer as a DNA fingerprinting tool for the purpose of identifying and differentiating fish species. The flexibility of PCR-RFLP lies in the unique endonucleases which can provide an increasing amount of differentiating ability. Coupled with chip based electrophoresis, PCR-RFLP can differentiate most fish species with three or fewer endonucleases quickly

and efficiently which makes it a great alternative to more expensive methods such as DNA bar-coding. In this study, the capabilities of chip based electrophoresis for differentiation of fish species was tested in two ways. First, fish species that are important from an economic or health standpoint have been analyzed and differentiated. Secondly, the ability of the bioanalyzer to differentiate mixed samples was tested by analyzing mixed extracts of red snapper and king mackerel. Snapper and mackerel were chosen because those samples were had in excess and also because red snapper has been reported by Oceana to be one of the most mislabeled fish species in America either by substitution with other fish species or other types of snapper.

Table 3 Sample Origins

Common Name	Scientific Name	Origin	Location	Sample Type	Correctly Labeled
Blue Catfish	*Ictaluras furcatus*	Wild	Memphis, TN	Fin-Clip	Yes
		Wild	Pascagoula, MS	Fin-Clip	Yes
Channel Catfish	*Ictaluras punctatus*	Farm-raised	Mississippi	Fillet	Yes
		Farm-raised	Mississippi	Fillet	Yes
		Wild	Memphis, TN	Fin-Clip	Yes
		Wild	Pascagoula, MS	Fin-Clip	Yes
Basa	*Pangasius bocourti*	Farm-raised	Asia	Fillet	No
Swai	*Pangasius hypothalmus*	Farm-raised	Asia	Fillet	Yes
Tilapia	*Oreochromis*	Farm-raised	Asia/Africa	Fillet	Yes

One of the advantages of PCR-RFLP is the source material (Table 3) that can be used for differentiation. Eggs, larvae, fillets, and fin clips can all be used in raw or cooked forms with less than a gram required for analysis. When comparing Pangasiid to Icatlaruid species, both fin-clips and fillets were used for analysis. No major differences were found between fin-clips and fillets other than natural degradation of fin-clips due to long term refrigeration. Differentiation of channel catfish and swai samples was still

possible with two of the three restriction enzymes (Figure 8). Agilent's database verified the identity of both fillets and fin-clips successfully.

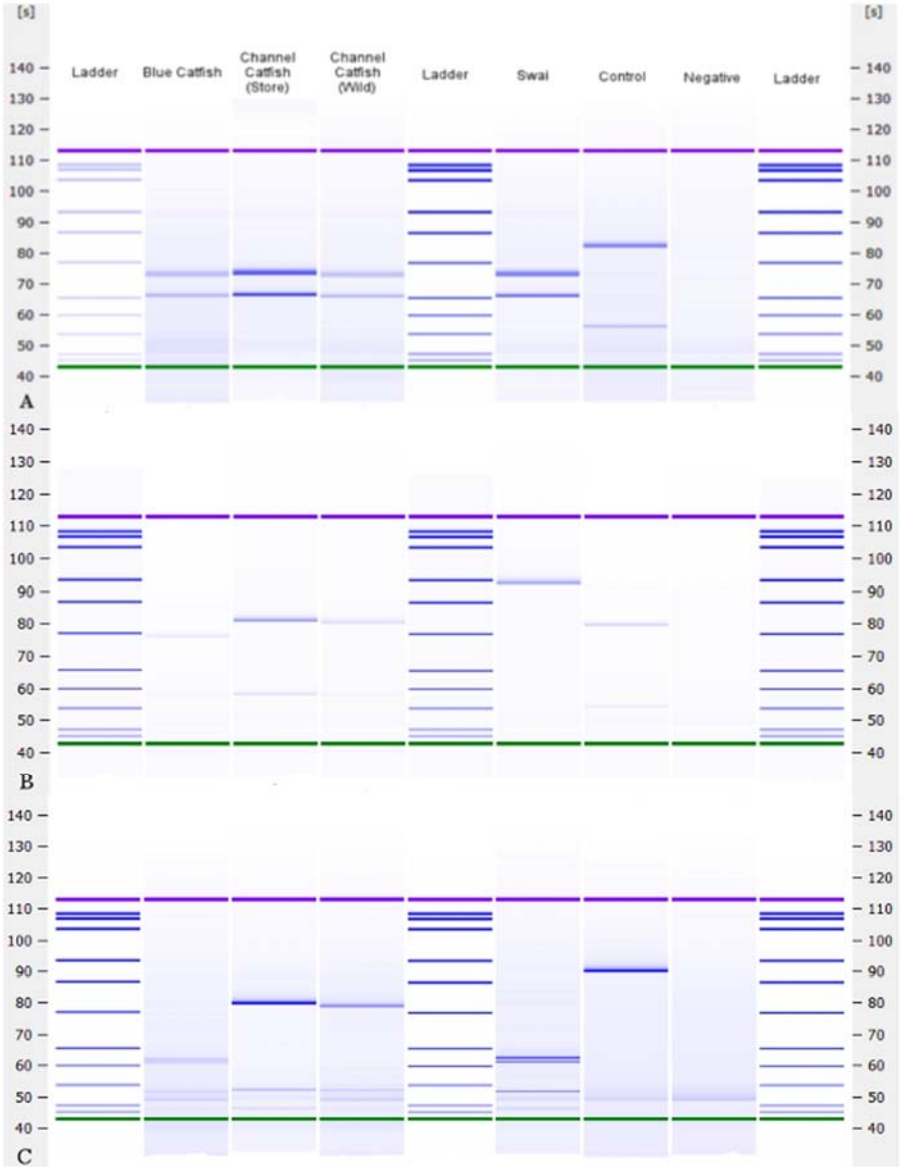

Figure 8 PCR-RFLP gel

Gels for chip-based electrophoresis of restriction digest products of (A) DdeI B) HaeIII C) and NlaIII for different fish species.

The next set of samples was those commonly considered to be important to the fishing industry. Only bonito and red drum were matched to Agilent's database, but many others such as white trout, Spanish mackerel, bonito, and blue runner are frequent gulf catches. Table 4 shows the average of multiple fragments (n=3) detected for each restriction enzyme including a standard deviation making each fish differentiable from the other. Whiting was the only fish which lacked any fragmentation patterns.

Table 4 Fragmentation patterns of Gulf fish species

Common Name	Scientifica Name	D(SD)	H(SD)	N(SD)	Database Match
Bonito	*Sarda sarda*	229 (0.5) 238 (2.38)	136 (0) 150 (0.5) 175(0.5)	122 (0.82) 245(1.26)	Yes
Cobia	*Rachycentron canadum*	132 (0) 348(1.53) 355 (1.0)	132 (0.58) 149 (0) 161 (0.58)	160 (0.58) 285(0.58)	No
Spanish Mackerel	*Scomberomorus maculatus*	441 (2.65)	130 (0.58) 149 (0) 182 (0)	183 (0.58) 281 (1.53)	No
Red Drum	*Sciaenops ocellatus*	450 (0.58)	75 (0.58) 117 (0.58) 130 (0.58) 148 (0)	92 (0) 107 (0) 289 (0.58)	Yes
Flounder (Store)	*Paralichthys*	158 (0) 277 (1.53) 285 (1.0)	134 (0.58) 294 (0.58)	180 (0.58) 290 (1.55)	Yes
Flounder (Wild)	*Paralichthys*	190 (0) 261 (1.15)	137 (0.58) 295(0)	290 (1.0)	Yes
Tripletail	*Lobotes surinamensis*	97 (0) 122 (0.58) 210 (1.0)	131 (0.58) 340 (0.58)	106 (0) 383 (1.0)	No
Blue Runner	*Caranx crysos*	472 (2.65)	47 (0) 137 (1.0) 164 (0)	124 (0) 174 (0) 189 (0)	No
White Trout	*Salmo / Cynoscion*	123 (0) 349 (1.26) 355 (1.41)	118 (0.5) 133 (0.82) 222 (0.5)	92 (0) 107 (0.5) 291 (0.82)	No

Although not matched to the database provided, samples can easily be matched to positively identified samples to verify authenticity when the database is found lacking. This removes the need for the screener to have any previous knowledge of DNA bar-coding or sequencing for that matter. One of the difficulties inherent in identifying fish is that most imported products have already been processed. Processes such as mixing and cooking alter the original DNA making identification more difficult. RFLP is advantageous because mitochondrial DNA which is the gene region of interest for most applications is quite resistant and still maintains interspecies differences after cooking. To test this, red snapper and king mackerel DNA was mixed at ratios ranging from 95% to 80%. With as little as 5% of source material, red snapper and king mackerel were both differentiable using DdeI and HaeIII restriction enzymes (Figure 9 and Figure 10)

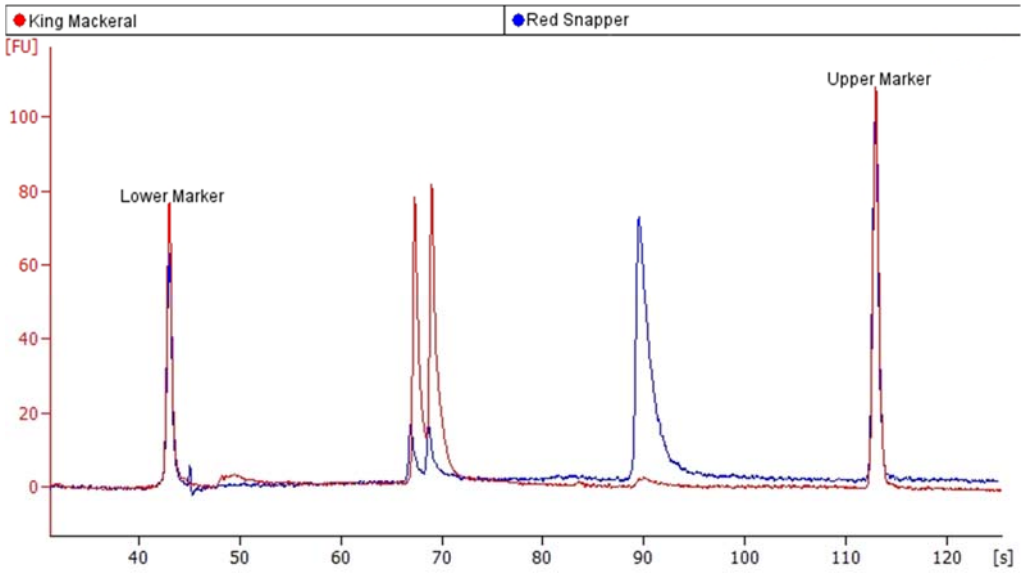

Figure 9 DdeI Electropherogram

Electroperhogram of DdeI restriction digestion products of king mackerel and red snapper mixed at a 95:5 ratio.

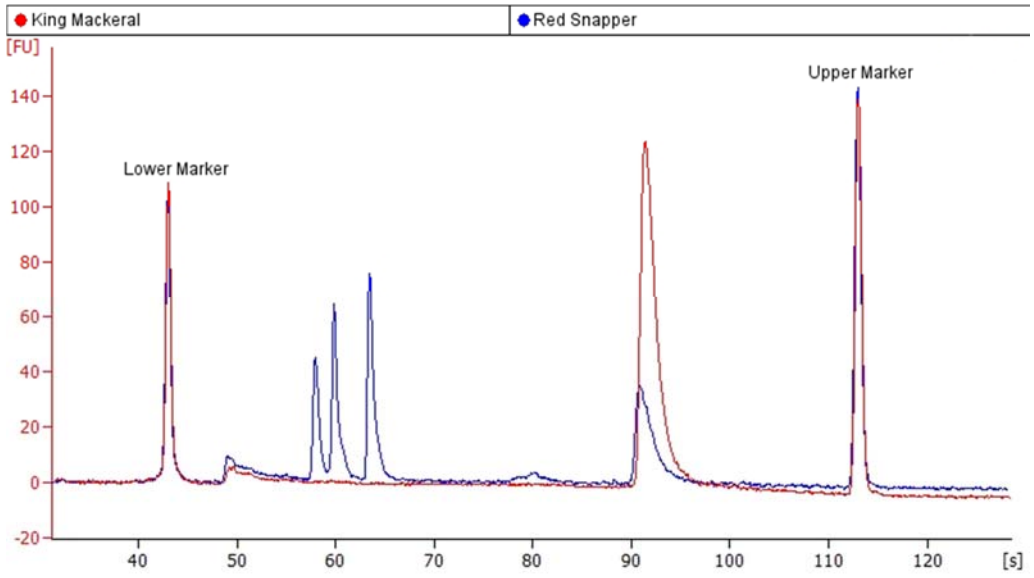

Figure 10 HaeIII Electropherogram

Electropherogram of HaeIII restriction digestion products of king mackeral and red snapper mixed at a 95:5% ratio.

The results of this study find that PCR-RFLP coupled with chip-based electrophoresis is a useful tool for the quick and efficient fingerprinting of fish species regardless if the fish has been filleted or mixed during processing. Taking less than a 24 period, the process can easily be used by those not familiar with sequencing or taxonomic identification allowing for the quick comparison to an already compiled database which also removes the need for reference samples.

Conclusion

Because importation of seafood products is continually increasing and the problem of mislabeling is also growing, a method for quick differentiation of fish species is needed to protect citizens' health and economic interests. This study concludes that the bioanalyzer is a useful tool for DNA fingerprinting. Capable of differentiating fish

species using restriction digestion products coupled with chip-based electrophoresis, the bioanalyzer simplifies the process of screening incoming seafood products. Not only is very little source material required, but individuals lacking expansive knowledge of sequencing or bar-coding protocols can still use the bioanlyzer for screening purposes. With an expansive database, chip-based electrophoresis stands out as a much needed tool to combat the growing mislabeling problem.

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